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Methods for quantifying intrafollicular drug delivery: a critical appraisal

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Importance of the field: In recent years there has been increasing awareness that the hair follicles and their associated pilosebaceous structures may act as significant permeation pathways and/or reservoirs for topically applied drugs. This has implications in terms of dermatological therapy for acne, hirsutism, alopecias or certain skin cancers as well as systemic drug delivery. As the processes modulating follicular drug penetration are poorly understood at present, there is an emergent need for methodologies that can quantify follicular drug penetration and deposition. So far, a review article specifically dedicated to these methodological aspects has not yet been written.

Areas covered in this review: This paper reviews the available quantitative follicular methodologies that have been developed over the years, describing the advantages and disadvantages of each approach. This review covers comparative techniques that are based on measuring drug flux through 'follicle-free' and 'follicle-containing' integuments, the skin sandwich, differential stripping and optical imaging-based technologies. Techniques for measuring drug–sebum interactions are also discussed.

What the reader will gain: The reader will develop an understanding of the complexities involved in quantifying drug delivery through follicles and pilosebaceous units. The Expert opinion section will give the reader insights into how more broad-ranging future research could allow identification of the most useful methods for quantifying follicular drug transport.

Take home message: This is still a poorly understood field. It clearly warrants much larger scale studies than have been performed so far involving multiple techniques and multiple drugs.

Keywords: drug delivery, evaluation, follicular route, hair follicles, quantification, sebum, transdermal, transfollicular

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1. Introduction

Recent years have witnessed increasing recognition that the hair follicles may act as significant penetration pathways and/or potential reservoirs for topically applied compounds [1-3]. This has important ramifications for both local drug treatments of acne, hirsutism, alopecias or follicle-associated tumours as well as systemic transdermal drug applications. The latter may be especially important for macromolecular and/or vaccine-delivery applications. In all cases, this emerging paradigm has led to a growing need for methodologies that allow quantification of follicular drug penetration – a process that is still poorly understood thus far [1].

Unfortunately, the complexity, small dimensions and location of pilosebaceous units combined with the inherent variability of skin tissue [4] mean quantitative measurements of drug transport and/or accumulation are difficult to implement.

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Article highlights.

- In this review, the diverse specialist methodologies that can be used to quantify the penetration of drug substances into hair follicles are discussed. Each methodology is imperfect in that each has its own specific advantages and disadvantages.
- The methodologies covered include comparative techniques based on measuring drug flux through 'follicle-free' and 'follicle-containing' skins, the skin sandwich, differential stripping and optical imaging-based technologies. Assessments of drug–sebum interactions are also discussed.
- Out of the comparative-based techniques, the selective blocking of follicles seems to constitute the most accurate and effective method overall. The sandwich approach is viewed with scepticism by certain research groups. The differential stripping approach yields drug amount rather than drug flux data like the other methods. Optical-based techniques continue to improve with continuing technological developments.
- Progress with respect to the role of sebum would be accelerated if the composition of a synthetic surrogate sebum could be agreed on by different groups at the international level.
- For various reasons, this topic remains a poorly understood and relatively unexplored research area. To enhance our understanding in this area, much larger scale studies than have been performed so far are clearly warranted. These should involve multiple techniques and multiple drugs.

This box summarises key points contained in the article.

One approach is simply to mechanically separate out the follicles and then assay the extracted drug. This can be done at different time points after topical drug application in order to yield solute concentration changes with time. However, mechanical separation protocols are highly problematic in that inter-tissue drug contamination is likely during the extensive excision and handling steps. The use of traditional autoradiography yields image signals that tend to represent tissue-bound residues rather than the freely diffusible substance [5].

Despite the myriad problems, over the years various specialist follicular methodologies have been developed that are indeed quantitative – at least in theory. Yet each of these techniques has its own distinct set of limitations. The aim of this paper is to review the different available quantitative systems, outlining the particular merits and drawbacks of each approach.

The first part of this review is a background section that covers the fundamental architecture and physiology of the pilosebaceous unit and hair follicles. This is necessary in order to put the subsequent methodological aspects in context. Then, there is a discussion of the established comparison-based methodologies, in which drug penetration through a follicle-containing skin is contrasted with that through some type of equivalent 'follicle-free' skin. This is followed by a detailed explanation of the *in vitro* skin sandwich technique and subsequently the differential stripping approach. The increasingly quantitative nature of certain optical imaging systems is then reviewed. The next section deals with sebaceous lipids and the methodologies available for probing drug–sebum interactions. This is followed by a brief account of how different authors have incorporated the follicular pathway within mathematical models of transdermal drug delivery. The Conclusions section summarises the different techniques discussed. Finally, this paper proposes some potential avenues for further research and outlines future objectives in this area.

2. The pilosebaceous unit: structure and function

The pilosebaceous unit (PSU) represents a complex and highly dynamic three-dimensional structure that exerts diverse biochemical, metabolic and immunological functions. As the anatomy and function of these structures have been discussed extensively in the literature [6], the details are covered relatively briefly here.

Structurally, each PSU consists of a hair follicle, hair shaft, connecting arrector pili muscle and adjoining sebaceous glands. The hair follicle consists of a hair bulb and shaft covered in an inner root sheath, an outer root sheath and an outermost acellular basement membrane known as the glassy membrane. Whereas the inner root sheath terminates about halfway up the follicle, the outer root sheath is a keratinised layer that merges with the epidermis. Each hair follicle is coupled with one or more multilobular sebaceous glands. These glands, which are outgrowths of epithelial cells, are joined by ducts to the upper segment of the follicular canal. **Figure 1** presents a simplified diagram of the hair follicle.

It should be stated that there are actually two separate types of human hair – terminal hairs and vellus hairs. Terminal hairs are long (> 2 cm), thick (> 0.03 mm), pigmented and usually incorporate a medullary cavity [6]. These hairs are anchored within the hypodermis to a depth of 3 mm or more. By contrast, vellus hairs are usually short (< 2 cm), thin (< 0.03 mm), non-pigmented and might be anchored in the dermis to a depth of just 1 mm. It is possible for hairs to exist in a vellus-to-terminal transition phase. In the scalp, the hair follicles typically occur in clusters, each composed of one to four terminal hairs and maybe one to two vellus hairs [7].

A characteristic feature of hair follicles is that they undergo a controlled cycle of alternating growth and rest stages. During the proliferative phase or anagen, the hair matrix cells rapidly divide and translate upwards to generate the hair shaft. Anagen is always followed by catagen. This is a brief phase associated with rapid morphological alterations including the end of mitosis, reabsorption and cell death in the lower follicle segment. The follicle then enters a rest phase termed telogen, after which the hair is shed. Anagen is then initiated

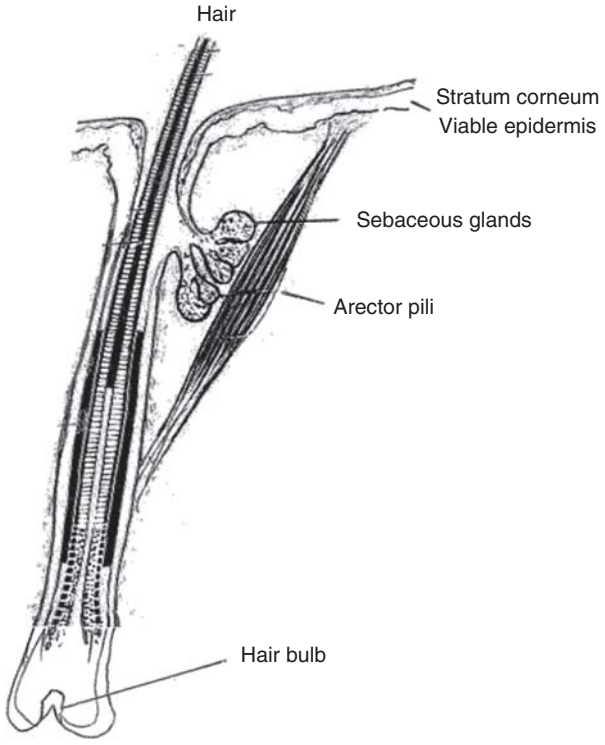


Figure 1. Cross-sectional diagram of the human hair follicle.

as the cycle restarts. Locally active inhibitors modulate this cycling process – a phenomenon termed the Chalone hypothesis [8]. In most human body sites the hair cycle lasts for a few months, but on the scalp it lasts for between 3 and 8 years. It should be noted that the duration of each growth phase as well as the percentage of hair in each growth phase differ significantly between vellus and terminal hairs. Also, hair growth is affected by seasonal factors. This is owing to regulation by the endocrine system, which acts by means of the pineal gland. Specifically, circulating prolactin levels [9] correlate inversely with melatonin levels, being higher in summer and lower in winter.

Of considerable interest are the sebaceous glands, which are designed to synthesise and release sebum. This is a bacteriostatic mixture of squalene, wax esters, triglycerides, fatty acids, cholesterol and its esters. The percentage composition is listed in **Table 1**. Sebum is produced during the programmed disintegration of the sebaceous glands. Gland rupturing is associated with sebum discharge into the ducts and subsequently its deposition in the upper follicular canal. This creates a hydrophobic lipid environment in this region of the PSU. Eventually, the sebum migrates upwards to reach the skin surface.

It is noteworthy that PSU density varies widely with body site. On the face and scalp, there are between 500 and 1000 pilosebaceous units per square centimetre. Follicular orifice diameters generally vary between 50 and 100 μm. It has been calculated that the amalgamated areas of such openings may represent as much as 10% of the total surface area of the face and scalp. In other sites, follicular openings typically account for ~ 0.1% of the total surface area, whereas certain regions such as the sole of the foot or the palm of the hand are devoid of hair follicles. There are also large regional variations in the distribution and activity of the sebaceous glands. Glandular activity is most intense in the facial region but is absent on the palms of the hands and soles of the feet. Once on the skin surface, excess sebum tends to flow in microfurrows from sebum-rich to sebum-poor sites. Also, excess sebum may be reabsorbed into the stratum corneum [10].

Effective drug delivery to the hair follicles is important for the therapy of PSU-associated disorders or diseases. The scheme presented in **Figure 2** lists some principal hair follicle-associated disorders.

3. Approaches based on comparing follicle-free and follicle-containing skins

Over the years, a diverse array of comparative methodologies have been devised that can be used, at least in theory, to quantify the follicular component of transdermal drug delivery. These models are run on a comparative basis in which drug penetration through some type of 'follicle-free' skin is compared with that through a control 'follicle-containing' skin. Each of these methods is described below.

3.1 Use of rodents showing differential follicular densities

A relatively simple approach is to compare drug penetration through skins showing different hair follicle densities. This can be implemented by using genetic rodent mutants that come in strains displaying different degrees of 'hairiness'. This approach has most commonly been applied *in vitro.* For example, one team [11] used a skin histoculture penetration chamber system to experiment with excised skins from both hairy and hairless mice. The group's aim was to elucidate the role mediated by hair follicles in the absorption of compounds having different partition coefficients. Also, Del Terzo and co-workers [12] used the excised skins of different Sprague-Dawley rat strains to determine the importance of follicles in the iontophoretic transport of diverse alkanolic acids. Later on, Hisoire and Bucks [13] considered retinoic acid transport via hair follicles and to this end compared this drug's *in vitro* penetration across hairy and hairless guinea-pig skin. In another study, follicular delivery of a liposome-encapsulated hydrophilic spin probe was investigated through comparative use of excised hairless and hairy mouse skin [14]. Furthermore, Shim and co-workers [15] tracked the *in vitro* follicular flux of minoxidil-loaded nanoparticles by comparing their permeation through hairy and hairless guinea-pig skins. The group observed that the follicles

Table 1. Relative compositions of real human sebum and two recently developed synthetic sebum-like mixtures.

Lipid class	Real sebum* (% w/w)	Synthetic sebum X‡ (% w/w)	Synthetic sebum Y§ (% w/w)
Triglycerides	45	45 [Olive oil 10] [Cotton seed oil 25] [Coconut oil 10]	44.7 [All as triolein]
Wax esters	25	25 [Paraffin wax 10] [Spermaceti wax 15]	25 [All as jojoba oil – a wax monoester]
Squalene	12	15	12.4
Free fatty acids	10	11.4 [Oleic acid 1.4] [Palmitic acid 5] [Palmitoleic acid 5]	17 [All as oleic acid]
Cholesterol and sterol esters	4	3.6 [Cholesterol 1.2] [Cholesterol oleate 2.4]	0
Other components	4	0	0.9

*Mean values derived from data cited in numerous past literature sources, as compiled by [62].
‡Artificial sebum developed and described in [27,28,72].
§Artificial sebum developed and described in [71].

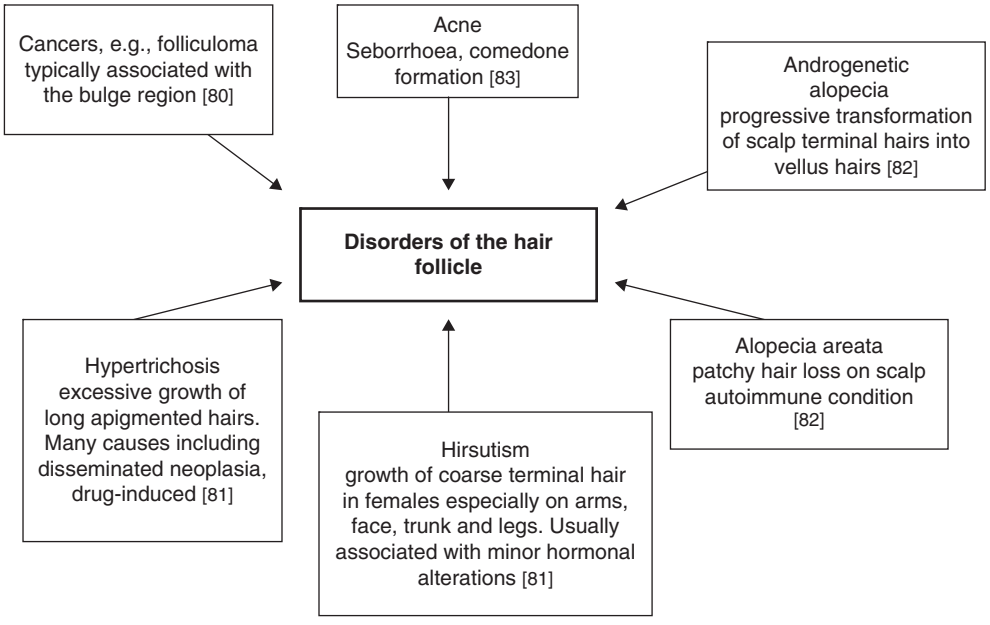


Figure 2. Scheme showing some of the principal disorders associated with hair follicle.

constitute a major particulate penetration route and that smaller nanoparticles showed increased permeation.

It is noteworthy that this methodology has also been applied *in vivo*. For example, Weiner's team [16] applied mannitol (hydrophilic) and progesterone (lipophilic) to the skins of both hairy and hairless rats. Urinary excretion data and skin concentration profiles were then used to assess the follicular role in the absorption of the two solutes. Similarly, Ogiso and colleagues [17] compared betahistine flux through the skin of Wistar rats and hairless rats *in vivo*.

The principal limitation of these studies is that the technique is capricious because skins with different follicular densities probably show other structural differences, apart from the presence of follicles [16]. Moreover, the skins of so-called hairless animals are not truly follicle-free but rather tend to incorporate some underdeveloped hair follicles. Despite these

major caveats, this type of approach may still yield some insights about relevant mechanisms and paradigms.

3.2 Use of follicle-free scar skin

Some three decades ago, it was shown that it is possible to create a genuinely follicle-free skin by exposing the skins of live rodents to water at 60°C for 1 min [18]. The epidermis is then allowed to regrow at the scalded site for several weeks until it forms a follicle-free scar skin. Crucially, this scar tissue shows the same transepidermal water loss (TEWL) rates as normal skin. There have been several past attempts to quantify follicular drug delivery by comparing solute flux through normal rodent skin with that through follicle-free regrown rodent skin. Some of these studies were performed only *in vitro* [19-21], whereas others also involved *in vivo* research [22,23]. Perhaps the most relevant research involved successfully adapting the scarring protocol for human skin application [24]. The group measured the *in vitro* penetration of hydrocortisone, estradiol, testosterone and progesterone across both follicle-free and normal human skin. It was found that for all four steroids, flux through follicle-free skin was greater than that through normal skin, suggesting that the follicular route is important for steroid penetration.

Unfortunately, regrown skin differs from normal skin in terms of both epidermal thickness and degree of dermal vascularisation. Also, the epidermal–dermal junction is flat in scar skin but is convoluted in normal skin [25]. Despite the normal TEWL rates, it is difficult to establish definitely that the continuous stratum corneum of regrown skin has equivalent barrier properties to that of regular skin. Hence, this method's validity is still rather under question.

3.3 The skin of newborn rats versus the skin of 5-day-old rats

The fact that the skin of newborn rats lacks hair follicles whereas the skin of 5-day-old rats has fully developed hair follicles has formed the basis of another comparative methodology. Illel and colleagues [20] measured the transdermal absorption of hydrocortisone through the skin of newborn rats and the skin of 5-day-old rats *in vitro*. It was found that steady-state flux through newborn rat skin was fivefold lower than that through 5-day-old rat skin. Hence, assuming that the continuous membranes have equivalent permeability, the authors concluded that the follicles were a major penetration route during steady-state flux. As before, the main question is whether the continuous membranes of the two types of skin have equivalent barrier properties. Yet it might be envisaged that the differences between these two tissues would be smaller than differences occurring in the scar skin system.

3.4 Use of sebum-filled follicles

This *in vitro* technique utilises the full-thickness skin of certain rodents such as Wistar rats or guinea-pigs. The excised skins are mildly heated so that their surface temperature reaches ~ 42°C. This is then followed by topical drug administration. Heating causes sebum to be released from the sebaceous glands so as to fill much of the hair follicle shafts. It was shown that such sebum deposition completely suppressed the transdermal flux of both sucrose and mannitol, which indicated that both these highly hydrophilic solutes penetrated exclusively by means of the follicular route [26]. However, sebum discharge merely reduced rather than completely inhibited the flux of hydrocortisone, which is less hydrophilic. A principal drawback of this approach is the fact that many molecules can partition into and diffuse through sebum to various extents [27,28]. Such effects need to be taken into account. Also, it is not known whether the sebum discharge occurs in other species, including human skin, and whether or not it can be induced *in vivo*.

3.5 Selective blocking of follicular orifices

One recently developed comparative methodology involves artificially and selectively sealing hair follicle openings with micro drops of a polymer adhesive. This essentially creates a 'follicle-free' skin area, and by comparing penetrant flux through this with that through adjacent normal skin it is possible to quantify appendageal drug transport.

The method was pioneered by Lademann's group, who used a varnish–wax mixture to seal selectively the orifices of porcine hair follicles [29]. *In vitro* diffusion studies were then run to assess the follicular penetration of 4-methylbenzylidene, curcumin or titanium dioxide microparticles. Subsequently, the technique was used on excised full-thickness human skin sections derived from female plastic surgery patients [30]. Following the topical deposition of caffeine-containing formulations, *in vitro* permeation experiments were performed on both follicle-blocked and normal skin samples. Flux data comparisons indicated that almost 59% of the permeated caffeine penetrated through the follicles. Abdulmajed and Heard adapted the protocol slightly as they applied tiny amounts of a water-resistant, fast-drying powder adhesive to seal porcine hair follicles selectively [31]. Both 'follicle-free' and untreated control skin samples were then mounted in diffusion cells and retinyl ascorbate solution was topically applied. Drug deposition in the epidermis was then evaluated by tape stripping at the end of the permeation period. It was found that under normal conditions, the follicles did not contribute to ascorbate absorption into the epidermis. However, the application of a controlled lateral tensional force produced a significant increase in follicular drug delivery into the epidermis. It was proposed that such skin stretching alters the follicle's morphology. Specifically, a disruption in intrafollicular sebum packing occurs in the line of the tensional force. Thus, sebum-free channels are formed through the pilosebaceous unit, allowing easier penetrant access.

Importantly, the follicle-blocking approach has also been deployed *in vivo*. Otberg and co-workers [32,33] conducted clinical trials examining caffeine delivery from various formulations into human hair follicles. Interestingly, blood

concentrations of caffeine were higher following its topical application to open follicles in comparison with sealed follicles. Crucially, these caffeine permeation data were similar to those derived from *in vitro* human skin studies [30].

The selective follicular blocking technique clearly requires considerable expertise. Appreciable practical skills are required to apply just sufficient sealant material to block the follicular orifices but not to deposit residues on the continuous skin surface areas surrounding them. Another complication is that capillary forces or fine cracks may develop in the polymeric seal and these might conceivably allow the follicular ingress of small molecules [29].

4. The skin sandwich approach

Devised by Barry and co-workers [34-36], the skin sandwich is an intriguing *in vitro* methodology that quantifies the follicular route contribution to total dermal drug penetration. Essentially, a 'sandwich' is formed by overlaying an extra stratum corneum membrane onto a human epidermal membrane. It is crucial that both layers originate from adjacent areas of the same donor. The idea is that because follicular shunt openings have such a tiny surface area in comparison with the total skin surface area, there is a negligible chance that orifices will overlap (see **Figure 3**). So shunt pathways leading from the sandwich's top to its bottom are effectively obstructed. Use of a Monte Carlo simulation indicated that this assumption was mathematically sound even considering that, in practice, adherence between the two membranes will be incomplete [35].

The calculations associated with this model are simple. At steady-state, passive drug flux through a solid homogenous membrane is inversely proportional to the permeant's pathlength. As the main permeation barrier in skin resides within the stratum corneum, flux through the sandwich should be half that through the single epidermis if the shunts make a negligible contribution to penetration. Conversely, if sandwich flux is significantly less than half the single epidermal flux then that would indicate that the shunt contribution is notable. Mathematically:

$$\%\text{Follicular Contribution} = [1 - (2 \times J_{\text{Sand}} / J_{\text{Ep}})] \times 100 \quad (1)$$

where J_{Sand} and J_{Ep} represent the steady-state drug flux values for the sandwich and single epidermis, respectively. The follicular contribution (FC) refers to drug flux into the follicular opening as a fraction of total drug flux into skin (see **Figure 4**). The methodology does not provide data on the drug's fate once inside the follicle.

Use of the skin sandwich involves making some key assumptions. First, the small resistance of the viable epidermis to permeation is ignored for the sake of simplicity. Second, it is assumed that the shunts represent just hair follicles because the tiny sweat duct orifices probably mediate a smaller role in absorption. Also, there is no sebum in this system. Finally, the approach is inapplicable to lipophilic molecules. Once the log octanol–water partition coefficient (log $K_{o/w}$) value exceeds ~ 2.7, transfer across the stratum corneum no longer constitutes the rate-limiting step for penetration.

The human skin sandwich has been deployed in several diverse experimental settings. Barry's group [34] tracked transepidermal estradiol penetration from ultradeformable liposomes. They found that the follicles made only a very minor contribution towards estradiol delivery. Comparisons between the passive permeation of mannitol (hydrophilic) and that of estradiol (slightly lipophilic) revealed that in the first 8 h mannitol transport was entirely mediated by follicles, whereas estradiol penetration was almost entirely non-follicular in nature [35]. Yet a more complex pattern emerged when mannitol flux was followed over longer timescales.

In the author's laboratory, the skin sandwich was used to quantify the effect of the octanol–water partition coefficient on follicular absorption in human skin [1]. To this end, estradiol, corticosterone, hydrocortisone, aldosterone, cimetidine, deoxyadenosine and adenosine were selected as candidates. These particular drugs showed a wide range of log $K_{o/w}$ values varying between 2.29 and -1.05 but comparable molecular masses (251 – 362 Da). The results showed an apparent parabolic relationship between %FC and log $K_{o/w}$. The FC to total flux was small at high log $K_{o/w}$ as well as at low log $K_{o/w}$ values but was maximal at intermediate log $K_{o/w}$ values. Mechanistically, it would be expected that the lipophilic molecules would preferentially partition into and diffuse through the lipoidal continuous stratum corneum, which has a much larger fractional surface area than the orifices. The low follicular absorption of the most hydrophilic candidates is possibly due to these molecules hydrogen bonding to follicular pores.

Later, the skin sandwich method was successfully extended for the first time to porcine skin [37]. Screening of the same seven drugs yielded a parabolic curve very similar to that described above. In fact, linear regression analysis of the FC data yielded a good correlation (r^2 = 0.87) between the two skin species. This indicates that pig skin is a good surrogate for human skin in terms of modelling follicular absorption. This finding fits in with the fact that porcine hair follicle anatomy is close to that of humans. In both species, similarly clustered hair follicles consist of internal and external root sheaths, shaft, bulge and bulb [38,39]. In both species, similarly structured and follicle-associated sebaceous glands are located in the superficial reticular layer. Hair follicle density is ~ 11 cm^{-2} in pigs [40] and 14 – 32 cm^{-2} in humans [41]. However, follicular openings in domestic pig skin typically account for just under ~ 1% of the skin surface area [42], whereas in human skin they have been shown to account for less [43]. Yet human skin shows much larger site-dependent variations in follicular density [44], so depending on human anatomical site, the difference may not be quite as marked.

Recently, human skin sandwich studies were performed on hydrocortisone in order to investigate formulation variable effects on one drug's follicular absorption [45]. It was found that topical pretreatment with chemical enhancers reduced

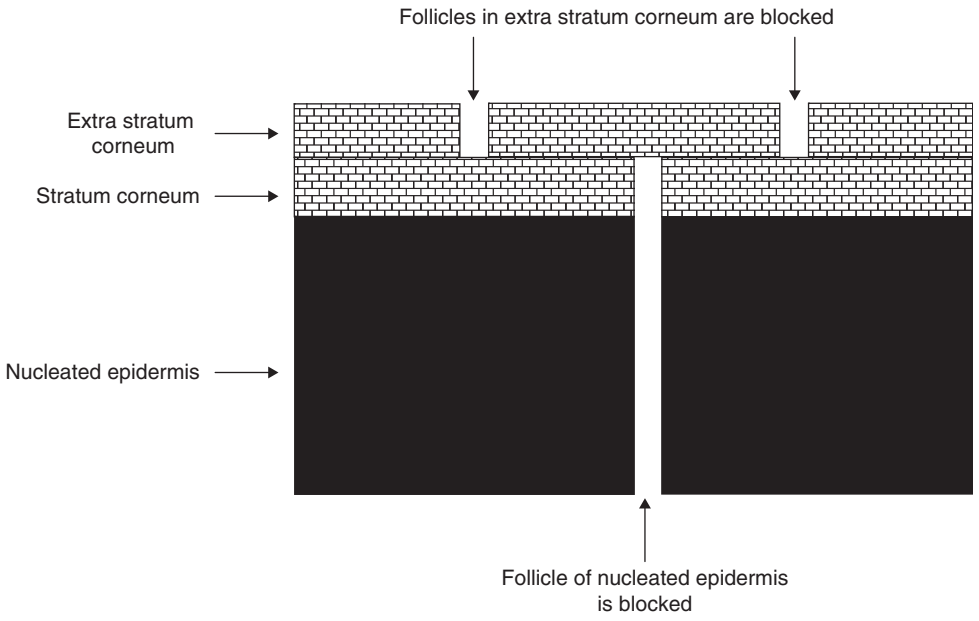
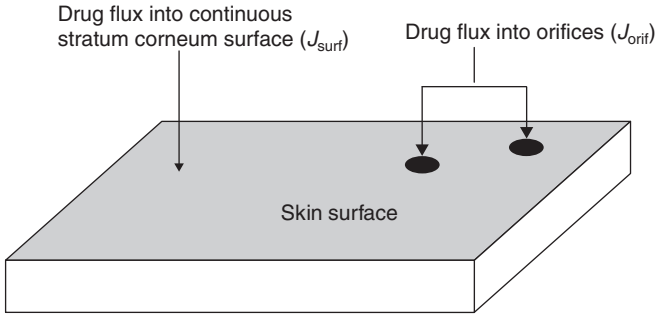


Figure 3. Scheme illustrating the basic principle of the skin sandwich technique. The sandwich is composed of an epidermal membrane with an extra stratum corneum on top that effectively blocks all follicles in the bottom epidermis.



% Follicular Contribution = $100 \times J_{orif}/J_{surf}$

Basic limitations of the system:

- Limited to drugs of log $K_{o/w} \sim \leq 2.6$.
- No information on the test drug's intrafollicular fate.
- No sebum.

Figure 4. Schematic diagram illustrating the type of data given by the skin sandwich technique as well as some of its limitations.

the follicular contribution to various extents. This is probably owing to permeabilisation of the continuous stratum corneum, making the follicular route less quantitatively significant. Also, using a polymeric gel vehicle rather than an aqueous solution markedly reduced follicular hydrocortisone absorption. This suggests that follicular flux is promoted when the drug-containing vehicle is sufficiently liquid to flow freely into orifices. If this finding is validated in further work, perhaps utilising other model systems, it would indicate that follicular absorption can be modulated by adjusting formulation viscosity.

5. The differential stripping approach

In differential stripping, adjacent or symmetrically opposite skin areas of the same donor are separately subjected to

adhesive tape stripping and cyanoacrylate surface biopsies [46]. Whereas application of cyanoacrylate-coated slide detaches the stratum corneum together with any associated upper pilosebaceous structures including the contents, tape stripping removes the stratum corneum alone. Hence, it is possible to quantify drug deposition in the upper hair follicle simply by calculating the difference in compound amounts that is extracted by each technique.

Lademann's group used differential stripping to quantify the penetration of fluorescent nanoparticles into human hair follicles *in vivo* [47]. They reported that the follicular delivery of nanoparticles was enhanced when a massage was applied to the skin surface. The authors proposed a mechanism involving the serrated relief of the hair surfaces, which in terms of size were the same order of magnitude as the nanoparticles. The idea is that the massage-induced motion of the serrated surfaces drives the nanoparticles into the follicles by a 'geared pumping' effect. Another suggestion was that massaging promoted mixing of the topically applied nanoparticles with sebum in the follicular canal. Massage-enhanced follicular delivery of these nanoparticles was also observed when the experiments were undertaken in porcine skin *in vitro*.

In another human *in vivo* study [48], investigations were performed on the delivery of the antifungal agent, brilliant green, to the hair follicle. The results indicated that follicular deposition may be an important consideration in topical antifungal therapy.

In key experiments, differential stripping was applied to compare *in vivo* and *in vitro* penetration of the same permeant [49]. Curcumin-loaded nanoparticles were utilised and their *in vitro* as well as *in vivo* penetration into human hair follicles was evaluated in the skins of patients undergoing surgeries. It was found that even for experiments performed on the same volunteers at the same skin site, the drug follicular reservoir following *in vitro* application was only ~ 10% the size of the drug follicular reservoir following *in vivo* application. The authors explained this surprising result by proposing that the elastic fibres surrounding the follicles contract during the cutting process [49]. Such a mechanism fits in with other observations on the differential role of integument-embedded muscle fibre types in mammalian skin [50]. Lademann and colleagues reported that it was not possible to reverse this follicular muscle fibre contraction by re-stretching the excised skin [2].

If the lack of *in vitro–in vivo* correlation described above is confirmed in further studies and with other drugs, this would mean that *in vitro* human skin is of only limited use for researching follicular penetration. Following on from this, it was proposed that the *in vitro* porcine ear skin system is actually a superior model system because its surface is intact but the integument as a whole is fixed in stretched form on underlying cartilage [2]. This is despite minor species-related differences such as the presence in porcine epidermis of interfollicular smooth muscle units that are absent in humans [51] or the fact that the lower regions of porcine hair follicles are much less heavily vascularised than in humans.

In summary, differential stripping is a powerful technique as it is relatively non-invasive and can be applied to all human skin sites except the scalp, where the follicles are too large and deep for cyanoacrylate removal [2]. For the same reasons, the methodology cannot be deployed on hairy animal skins.

6. Approaches based on optical imaging

Over the years, diverse optical methodologies have been applied to visualise the permeation pathways of topically applied compounds. Although such techniques started off qualitative in nature, technological improvements have allowed systems to become increasingly more quantitative with time.

Fabin and Touitou [52] devised a quantitative autoradiographic method to quantify the delivery of two lipophilic compounds to the hair follicles of hairless rats *in vitro.* By applying a computerised image analysis system, they evaluated drug concentrations in the hair follicles at different time points. Quantitative autoradiography was also used to assess the deposition of caffeine within the follicles of hairless rat skin [53]. A different team was able fabricate a device that could directly quantify skin distributions of a radioactively labelled compound on histological sections [54].

Confocal laser scanning microscopy (CLSM) is a well-established, non-invasive imaging technique that has been used to derive high-resolution images of skin. The principal advantages of CLSM are good time-resolution, potential *in vivo* applicability, and multi-depth imaging parallel to the skin surface without a need for mechanical sectioning. In a key step, Bouwstra's group successfully extended CLSM so that it could be used to track the *in vitro* penetration of a fluorescent lipophilic model dye through fresh human scalp skin [55-57]. This system allowed online visualisation and quantification of the dye as it penetrated through the scalp tissue. Dye amounts in the hair follicles and other sites were determined by calculating relative fluorescent intensity values at each time point over different skin regions [56]. CSLM was used to follow the topical deposition of polylactic acid-based polymeric microspheres on human skin explants [58]. The fluorescent dye-loaded microspheres penetrated into half of all the observed vellus hair follicles, whereas in 12 – 15% of all follicles the particles reached a depth equivalent to the branching off of the sebaceous glands. CSLM was used recently to show that after topical application to porcine skin, polymeric nanoparticles did not penetrate beyond the superficial stratum corneum, although the particles showed some affinity for hair follicle openings [59].

Some workers have developed systems that work by combining two or more different technologies. An imaging technique that incorporates both CLSM and confocal Raman spectroscopy was described as a promising probe for quantitatively assessing the delivery of a compound to the hair follicle [60]. Lademann's group [61] used optical coherence tomography in conjunction with laser scanning microscopy

to track the delivery of a fluorescent dye to human hair follicles *in vivo*. This approach was used to distinguish between open hair follicle openings and those plugged with corneocytes.

7. Approaches for evaluating the role of sebum

A primary function of human pilosebaceous units is the synthesis and release of sebum, which is a fungistatic and bacteriostatic blend of short chain fatty acids. The secretion, formed by the disintegration of the sebaceous glandular cells, passes through ducts into the upper third of the follicular canal [62,63]. This creates a milieu rich in neutral, nonpolar lipids in this portion of the follicle. The human sebaceous glands take 3 weeks to produce sebum, but this varies with anatomical site and age-related hormonal activity. Nevertheless, typical excretion rates of 0.1 mg/(cm^2 h) leading to a skin surface content of 0.5 mg/cm^2 have been recorded. It is still uncertain whether secretion depends on environmental temperature [64].

The presence of sebum in the upper third of the follicular canal would be expected to affect follicular drug penetration greatly. However, there is contradictory evidence regarding sebum's effect on drug penetration rates, with both suppression and enhancement effects being claimed by different groups [65,66].

Clearly, the synthesis and testing of artificial sebum represents one way forward for investigating sebum–drug interactions. Zatz's group [67] formulated a surrogate sebum and used differential scanning calorimetry (DSC) to determine how chemical modifications affect the relative solid and fluid components of the mixture. Later, it was shown that DSC could be used to identify sebum-miscible vehicles that were deemed useful for delivering drugs preferentially to the sebaceous follicle [68]. Others examined the effect of alginic acid buffer on triethanolamine penetration through test tubes filled with synthetic sebum [69].

One of the problems in this area is that different groups have synthesised various different sebum-like mixtures, each having different compositions and properties. Stefaniak and Harvey actually documented 18 distinct synthetic sebum formulations that have been developed and tested over the years [70]. However, many of these compositions did not closely resemble real human sebum. Recently, Wertz [71] synthesised a new lipid mixture composed of 17% fatty acid, 44.7% triglyceride, 25% wax monoester and 12.4% squalene. **Table 1** presents a comparison between this mixture and real sebum. Not only was this formulation inexpensive to make and close to human sebum in composition, but it was also stable on storage for 6 months. Moreover, the lipids were miscible with moisture, thus simulating equilibration with sweat. Wertz proposed that this particular formulation could be utilised as a 'standardised' artificial sebum for follicular research purposes.

Perhaps one of the most relevant set of studies was published by Lu and colleagues, who initially considered drug partitioning from aqueous solution into artificial sebum because this process constitutes the first step in selecting sebum-targeting drug candidates [28]. The group initially formulated an artificial sebum that closely resembled real human sebum (see **Table 1**). Then, determinations were made of sebum–water partition coefficients for 12 compounds showing diverse chemical structures. Interestingly, it was found that sebum–water partition coefficients poorly correlated with octanol–water partition coefficients. However, a linear trend between the two parameters was identified when different 4-hydroxybenzoate straight chain series compounds were screened, thus indicating that sebum partition coefficients depend on both lipophilicity and chemical structure. Subsequently, the team measured the flux rates of a large number of candidate drugs through polycarbonate filter membranes loaded with surrogate sebum [27]. Again, screening of both diverse and homologous series molecules was undertaken. It was reported that sebum diffusion rates were mostly a function of the drug's lipophilicity and solubility, whereas acidity, charge, molecular mass and molecular orientation exerted minor effects. For the homologous series molecules, plots of log diffusion rate as a function of carbon chain length yielded a bell-shaped curve. The authors concluded that based on differences in skin flux and sebum flux data for each of the molecules, it was possible to choose those compounds that were most suitable for sebum-targeted delivery. Finally, the surrogate sebum used in the previous studies was compared with natural human sebum, hamster sebum and other artificial sebum-like mixtures [72]. In terms of DSC, NMR as well as the partitioning and diffusion behaviour of three model compounds, it was found that the group's developed artificial sebum was indeed the closest to real human sebum.

8. The follicular route in mathematical models

Over the years, numerous mathematical models have been developed for describing various facets of percutaneous drug delivery [73]. It is interesting to examine how different researchers have incorporated the follicular route into their models. Back in 1978, Wallace and Barnett computer-fitted penetration data describing methotrexate flux through hairless mouse skin *in vitro* [74]. This generated estimated values of several key model parameters. The authors demonstrated that methotrexate flux through modelled parallel penetration pathways was influenced by vehicle pH and the state of drug ionisation. Several years later, Keister and Kasting [75] identified the transport pathways of ibuprofen through excised human skin by mathematically analysing transient diffusion data. Calculations showed that the hair follicles contributed to ~ 25% of total drug absorption, with only the ionised form of ibuprofen permeating through the follicles.

One question that still remains unclear is whether follicular absorption essentially constitutes a stratum corneum-bypassing hydrophilic route or whether it is highly lipophilic owing to

the presence of sebum [65]. This contradiction is reflected in the mathematical models. So, for example, Mitragotri modelled drug absorption as occurring through up to four distinct permeation pathways [76]. In this scheme, follicular permeation was modelled as an aqueous shunt route that was preferentially selected by large hydrophilic drug molecules. By contrast, Ho considered the follicles to be filled with sebum [77]. He therefore treated the follicular route as an oil phase-loaded diffusion pathway in his probabilistic, transient, three-pathway model of percutaneous absorption.

9. Conclusions

Quantitative techniques based on comparing some type of 'follicle-free' and 'follicle-containing' skins have certainly been available for a long time. Some of these, such as the scar skin system, have been utilised fairly extensively, whereas others, such as the sebum-filled follicle system, have not. The original comparative technique involved the use of hairy rodent and hairless rodent skins. As discussed previously, this approach is probably not effective. The last few years have witnessed considerable interest in the follicle-sealing comparative technique, which is in many ways perhaps the simplest and most obvious method. The skin sandwich method is by definition an *in vitro* approach and it has several key limitations that have been mentioned. Yet the technique has been validated statistically, and it does have a certain mathematical elegance. Differential stripping has aroused considerable interest in recent years. Imaging-based techniques are continuously improving and the fresh tissue-adapted CSLM developed by the Bouwstra group offers great potential. Finally, the penetration and interactions of drugs through surrogate sebum systems have already started to attract major research interest.

10. Expert opinion

Table 2 lists some of the specialist follicular techniques discussed above and summarises key features of each. It should be noted that some of these techniques are applicable both *in vivo* and *in vitro,* whereas others are limited to *in vitro* only. It is also noteworthy that whereas differential stripping yields follicular drug amounts, all the other methods provide follicular flux data – although image-based analysis may provide both types of information. Out of the comparative-based methodologies, the selective blocking of follicles is probably the most reliable. In this model, the 'follicle-containing' and 'follicle-free' skins are closest in terms of continuous membrane structure. However, this particular technique needs further work in order to validate that the volume and relative distribution of manual skin coating does not vary between individual workers. Furthermore, the issue of polymer cracking and potential for capillary forces needs to be addressed. Continuing innovations and refinements to imaging approaches mean these are becoming increasingly rapid, quantitative, user-friendly and inexpensive.

Unfortunately, current literature reports do not allow us to focus on a single drug and reliably compare its follicular pathway contribution as measured by different methodologies. This is because negligible research has involved different follicular techniques performed on the same drug. Furthermore, even where different groups have applied distinct quantitative techniques to study the same drug, those groups tend invariably to formulate the drug in different vehicles, use different application protocols and test different skin sites. Another complication is that publications by their very nature tend to report positive effects selectively. So it is possible that many studies conducted over the years showed a negligible role for the follicular route, yet those observations were not published.

Many of the studies performed so far have involved the use of a single methodology and single drug. Consequently, there is sparse information about how a molecule's physicochemical properties influence its preference for the follicular route. An exception to this was the skin sandwich study carried out on seven candidate drugs representing a wide range of partition coefficients [1]. The parabolic trend identified thus far would need to be validated by extending the sandwich studies to more candidate drugs. Furthermore, it would be very useful to screen all these candidates with one or more of the other follicular methodologies. This point is particularly crucial because the sandwich approach is still regarded with scepticism by some workers. In particular, the model's lack of sebum and hydrated tissue status has been critiqued. In a sense, as no technique is perfect, using multiple methodologies and obtaining similar results or at least similar trends will tend to validate all the methods. If any technique provides data that are radically different from the others, then it is likely that that technique is probably significantly deficient for some reason. Also, results obtained from *in vivo* approaches would clearly be preferred to data derived from *in vitro* approaches. For all research, it would be important to use the same body regions throughout because it is known that follicular dimensions vary with site [41].

In recent years, evidence derived from the selective follicle-sealing approach has indicated that skin stretching may affect hair follicle morphology and thus follicular drug penetration. Although it is known that skin is elastic [78], how this relates to follicles is still poorly understood. Research is required to determine how the direction, strength and duration of the applied stretching affect follicular dimensions. Moreover, the reversibility or irreversibility of any induced dimensional changes needs investigation. A related aspect is whether and how such changes may affect the internal packing of sebum.

As mentioned previously, the Lademann group found evidence that human skin excision radically reduces the follicular drug reservoir size. Thus, it was proposed that the intact porcine ear skin actually offers a closer model to the clinical situation than does *in vitro* human skin. This particular idea still needs further investigation.

Table 2. Methodologies available for evaluating the follicular delivery of drugs.

Technique [example Ref.]	*In vitro*/*in vivo*	Limitation(s)
Rodent skins of different follicular densities [6-10,80,81]	Both	Skins may differ in other respects apart from follicles. Hairless rodents are not truly follicle-free
Follicle-free scar skin [11-15,82]	Both	Scarred and unscarred skins may differ in other respects apart from follicles
Newborn rat skin versus 5-day-old rat skin [11]	Both	Skins may differ in other respects apart from follicles
Sebum-filled follicles [17]	So far, *in vitro* only	Discharged sebum does not completely suppress the delivery of lipophilic drugs. Species specificity not yet determined
Selective sealing of follicular orifices [20-24]	Both	Requires specialised expertise. Fine cracks and capillary forces may occur in the polymeric follicular seal
Skin sandwich [1,25-28]	*In vitro* only	Hydrophilic drugs only. Intrafollicular fate undetermined. Skin is highly hydrated
Differential stripping [2,37-40]	Both	End point yields drug mass rather than drug flux data. Some evidence of poor *in vitro–in vivo* correlation
Skin imaging [43-52]	Both	Image interpretation may be quasi-quantitative. Signals strengths may reflect drug affinity for follicular structures rather than quantitative measurement of drug mass or flux

Apart from aspects relating to the follicles themselves, there is also the crucial question of the role of sebum. As has already been proposed by Wertz [71], it would be a good idea if a specific synthetic lipid mixture could be agreed on by all groups as a standardised artificial sebum for research purposes. *In vitro* screening could then be undertaken to elucidate the sebum partition and diffusion coefficients for a wide range of different drug molecules. As a secondary point, it should be mentioned that one neglected aspect in this area is the composition of porcine sebum. So far, this has been poorly documented. Given the plethora of porcine follicular studies, it would be a good idea if the composition of porcine sebum could be determined.

By combining the principles determined from the quantitative follicular models with those derived from artificial sebum studies, it should be possible to develop a new generation of mathematical models describing follicular drug delivery. These would relate drug physicochemical properties to the drug's preference for follicular penetration. The ultimate goal would be for such mathematical models to be sufficiently accurate so that they would give a reasonable indication of the follicular affinity of any given drug molecule. Finally, apart from free drug molecules, nanoparticulate-based drug delivery has attracted growing interest in recent years [79]. So, it is recommended that the proposed research methodology paradigms stated above should also be applied to nanoparticle systems.

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

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