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Transdermal drug carriers: basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides

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Abstract

In order to get across the intact mammalian skin, drug carriers must pass through a series of very fine pores with a diameter of typically less than 50 nm, under the influence of a suitable transdermal gradient. Well-known agent carriers, such as liposomes, fail to do this owing to their large (minimum) size. Special, composite carrying bodies called Transfersomes™, achieve this goal by virtue of their very high and self-optimizing deformability. (The efficiency of passage for 500-nm Transfersomes through pores of 100 nm diameter is as high as that of pure water, 1500 times smaller than the former.) When applied onto the intact skin surface non-occlusively, Transfersomes penetrate the skin permeability barrier spontaneously. Subsequently, they are distributed, probably via the lymphatic system, throughout the whole body. Drug exchange between the Transfersomes and the biological surroundings may occur at this or any later stage. This permits regio-selective drug delivery by means of Transfersomes. (Topically applied corticosteroids, for example, can be confined to the viable skin with an efficiency of 99.999%.) Meticulous optimization ensures the Transfersome-mediated flux of lipids to exceed 0.1 mg cm^{-2} h^{-1}, in the murine test system. Inulin, which has a low propensity for the association with Transfersomes, is carried across the skin less efficiently (10–20%). In spite of this, comparable serum values are measured 7 h after the epicutaneous or subcutaneous application of this compound in the form of Transfersomes. Combinations of peptides and Transfersomes provide a very successful means, however, for the non-invasive therapeutic use of such large molecular weight drugs on the skin. Transfersome-associated insulin is carried across the skin with an efficacy of $\geq 50\%$ (and often $\geq 80\%$), for example; human trials with a number of preparations and formulations have proven this. The results measured with insulin are claimed to be representative of all substances with high encapsulation efficacy into and/or association capability with Transfersomes.

1. Introduction

The delivery of large biogenic molecules such as peptides or proteins into the body is notoriously difficult. When applied orally in a solution, peptides are almost completely degraded in the gastrointestinal tract. When used in a degradation-preventing formulation, their uptake in the gut remains problematic and extremely inefficient; even with the best currently available formulations, the total uptake efficiency does not exceed a few percent. In addition to this, hepatic metabolism always causes an appreciable first-pass effect. These are the reasons why nearly all therapeutic peptides still have to be introduced into the body through an injection needle, in spite of the inconvenience of this method. Numerous attempts have therefore been made to find acceptable alternatives. In particular, the delivery of drugs across the skin or

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mucosa, has attracted a great deal of attention in recent years [1,2].

Currently, there are several products on the market, for example, which make use of the transnasal route for the delivery of intermediate size peptides for the therapeutic purposes; the transnasal desmopressin was the first and the calcitonin spray is perhaps the best known example of this.[1] This notwithstanding, it is as yet unclear how many transnasal peptide formulations will be introduced in the future or how successful they will be. The recent failure of several transnasal insulin developments calls for caution in this respect.

Compared to the skin permeability barrier, nasal mucosa is a rather small obstacle to the diffusion of drugs. This explains why the efficacy of peptide delivery across the skin is typically far lower than through the nose: normally, only a fraction of a percent of the applied drug amount gets across the intact mammalian skin, except for the selected low-molecular weight molecules. The use of supporting treatment, such as ultrasound [3], iontophoresis [4] or even electroporation [5] improves this situation somewhat. However, even the most efficient established methods for transdermal peptide delivery to date only achieve a transport efficiency of a few percent.

The reason for the difficulty of bringing polypeptides through the normal skin is clear: skin is our outermost protective shield against the detrimental environmental agents: consequently, it *must* be a good permeability barrier. In its outermost region, the horny layer or stratum corneum, human skin contains some 15–20 layers of dead or dying keratinized cells, corneocytes. These flaccid cells are put together like a wall: corneocytes are separated by narrow interstices which are largely filled up with lipids in a crystalline state [6]. Such 'brick-and-mortar' architecture of the outer skin layers effectively prevents transdermal drug permeation. Indeed, the interstices in the horny layer, which seldom exceed 20 nm in width, are extremely impermeable but to the smallest compounds. Even water trespasses the skin at the rate of 0.4 mg cm^{-2} h^{-1} only [7].

Attempts to lower the skin permeability barrier have been both numerous and inventive. They range from the application of high-voltage and laser-light pulses through to the abrasive action of fine sand; the overwhelming majority of skin penetration enhancing protocols, however, involve chemicals [8]. Such chemical enhancers typically increase the fluidity of lipids in the stratum corneum layer and thus increase the permeability of the skin to the superficially added agents. Solvents, such as dimethylsulfoxide [9], glycerol [10], azone [11], or highly concentrated surfactant solutions [12] are frequently used with this idea in mind. One of the difficulties with such chemical skin treatment is the danger of side effects upon chronic use [13]. Skin fluidizers, moreover, can at best facilitate the permeation of small agents across the intact cutis; it is as yet impossible to enhance significantly the transport of large drugs through the intact skin by chemical means.

In a step toward this goal, occlusive patches containing drug-laden lipid vesicles (liposomes) have been also used dermally (for reviews, see [14,16]). The initial rationale for this was not the same as it is today, however. Initially, liposomes were used in combination with classical penetration enhancers and were expected to act as excipients or a local drug depot [14]. It is, indeed, difficult to perceive why any liposome-drug complex should diffuse across the skin more easily than an individual drug molecule, as long as the material transport is only driven by drug or liposome concentration gradients. The question has only recently been asked why and when a liposome and/or liposome-mediated drug penetration across the skin should occur at all [15]. Furthermore, no consensus has yet been reached as to whether or not the epicutaneously applied liposomes really penetrate the intact mammalian skin to any significant extent [16].[2]

We have thus decided to address some of these problems within the framework of our concept of rational membrane design [17,18]. We also wish to explain the mechanism of the lipid vesicle penetration across the skin in some detail. Consequently, we have first attempted to identify the forces that can be made responsible for the process of vesicle penetration across the intact skin. We have next tried to determine those properties of lipid vesicles that have the highest impact

[1] According to a recent prediction, the growth of transdermal drug market will be with nearly 30% by more than a factor of two above the average in the pharmaceutical industry.

[2] Even when encouraging results were reported in the literature for dermally used drug-laden liposomes, the overall efficiency of the resulting transdermal material transport for large molecules was low, occasionally in the order of 1–5% and typically much less. Moreover, the published evidence in favor of intact liposome penetration has not been generally accepted.

on the efficiency of transdermal carrier penetration. Finally, it was our desire to use the knowledge gained for the design of innovative transdermal drug-carriers with unprecedented material transport efficacy, which we call Transfersomes™.[3]

Here we show that Transfersomes optimally fulfill the requirements for the efficient particle penetration across the permeability barriers, including the intact mammalian skin. We present some data which illustrate the high efficiency of Transfersome-mediated peptide delivery across such skin. We suggest that this delivery depends on the transdermal osmotic pressure difference and we show that it can reach efficiency higher than 80%. Last but not least, we argue that such very efficient material transport across the skin is not a consequence of the modified skin properties but rather depends on the penetration of the intact-but highly deformed-carriers across the essentially unchanged stratum corneum.

2. Materials and methods

2.1. Materials

Phosphatidylcholine from soya bean (SPC; purity ~98%) was either purchased from Nattermann Phospholipids (Cologne, Germany) or from Lipoid KG (Ludwigshafen, Germany). Sodium cholate and deoxycholate were from Merck (Darmstadt, Germany) and Rhodamine 123 was from Kodak (Rochester, NY). Desoxymetasone was supplied by Sigma (St.Louis, MO) and tritiurated radiochemicals ([^{3}H]inulin and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[^{3}H]trimethyl=dipalmitoylphosphatidylchlorine) by Amersham-Buchler (Braunschweig, Germany). ^{125}I-Insulin was a kind gift of Novo-Nordisk. With the exception of phospholipids, all chemicals were of the highest available purity and were used as received. All solvents were of analytical grade. Water (18 MΩ/cm) was doubly distilled in an all glass apparatus and reprocessed by a water purification unit (Elgastat HQ; Elga, UK) including filtration just before the preparation of the samples for the dynamic light scattering experiments.

[3] Transfersome and Transfersulin are registered trademarks of IDEA GmbH.

2.2. Animals

For all animal studies, NMRI mice were used. At the time of experiment they were typically 8–12 weeks old, weighed 32 ± 2 g, and were kept in cages in groups of 2–4 with water and chow ad libitum. Prior to epicutaneous treatment, the selected area on the mouse back was either plucked (on day −4, unless otherwise stated) or clipped (on days −2 and −1) free of hair. Each test animal was then numbered and placed into an individual cage.

All manipulations on animals were done under general anaesthesia performed with a mixture of Ketavet and Rompun (0.3 ml per mouse of an isotonic NaCl solution containing 0.0071% Rompun (Bayer, Leverkusen, Germany) and 14.3 mg/ml Ketavet (Parke-Davis, Rochester, USA).

2.3. Liposome preparation

Liposomes consisting of SPC and appropriate amounts of the radioactive or fluorescent labels were prepared by standard methods. In brief, an organic solution of the required lipid mixture was first dried under vacuum (10 Pa) overnight. The resulting lipid film was then hydrated with triethanolamine-HCl buffer (pH 6.5) to make a 10% lipid suspension. This suspension was sonicated for 60 min at 4°C until the desired vesicle radius was achieved. The latter was measured by the dynamic light scattering.

2.4. Transfersome preparation

Transfersomes were prepared as described in ref. [22]. In short, an ethanolic SPC solution was mixed with the appropriate amount of sodium cholate and the corresponding lipophilic label, when required. This solution (which typically contained 8.7 w% SPC and 1.3 w% cholate as well as approx. 8.5 v% ethanol) was mixed with triethanolamine-HCl buffer (pH 6.5, with the hydrophilic label, when appropriate) to yield a total lipid concentration of 10 w%. The resulting suspension was sonicated, frozen, and thawed (2–3×) and brought to the desired size (as measured by means of photon correlation spectroscopy) by ultrasonication or intermediate-pressure homogenization. Vesicle suspension was then sterilized by filtration through a 0.2 μm microporous filter (Poretics, CA, USA). The final

vesicle size was checked by the dynamic light scattering.

2.5. *Transfersulin preparation*

was based on a common pharmaceutical formulation of human recombinant insulin (HRI: Actrapid 100 HM or 45 HM; Boehringer-Novo, Mannheim, Germany). Starting insulin solution thus contained approximately 4 mg or 2 mg of polypeptide, and 3 mg (1.5 mg) *m*-cresol per ml. To prepare 1 ml of a final Transfersulin™ preparation, 0.84 ml of the original solution was mixed with 88 mg of SPC and approx. 12 mg sodium cholate or deoxycholate (p.a.) in 0.1 ml ethanol. (In experiments with Actrapid 45 HM, lipid concentration was halved and cholate concentration was increased by 10 mol%.) The resulting crude suspension was homogenized to yield a mixture of vesicles with a radius of approximately 90–110 nm. These transfersomes were sterilized by filtration through 0.22 μm pores (Sartorius, Göttingen, Germany) and used for experiments within less than a day. The final concentration of insulin in all transfersomal preparations was nominally 84 ± 2 IU/ml (or 40 IU/ml).

2.6. *Vesicle size determinations*

The dynamic light scattering (DLS) measurements were done in triplicate with a Zetasizer 2C instrument (Malvern Instruments, Malvern, UK). They were analyzed by the multi-tau procedure from the Autosizer software package.

2.7. *Permeability measurements*

These were done with a dedicated, home-built device (D. Gebauer and G. Cevc, to be published). In short, the flux of vesicle suspension through a large number of pores of known size (e.g. through a sandwich of different microporous filters (with pore diameters between 50 nm and 400 nm, depending on the starting transfersome suspension) was driven by an external pressure (originally 3 MPa, later typically ≥ 0.5 MPa and measured as a function of time. From this the relative permeation capability was calculated by using standard hydrostatic-flow expression. Calibration was done in comparison with pure water. DLS measurements just before and after each experiments were used to control the degree of filtration and the changes in particle size.

2.8. *Skin-penetration measurements with CLSM*

These were used to optically (this is, non-invasively) section the native skin samples. For this purpose, Transfersomes were labeled with 0.2 mol% of 1,2-dihexadecyl-*rac*-glycero-3-phosphoethanolamine-*N*-rhodamine (Molecular Probes, Eugene, OR) and then applied non-occlusively to the back of anesthetized NMRI mice. After 8 h, the superfluous material was washed from the skin surface with distilled water and the mice were then killed. Treated skin areas were excised and the subcutaneous tissue was carefully removed with a scalpel. The resulting, approx. 0.5-mm-thick, specimens were mounted onto the objective glass with the skin surface facing the lens. To take the CLSM pictures, a combination of Leitz microscope and Zeiss software was used at EMBL in Heidelberg by the courtesy of Dr. Ernst Stelzer.

2.9. *Animal trials*

Carrier formulations were applied on the skin surface with a pipette to give dose per area between 0.25 and 5 mg cm^{-2} (depending on the duration and type of experiment). They were smeared out uniformly over the pre-marked area and left to dry out. (In one experiment with inulin, the application area was covered with a piece of dry cotton material at $t = 30$ min.) Blood samples (~ 20 μl) were taken at given intervals by tail puncture. At appropriate time, mice were killed and skin specimen were first collected by stripping the application area five times with a Scotch tape. The remaining skin and other organs of interest were then cut out with a scalpel. ('Surface' values from 'inulin experiment' give the radioactivity counts obtained from the covering (cotton) material.) For the measurements with a beta-scintillation counter (Berthold, Wildbad, FRG), blood and tissue samples were discolored with 0.4 ml H_2O_2 and 0.2 ml $HClO_4$ at 80°C overnight. Subsequently, all probes were neutralized

with 0.2 ml CH_3COOH. After the addition of 10 ml Aquasol-2 (NEN-DuPont, Dreieich, FRG), total activity of the samples was determined.

2.10. *Human trials*

The biological activity of transfersulin preparations, to date, was checked on seven healthy volunteers. Although the inter-individual variability was observed to be quite large $\geq 100\%$ before the correction to insulin sensitivity), the biological responses for the similarly responsive individuals ($\leq 30\%$) as well the experimental reproducibility ($\leq 20\%$) were quite acceptable. A detailed report of the results of these investigations will be published separately, but representative examples measured with one healthy male volunteer (70 kg, age 40, normo-glycemic), fasting for 20 ± 2 h, are given below. Prior to each Transfersulin application, the blood glucose level was monitored for at least 40 min. For this purpose, 2.25 ml blood samples were drawn every 10–15 min through a soft intravenous catheter placed in one arm. After an initial period, appropriate amount of the transfersomal preparation was applied on the inner side of the contralateral forearm and smeared uniformly over an area of 10–20 cm^2. Care was taken to avoid any surface of skin with abrasions or other deviations from normal skin architecture. A few minutes after the Transfersome application, the skin surface appeared macroscopically dry; 60–90 min later, only faint traces of the Transfersome suspension were still visible.

The blood sugar concentration was determined by a standard glucose-dehydrogenase assay (Gluc-DH; Merck, Darmstadt, Germany). To ensure good blood flow, the catheter was flushed with 0.25 ml of heparin (2 500 I.U.) every 2–3 h. The first 0.25 ml of each sample were discarded, except when heparin was applied; in the latter case, the discarded volume was increased to 1 ml. Each specimen (2.5 ml) was split in three samples and each measurement was made at least in triplicate. This ensured the standard deviation of the mean never to exceeded 2 mg glucose/dl, the typical error being on the order of 0.5 mg/dl and thus smaller than the displayed data symbols.

C-peptide concentration in the serum samples was determined (by courtesy of Prof. H. Botterman) in the laboratory of the Department for Inner Medicine (Klinikum r.d.I., TUM) by a routine RIA-based procedure.

3. Results and discussion

3.1. *Vesicle characteristics and permeation capability*

The efficacy of trans-pore movement of Transfersomes is quite high: a suspension of Transfersomes with an average diameter of 500 nm can be transported through the pores 5 times smaller nearly as rapidly and efficiently as pure water, in spite of the 1500× size difference between the characteristic (molecular or vesicle) dimensions in both systems. The bottom panel of Fig. 1 illustrates this. Such a high penetration capability is only seen, however, when the stress suffered by Transfersomes (e.g. the flow-driving pressure) is sufficiently high. This is a key characteristic of Transfersomes, with roots in the self-optimizing capability of the Transfersome body or membrane.

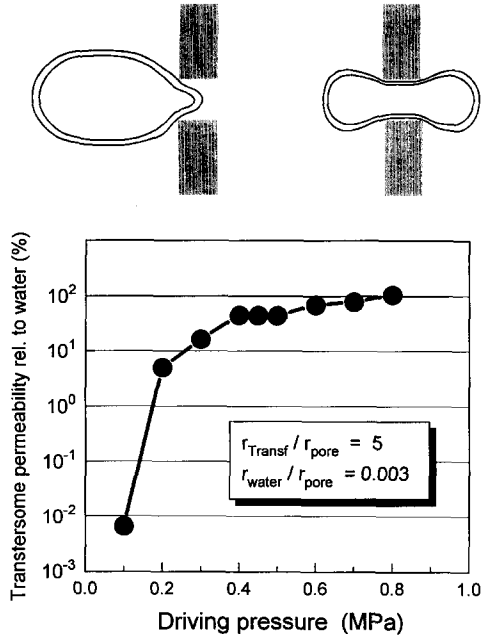

Fig. 1. Top: Schematic representation of a Transfersome approaching or passing through a pore. Bottom: Relative permeation capability of the ultradeformable lipid vesicles, Transfersomes, through the narrow pores under the influence of a trans-pore pressure difference. To obtain the plotted values, the measured permeabilities of Transfersomes were divided by the corresponding values measured with pure water.

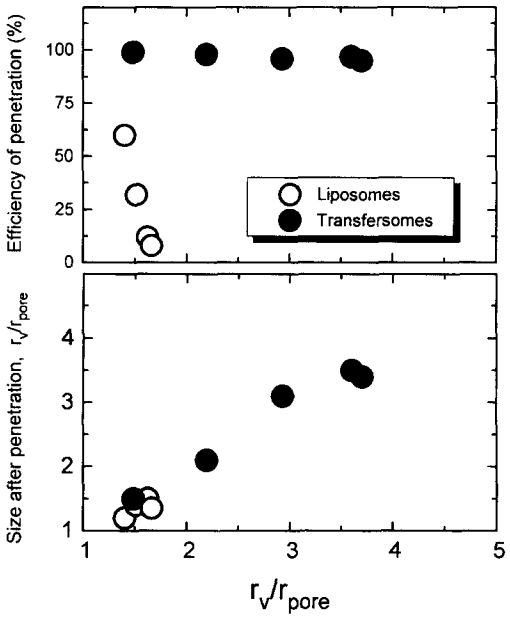

Fig. 2. Characteristics of penetration of liposomes (open symbols) or Transfersomes (closed symbols) with a radius r_v through the pores of various relative sizes, r_{pore}. Top: The efficiency of vesicle penetration through the pores. Bottom: The penetration (and deformation) induced changes in vesicle sizes. Owing to the vesicle and pore polydispersity, the given relative sizes are only accurate to within 50%.

In comparison to this, simple liposomes are far less deformable [18]. Consequently, they are also less capable of passing through the barriers with pores smaller than their own diameter. When liposomes are slightly larger than the pore diameter, sieving therefore sets in. Liposome exclusion from the flowing material is then complete when this mismatch exceeds a factor of approx. 1.7. Data shown in the upper panel of Fig. 2 demonstrate this. In contrast to this, the passage of Transfersomes through pores that are 'too narrow' is nearly perfect, even when their size exceeds the pore diameter by a factor of approx. 4 (see Fig. 2) or even 10 (not shown).

In principle, this difference could be due to the Transfersome fragmentation inside a pore, but data given in Fig. 2 (bottom) disprove such a speculation. The size of Transfersomes before and after the pore passage is nearly the same, unless their size is not only relatively but also absolutely quite large ($\geq$200–300 nm). The reason for this latter (composition dependent!) maximum size limit is not the insufficient Transfersome deformability, however. Rather, the fragmentation of large vesicles during the penetration step is indicative of the fact that many Transfersomes have an inherent tendency to be small [23]. Consequently, they tend to diminish their size and reach an optimum whenever they get an opportunity for doing this. Passage through constrictions offers such a chance to them.

The crucial feature of all transfersomal drug formulations, in comparison with the standard liposomes and other types of the drug-laden lipid suspensions, is the flexibility of the former: whenever required, a Transfersome can get more deformable than any other commonly used lipid vesicle by up to 5 orders of magnitude [23]. The reason for this is that the different components in each Transfersome are chosen so as to be able to accommodate to particle shape changes. In the Transfersomes described in this work, for example, bile molecules tend to accumulate at the sites of highest stress (and thus deformation). Owing to the higher propensity of such molecules for the highly-curved structures (e.g. micelles) this rearrangement diminishes the energy cost of particle deformation and thus promotes the Transfersome deformability. Transfersomes, consequently, undergo shape changes whenever such deformations are needed, this is, when such deformations are enforced by the surrounding stress or space confinements (Fig. 1: top). Ultra-flexible transfersomal carriers, consequently, can penetrate relatively easily even through the pores that are much smaller than their own diameter.[4] In doing so, Transfersomes can efficiently mediate the transport of such macromolecular or other large materials that are associated with the carriers across the pores. This justifies their name: 'carrying bodies' or Transfersomes.

3.2. *Vesicle penetration through the intact skin*

We have previously argued [15] that non-chemical gradients across the outer skin layers may enforce a flow of suitably designed lipid vesicles into or through the intact skin. The proviso for this is that vesicles are sufficiently deformable.

[4] Their precise permeation capability depends on the carrier-pore size mismatch as well as on the characteristics of the pore; application details also play a role. Comparison with water is slightly ($\leq$20%) less favorable than indicated in Fig. 1 when a strong micro-filter is used.

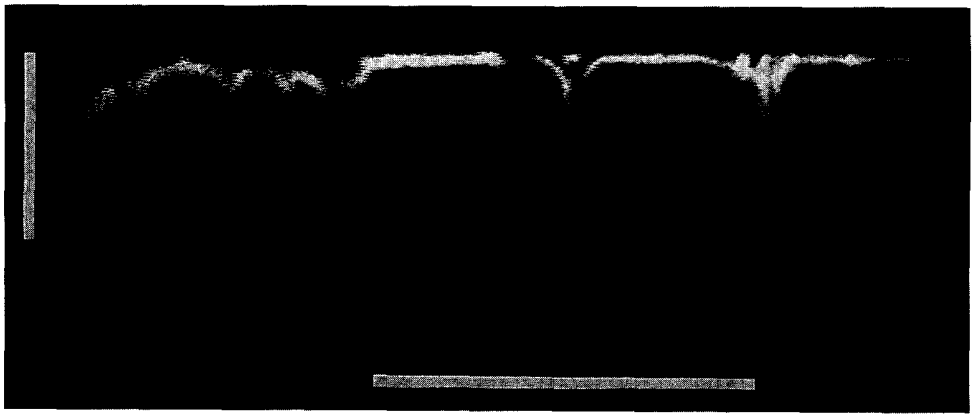

Fig. 3. Transfersome penetration into the intact murine skin, as visualized by means of the confocal scanning laser microscopy (CSLM) for the vesicles filled with a water-soluble marker, rhodamine 123. Highly brilliant skin surface is on the top and is seen to have deep invaginations at which the stratum corneum folds into the skin depth. Transfersome-mediated material transport reaches at least to a depth of 30 μm; fluorescence near the skin surface partly stems from the non-encapsulated fluorophores. Horizontal and vertical bars denote 50 μm and 100 μm, respectively.

One naturally occurring transdermal gradient is osmotic gradient. Such a gradient is created by the difference in the total water concentrations between the skin surface and the skin interior. When a lipid suspension is placed on the skin surface and partly dehydrated by the water evaporation loss, the lipid vesicles feel this gradient and try to escape complete drying by moving along this gradient. They can only achieve this if they are sufficiently deformable to pass through the narrow pores in the skin. Less deformable vesicles, including standard liposomes, are confined to the skin surface where they dehydrate completely and fuse. Lipid concentration gradient, according to our experimental data, is too weak to contribute significantly to the lipid flux into and through the epidermis. Occlusion thus prevents vesicle penetration into and through the intact skin, as it deprives the vesicles from their chief driving force.[5]

Phosphatidylcholine liposomes applied to the intact skin surface hence only show a very limited skin penetration capability. Consequently, they normally realize an intradermal lipid transport efficacy of less than 2% (in mice) only, if the rate-limiting stratum corneum is intact. The results for water soluble materials are even worse ($\leq$0.05%). In both cases, the depth of liposomal carrier penetration is more or less limited to the outermost stratum corneum layers [15].

In contrast to this, the ultradeformable material carriers, Transfersomes, may bring as much as $\geq$95% of the applied lipid mass into or through the normal horny layer of the murine skin, when applied non-occlusively [15]. Fig. 3 illustrates this for the Transfersomes loaded with a hydrophilic fluorescent marker, Rhodamine 123. This marker (381 Da) does not normally get into the skin in large quantities when applied in the form of a liposomal suspension (data not shown). When used in the form of a transfersomal suspension, however, this dye is transported extensively and reaches a depth of at least 30 μm to then also label the cells in the viable skin tissues (see Fig. 3). The transdermal fluorescence distribution profile is not uniform, however. The measured fluorescence intensity drops relatively abruptly at a depth of approx. 30 μm in the illustrated case and at depths between 10 μm and 15 μm in most other murine studies. This is due to the fact that Transfersomes (and their labels) are facing the nearly infinite sink below the stratum corneum, which results in extensive dye dilution and elimination through the lymphatic drainage system.

[5] The minimum force needed to push a vesicle through the pores in the skin permeability barrier depends on vesicle properties. Vesicles with an intermediate deformability, such as sub-optimal Transfersomes, may still get across the skin if they are driven by a very large force. In contrast to this, the even less deformable vesicles, including most of those commonly used, only become fragmented or else clog the pore when they are exposed to such very high transpore pressure differences. This happens, for example, with pure phosphatidylchloline liposomes that are pressed through the small pores, and provides a basis for certain liposome manufacturing procedures [24].

By means of Transfersomes, a comparably effective transfer is also accomplished for other, much larger, water-soluble agents such as peptides, polysaccharides and proteins. The total efficiency of this transfer for the water-soluble drugs is 10–90%, depending on the efficacy of drug partitioning into the Transfersome (see Ref. [25] and further discussion).

3.3. Transfersome distribution in the body

After having penetrated through the outermost skin layers, Transfersomes reach the deeper skin stratum, the so called dermis (cf. Fig. 3). From this latter skin region they are normally washed-out, via the lymph, into the blood circulation and through the latter throughout the body. If applied under suitable conditions, Transfersomes can thus reach all such body tissues that are accessible to the subcutaneously injected liposomes.

Fig. 4 corroborates this claim. It presents the biodistribution of the tritiurated phosphatidylcholine ([^{3}H]DPPC) in the body of test mice at various times after the epicutaneous and subcutaneous [^{3}H]DPPC-Transfersome application. The radioactivity derived from the Transfersomes injected subcutaneously (s.c.) are seen to be concentrated in the skin at $t = 8$ h but then to move gradually into the liver, the main 'target' organ of particulate materials, and to the kidneys, the termination point for the excretion of the degraded extraneous material.

The estimated total radioactivity in the muscle is also rather high. Its value first increases (accumulation) and then decreases (degradation?) with time. The specific radioactivity of the blood compartment is essentially constant, $3 \pm 0.7\%$, at $t \geq 8$ h.

Application of the same amount of identical Transfersome preparation to a similarly large area *on* the skin (e.c.) ultimately gives rise to a very similar biodistribution to an injection. In the beginning, however, notable exceptions are observed. In particular, the total radioactivity in the muscle and skin tissues (the latter including dermis as well as stratum corneum) show precisely the opposite time dependence to the radioactivity distribution after s.c. injection.

These data clearly indicate that Transfersome penetration through the skin is very efficient but preceded by a time-lag. Such time-delay must originate partly from the need for the excess water evaporation from the skin surface before the actual start of Transfersome penetration. The other cause of the time lag is the extra time for carrier passage through the skin. Nonetheless, 24 h after the Transfersome application, no significant difference is seen between the [^{3}H]DPPC-derived radioactivity biodistribution resulting from the injected or epicutaneously applied Transfersomes (Fig. 4, bottom panel).

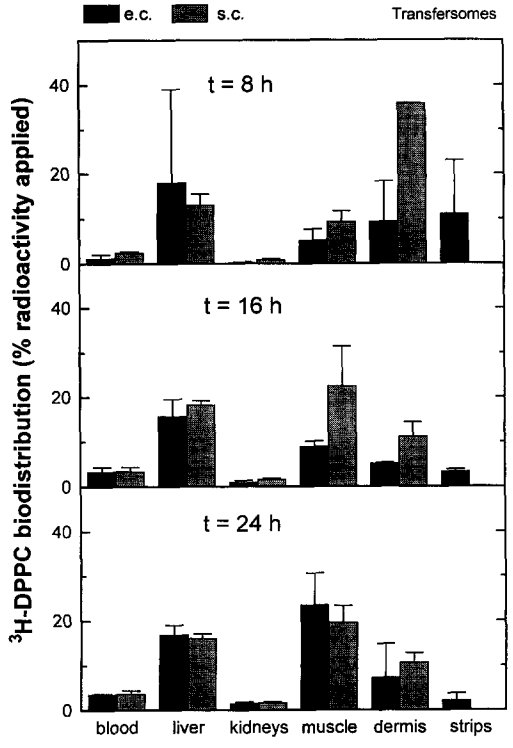

Fig. 4. Temporal dependence of the biodistribution of the carrier-derived [^{3}H]phosphatidylcholine radioactivity after Transfersome application onto the intact murine skin surface (e.c.) or after the injection of the corresponding preparation amount under the skin (s.c.). One day after the carrier application the biodistribution is the same whether Transfersomes were injected subcutaneously or applied epicutaneously.

3.4. Drug distribution in the skin and body

The precise reach, as well as the kinetics, of Transfersome penetration through the skin depends on the carrier type, total mass applied, and the detailed application conditions.

Agent or marker exchange between the carrier and its biological surroundings always plays an important role. In order to achieve an optimum carrier efficacy

and the best possible therapeutic results, one must therefore simultaneously consider and bring to perfection a number of the carrier system properties. This includes the distribution of pharmaceutical agents between the carrier and the body compartments, as well as the changing carrier composition and function during the course of penetration. Such multiple interdependences bring many formulation difficulties but also offer means for achieving special, and frequently quite desirable, drug distributions.

In general, the total reach of agents that are brought across the skin by means of ultradeformable carriers is affected by:

- Skin characteristics
- Carrier characteristics
- Drug-carrier interactions
- Drug-skin interactions
- Applied dose
- Application form
- Post-treatment

Agents with a low carrier affinity are more difficult to target into the selected tissue, of course. Such agents are also burdened with a lower efficiency of transfer across the intact skin.

Fig. 5 gives one example of this. It presents the data measured with inulin, a polyfructoside of approx. 5 kDa, which is entrapped only inefficiently into the vesicle interior. This results in formulations that contain most of the 'agent' outside the carriers. After epicutaneous application in the form of Transfersomes, such radiolabeled inulin is thus not co-transported through the skin: the non-encapsulated part remains on the skin surface and is recovered in the skin strippings; 70–80% of the inulin-derived radioactivity are thus found on the skin surface. Indirect support for this conclusion comes from the biodistribution data for the drug that was initially encapsulated into carriers. If one takes into account that some 10% of total inulin is entrapped in the Transfersomes, the calculated biodistribution data for such encapsulated material (insert to Fig. 5) are rather similar to those shown in Fig. 4 for the empty carrier distribution.

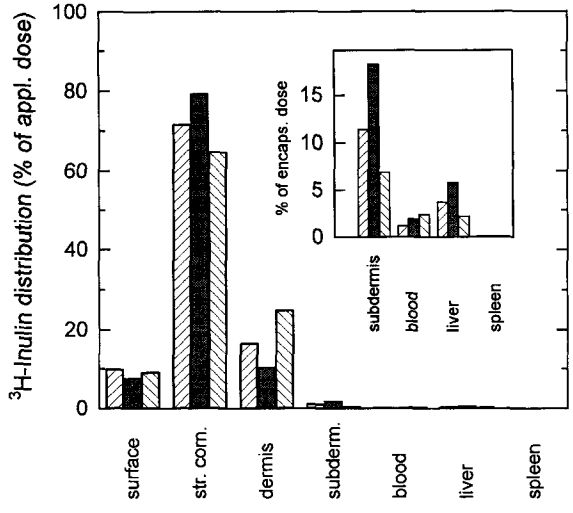

Fig. 5. Biodistribution of the [^{3}H]inulin derived radioactivity 8 h after the application of this polyfructoside (≈ 5 kDa) in three different Transfersome formulations onto the intact murine skin. Insert shows the corresponding tentative data for the encapsulated drug, which were calculated by multiplying the directly measured values by a factor of 10.

Another observation is also noteworthy in this context. The significant, albeit low, inulin-derived radioactivity measured in the liver provides circumstantial evidence for the conclusion that at least some of the inulin-carrying Transfersomes must have passed through the skin intact. If they fell apart in the skin inulin would have been liberated from the carrier interior. It would then have been excreted as a free substance, i.e., renally rather then hepatically, the liver being known to accumulate and process mainly particulate materials. The fact that the specific radioactivity in the liver is higher than in the blood provides further support to this claim.

Independent studies with CLSM provide further indirect support for the claim that some (in our oppinion most) of the dermally applied Transfersomes pass through the skin barrier intact: the distribution of two fluorescent labels (PE-NBD and PE-Rhodamine) incorporated into Transfersomes is essentially the same throughout the whole stratum corneum region [32]. If extensive vesicle fragmentation would have taken place, one would expect this no longer to be true.

Similar measurements also reveal the existence of 'virtual pores' in the skin, with an apparent, effective size of 30 nm (A. Schätzlein and G. Cevc, paper in preparation). This means that data given in Fig. 2, which prove beyond any doubt that Transfersomes are not fragmented in the artificial system, are also relevant for the skin situation. These data and the circumstantial information given in previous paragraph thus provide a rather strong evidence in favor of the claimed Transfersome integrity upon skin penetration. Further, and

more direct, information will be given in our forthcoming papers (D. Gebauer and G. Cevc, in preparation).

3.5. Kinetics of carrier penetration and drug distribution

The lag between the time at which epicutaneously applied or injected drugs appear in the body has been mentioned already. This lag, on the one hand, may be as short as 15 min, if rapidly exchanging agents, such as local analgesics, are looked for immediately after the skin permeability barrier [26]. On the other hand, we have also measured lag times of more than 6 h for the highly lipophilic molecules, which do not diffuse readily from the transfersomal carriers, when relatively inflexible vesicles were used.

In general, the kinetics of therapeutic action is a function of the velocity of the transdermal carrier penetration and of the speed of drug (re)distribution and action. The most important single factors in this process include:

- Carrier inflow
- Carrier accumulation at the target site
- Carrier elimination
- Drug release from the carrier
- Drug uptake by the target
- Biological action of the drug
- Drug elimination

Carrier elimination from the subcutis under the application site is mainly affected by the lymphatic flow. In contrast to this, the inflow rate and, to some extent, the accumulation of carriers in such a site depend on the characteristics of the skin, but also on the carrier membrane properties.

Some of the consequences of these latter two variabilities are illustrated in Fig. 6. The lower panel in this picture illustrates the effect of changing Transfersome properties on the time-lag for carrier penetration across the skin: the appearance of inulin radioactivity in the blood of test animals as a function of time is used to monitor this. The corresponding data reveal that carriers of higher deformability get into the blood compartment earlier than more rigid Transfersomes.[6] The

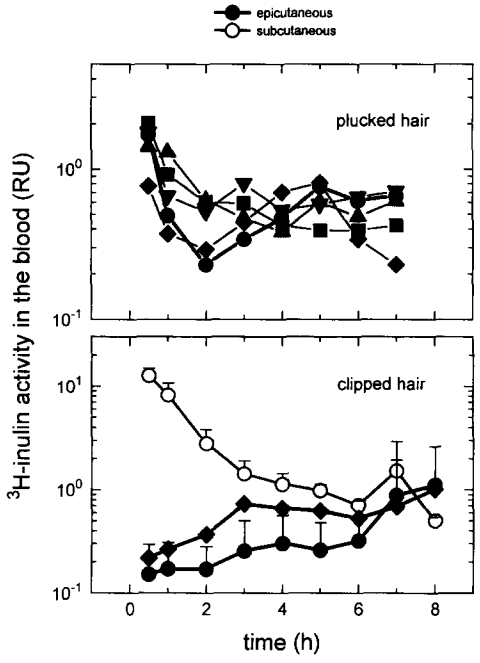

Fig. 6. Time dependence of the [^{3}H]inulin derived radioactivity in the blood of test animals after the application of marker-carrying Transfersomes onto the murine skin plucked (top) or clipped (bottom) free of hair (solid symbols). Open symbols give the corresponding time profile of radioactivity concentration after the subcutaneous injection of Transfersomes. (To facilitate data comparison, all results in the lower panel were normalized such that the highest value measured after an epicutaneous Transfersome application was set to 1.)

former thus also lead to a faster system equilibration. While the first type of drug carriers gives rise to the drug concentrations in the blood that are similar to those mediated by a subcutaneous injection within 3 h, the less flexible Transfersomes reach the same goal after only 7 h.

Increasing skin permeability has similar, but on the whole less potent, effects. This is seen from the data shown in upper panel of Fig. 6. These data have been measured in experiments in which the backs of test mice have been plucked, rather than clipped, free of hair 24 h before the Transfersome application. The empty hair follicles in the former case appear to have acted as permeation shunts. They have thus lowered the skin permeability barrier: pulsatile appearance of the drug-derived radioactivity in the blood during the first 2 h of an experiment is diagnostic of this.

Such shunts in the skin have long been known [27]. Unfortunately, even ample shunts found in hairy ani-

[6] While, at first glance, it appears that the measured difference is insignificant in light of the relatively large experimental errors, the statistical test shows the two curves to differ significantly at the level of $P = 0.05$.

mals only let a few percent of the dermally applied hydrophilic or small amphiphilic agents into and across the skin.[7] Their permeability for the more lipophilic substances is very low, however. Such shunts thus do not provide a viable basis for the transdermal agent delivery.

In contrast to this, the epicutaneously applied Transfersomes can deliver drugs with a much higher total efficiency at nearly constant rates. This is certainly true after the steady-state conditions are established. Comparison of the blood concentration values achieved after epicutaneous or subcutaneous applications (see Fig. 6: bottom) suggest this to be true even for inulin, which has a rather low overall transport efficiency. The efficacy of transdermal transport by means of Transfersomes for the other, more relevant molecules, such as polypeptides, is much higher. Next section gives evidence for this.

3.6. Therapeutic potential of Transfersome formulations

Hitherto, only the results of experiments with radiolabels were described. In order to assess the therapeutic potential of various drug formulations on the basis of Transfersomes it is also important to know how efficient are the epicutaneous applications of such formulations. In this section we thus describe the results of a few representative trials in humans with insulin, with a molecular mass of approximately 6 kDa, as a test drug.

Insulin normally does not penetrate the skin. Even when used in the combination with iontophoresis the relative efficiency of this drug transport across the intact stratum corneum is $\leq 5\%/h$, in the best case, and normally lower than 3%/h [19]. Part of this effect may even be due to the skin damage [20].

The performance of Transfersomes as insulin carriers is much higher than this. The efficacy of drug transport across the intact skin may reach 50%, and often more than 80%, when mediated by properly optimized Transfersomes. Fig. 7 and Refs. [22,28,31] provide ample evidence for this.

Specific data from one human experiment are given in Fig. 8. They were achieved by using 30 IU of transfersomal insulin, Transfersulin, epicutaneously. Such

[7] Our value of approx. 1% of the totally applied drug is in qualitative agreement with this.

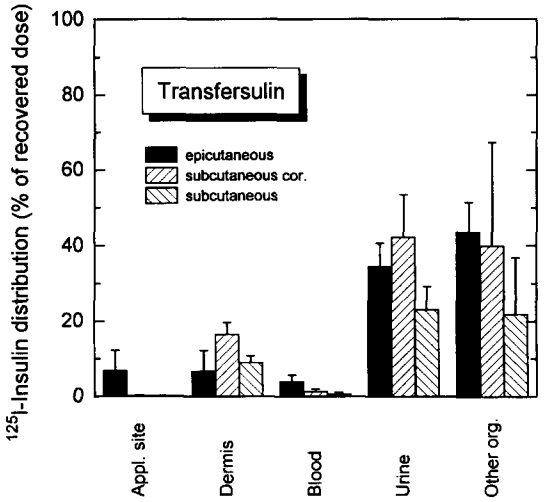

Fig. 7. Biodistribution of the radioactivity derived from ^{125}I-insulin applied with Transfersomes either on the intact skin (e.c.) or subcutaneously (s.c.). Total measured radioactivity was $95.7 \pm 7.9\%$ and $54.7 \pm 15\%$, for the e.c. and s.c. applications, respectively. s.c. values (subcutaneous cor.; middle columns) are presented in the corrected, normalized (to 100%) form.

an insulin amount is seen to have lowered the blood glucose concentration relative to the starting value (84.6 ± 3.5 mg/dl) by approx. 20% within 3–4 h (Fig. 8: lower panel). This corresponds to a time lag of approx. 150–180 min, which is similar to the values measured with radioactive labels (see Fig. 6).

After this period of time, the presence of transfersulin on the skin keeps the blood-sugar concentration constantly lowered by more than 15 mg/dl. This effect, according to Fig. 8, persists for at least 10 h (Fig. 8: top). The Transfersulin-induced hypoglycemia is also accompanied by a reduction of the native insulin production, as indicated by the concomitant reduction of the c-peptide concentration in the serum (Fig. 8: top). The parallel temporal profiles of the glucose and c-peptide concentration permit the conclusion that the observed effects are, indeed, caused by the applied Transfersulin.

For a healthy subject this is a remarkable glucose-lowering action: a subcutaneous injection of 0.215 IU/kg of the ordinary, non-encapsulated insulin (Actrapid HM) lowers the blood glucose level in the same test person by approximately 23 mg/dl. Moreover, this effect, which sets in approximately 45 min after the insulin injection, only lasts for 3–4 h (not shown). Likewise, a subcutaneous injection of the transfersomal

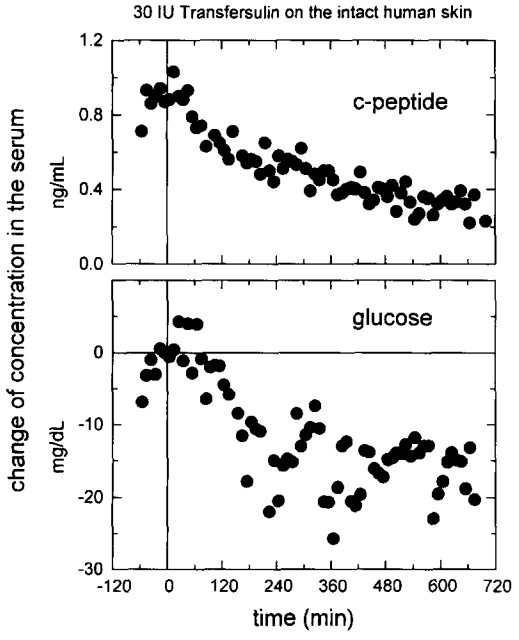

Fig. 8. Bottom: Transfersulin induced change in blood glucose concentration as a function of time before and after an application onto intact skin of 0.425 IU insulin per kg body weight in Transfersomes containing 8.7 w% SPC and 1.3 w% deoxycholate as well as 8 V% ethanol. Top: Corresponding c-peptide concentrations in the blood samples, as measured by means of specific radioimmunoassay; these values are indicative of the body's own decreased production of insulin.

preparation with 0.115 IU/kg of insulin diminishes the blood glucose level by approx. 10 mg/dl for just 2 h [28].

The overall efficacy of the dermally applied insulin is strongly affected by the detailed carrier design and composition. Take, for example, the blood glucose level measured after a topical application of various insulin vehicles. Simple insulin-loaded lipid vesicles, liposomes, for example, fail to elicit a measurable hypoglycemia for at least 5 h after the epicutaneous application [28]. Skin pretreatment with a solution of the surfactant micelles and subsequent use of standard liposomes yields equally poor results [22]. The use of particulate carriers or the manipulation of skin-lipid fluidity thus do not suffice per se for bringing peptides efficiently across the skin. If this were the case, standard liposomes should be as good insulin carriers as the Transfersomes of comparable dimensions. In fact, they are not.

The situation with lipid suspensions containing suboptimal phospholipid-surfactant mixtures is not much better. A suspension of lipid vesicles with 66 mol% phosphatidylcholine and 33 mol% deoxycholate and a total of 0.425 IU/kg of human recombinant insulin causes no hypoglycemia [28]. The preparation containing a total of 25 mol% deoxycholate is also almost inactive: 0.425 IU/kg of insulin in such 'Transfersomes' can only change the blood glucose-concentration by ≤ 3 mg/dl, which is an insignificant amount [28].

Systemic hypoglycemia caused by the insulin-containing Transfersomes thus cannot be primarily due to the detergent-mediated skin fluidization, similar in origin to the effect of transnasal detergent-containing preparations [21,29]. If it was, the biological response should increase with the bulk desoxycholate concentration [30]. This is precisely the opposite of what we observe [22].

Drug pharmacokinetics in Transfersulin experiments is seen to depend somewhat on the formulation details. But the temporal evolution of hypoglycemia is always well behaved and reproducible. Different dermal preparations of the same basic form of transfersulin, moreover, induce hypoglycemia after comparable delay times. All data points measured with one test person but different transfersulin preparations give a nearly linear dose-action line in the dose range between zero and 0.5 IU/kg [22].

4. Conclusions

Transfersomes are specially optimized particles or vesicles which can respond to an external stress by rapid and energetically inexpensive shape transformations. Such highly deformable particles can thus be used to bring drugs across the biological permeability barriers, such as skin. When tested in artificial systems, Transfersomes can pass through even tiny pores (100 nm) nearly as efficiently as water, which is 1500 times smaller.

Drug-laden Transfersomes can carry unprecedented amounts of drug per unit time across the skin (up to 100 $\mu g\, cm^{-2} h^{-1}$). The systemic drug availability thus mediated is frequently higher than, or at least approaches, 80–90%. The biodistribution of radioactively labeled phospholipids applied in the form of Transfersomes after 24 h is essentially the same after an epicutaneous application or subcutaneous injection

of the preparation. When used under different application conditions, Transfersomes can also be positioned nearly exclusively and essentially quantitatively into the viable skin region.

Such a high efficacy of skin passage by Transfersomes is quite reproducible, even when carriers are loaded with the peptidic molecules with a molecular weight of several kDa. Increasing the carrier deformability, however, does speed up the skin penetration by Transfersomes.

Transfersomes thus offer a singularly good opportunity for the non-invasive delivery of small, as well as medium and large sized drugs. The results of the first human trials with the epicutaneously applied transfersomal insulin support this conclusion. The transfersulin-induced systemic hypoglycemia in these experiments is found to reach approx. 30% of that induced by subcutaneous insulin injections the cumulative efficiency over a period of 12 h being comparable ($\geq$ 75–100%) in both cases. The former has a much slower action, however. Several unrelated Transfersulin formulations were proven to be active hypoglycemically when applied on the intact skin.

References not mentioned in text: [21,31]

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