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Research paper

Nanoparticles – An efficient carrier for drug delivery into the hair follicles

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Abstract

The penetration and storage behavior of dye-containing nanoparticles (diameter 320 nm) into the hair follicles was investigated. The results were compared to the findings obtained with the same amount of dye in the non-particle form.

In the first part of the experiments, the penetration of the dye into the hair follicles was investigated in vitro on porcine skin, which is an appropriate model for human tissue. It was found that the nanoparticles penetrate much deeper into the hair follicles than the dye in the non-particle form, if a massage had been applied. Without massage, similar results were obtained for both formulations.

Subsequently, the storage behavior of both formulations in the hair follicles was analyzed in vivo on human skin by differential stripping. Using the same application protocol, the nanoparticles were stored in the hair follicles up to 10 days, while the non-particle form could be detected only up to 4 days.

Taking into consideration the surface structure of the hair follicles, it was assumed that the movement of the hairs may act as a pumping mechanism pushing the nanoparticles deep into the hair follicles.

Keywords: Particles; Hair follicles; Percutaneous penetration; Reservoir function; Drug delivery

1. Introduction

The knowledge of the penetration efficacy of topically applied substances into and through the skin is important for the development and optimization of cosmetic products and drugs. Whilst in the last decades, the penetration of topically applied substances through the skin barrier (stratum corneum) was assumed to be diffusion inside the lipid layers surrounding the corneocytes [1–3], recent investigations have attributed that the hair follicles perform a significant part in skin penetration [4–7]. Several in vivo and in vitro investigations have revealed a significant influence of the hair follicles on the penetration process [8–11]. Feldmann and Maibach [8] showed higher absorption rates in skin areas with higher follicle density. Hueber et al. [9] and Tenjarla et al. [10] found a decrease in percutaneous absorption of appendage-free scarred skin compared to normal skin.

Barry [11] explored a novel in vitro technique by comparing drug delivery through epidermal membranes with

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penetration through a sandwich model, assuming that the top epidermal membrane essentially blocks all shunts in the lower membrane.

Furthermore, the hair follicles represent an efficient reservoir for topically applied substances, which is comparable to the reservoir of the stratum corneum on a number of body sites [12]. The highest volume of hair follicles can be found on the scalp, calf and forehead regions. In contrast to the reservoir of the stratum corneum, which is located in the uppermost cell layers of the horny layer (approximately 5 μm) [13], the reservoir of the hair follicles is usually extended deep into the tissue up to 2000 μm [14]. Here, dendritic cells and a close network of blood capillaries surround the hair follicles. Whilst the storage of substances in the stratum corneum is rather short, due to its high turnover, the reservoir of the hair follicles can be depleted only by penetration into living tissue, or by leaving the hair follicles, with sebum flow and active hair growth [6]. Actually, the hair follicles appear to be an efficient long-term reservoir for topically applied substances; especially particles play an important role in follicular penetration [6,14]. If the size of the particles is higher than 5 μm, they do not penetrate into the lipid layers of the stratum corneum but only into the infundibula of the hair follicles [15]. TiO_2 particles with a diameter of approximately 100 nm, often used in sunscreens, penetrate into the hair follicles but do not pass out of the follicles into living tissue [16]. Additionally, 100-nm particles do not penetrate into all hair follicles, but it must be distinguished between "open" and "closed" hair follicles. Closed hair follicles are covered by a plug, which can easily be removed by cyanoacrylate surface biopsy or by peeling [17,18]. Toll et al. [14] used this procedure to open all follicles of excised human skin samples, and investigated the penetration efficacy into the hair follicles of fluorescent dye-labeled microspheres in different sizes (1.5–0.75 μm). Small particles at a diameter of approximately 750 nm penetrated most efficiently into the follicles, a process which could even be enhanced by mechanical massage of the particles into the skin.

The aim of the present investigation was the comparison of the efficacy of the penetration and storage of substances in particle and non-particle form into hair follicles. Penetration experiments were carried out using the fluorescent dye sodium fluorescein in vitro on porcine ear-skin, because of the necessity to analyze a high number of biopsies. Porcine skin is a highly suitable substitute for human tissue [19]. Subsequently, the storage behavior of substances in particle and non-particle form was investigated in vivo on human skin, using the recently developed method of differential stripping [20].

Additionally, the structure of the hair surface and the hair follicles was analyzed to explain the observed differences in penetration and storage behavior of particle and non-particle formulations.

2. Materials and methods

2.1. Formulations

The Department of Biopharmaceutics and Pharmaceutical Technology, University of Saarland, Saarbruecken, prepared two formulations based on the same hydrogel. The formulations of both, the particle and non-particle form, contained the same concentration of the fluorescent dye, sodium fluorescein. This food dye was used on account of its good fluorescent properties and efficient penetration into and through the skin, depending on the formulation [21].

Fluorescent particles: nanoparticles (average diameter 320 nm, PI 0.06) were prepared by Resomer® RG 50:50H (Boehringer Ingelheim, Ingelheim, Germany) and covalently labeled with 5-fluoresceinamine (Sigma Chemical Co., St. Louis, MO, USA).

The polymer dissolved in acetone was pumped into a 0.5% solution of polyvinylalcohol (PVA) Mowiol® 4-88 (Kuraray Specialities Europe GmbH, Frankfurt, Germany) and stirred during nanoprecipitation. Organic solvent was removed using a rotary evaporator. Particles were freeze-dried.

Fluorescent particle-containing hydrogel: a 1% suspension of fluorescein labeled nanoparticles (average diameter 320 nm, PI 0.06) was prepared in water. A 3% hydroxyethylcellulose hydrogel (Natrosol® type 250 M pharma, Aqualon, Duesseldorf, Germany) was prepared separately. The polymer was dispersed in water under vigorous stirring (800 rpm) until it was homogeneously distributed; later on, the polymer was allowed to swell under low speed stirring (100 rpm) overnight. Both preparations were mixed at a proportion 1:1 and shaken until a homogeneous distribution of the particles in the gel was obtained, resulting in a nanoparticle 0.5% w/w hydrogel.

Fluorescein-containing hydrogel: a 0.003% sodium fluorescein-containing hydrogel (equivalent to the fluoresceinamine amount linked to the polymer) was prepared by dissolution of the dye in water and addition of the polymer under stirring. Again, the preparation was stirred overnight at low speed (100 rpm) to allow swelling of the polymer. The preparation was stirred overnight until the polymer was completely swollen. Both gels showed similar viscosities.

2.2. Pre-treatment of volunteers/porcine skin

The in vivo experiments were performed on the calves of six volunteers, and the in vitro investigations on ear-skin of 12 freshly slaughtered pigs (6-month-old, German domestic pigs). The calves of the volunteers and the ear-skin of the pigs were prepared by washing, drying with soft tissue, and abscising the hairs; defined skin areas of 10 cm × 4 cm were demarcated using a permanent marker. Approval for this study had been obtained from the Ethics Committee of the Charité and from the Veterinary Board of Control,

Berlin, Treptow-Köpenick. The study was conducted according to the ethical rules stated in the Declaration of Helsinki Principles. The volunteers participating in the study had given their written consent.

2.3. Study designs

- Study design A: In vitro investigation of the follicular penetration depth of a topically applied dye in particle and non-particle form by analyzing biopsies.
- Study design B: In vivo analysis of the storage effect of the topically applied dye in particle and non-particle form by differential stripping.

2.4. Study design A

2.4.1. Application

Two microgram per centimeter square of each of the two formulations was applied homogeneously onto the ear-skin of 12 pigs. In the case of six pig ears, the formulations were massaged into the tissue for 3 min by means of a massage appliance (Massage Gerät PC60, Petra electric, Burgau, Germany). In the case of the other six pig ears, the formulations were applied lightly without massage.

2.4.2. Biopsies and microscopy

After a penetration time of 1 h, biopsies were taken from the pretreated porcine tissue, using 3 mm punch biopsies. Subsequently, the samples were frozen and histological sections were obtained. These were analyzed using a laser scanning microscope LSM 2000 (Carl Zeiss, Jena, Germany). The samples were measured in the transmission and the fluorescent modus. By superposition of the images, it was possible to investigate the distribution of the fluorescent dye inside the hair follicles as previously described by Toll et al. [14]. Fifty hair follicles from skin areas treated with the particle-containing formulation and 50 hair follicles from skin areas treated with the non-particle formulation were investigated. The penetration depths of the fluorescence were measured; mean values and standard deviations were calculated and compared.

2.5. Study design B

2.5.1. Application

Two microgram per centimeter square of both formulations was applied homogeneously on the calves of six volunteers. The formulations were massaged into the skin for 3 min by means of a massage appliance.

2.5.2. Differential stripping

After increasing penetration times of 24, 72 and 120 h, differential stripping was applied on adjacent pretreated skin areas of each volunteer as described previously by Teichmann et al. [20]. Therefore, the upper part of the stratum corneum, which serves as a reservoir for topically applied substances, was removed by tape stripping as described by Weigmann et al. [22]. A roller was used to press the adhesive film (*tesa* Film No. 5529, Beiersdorf, Hamburg, Germany). During the rolling movement, the skin became stretched and the adhesive film was in contact with the flat skin surface. In this way, the influence of the furrows and wrinkles of the skin surface on the tape stripping procedure could be avoided [23]. Fifteen to 20 tape strips were removed from the skin. The tape strips were checked using a laser scanning microscope, in order to ensure that the fluorescent dye had been removed completely from the stratum corneum and was located only in the orifices of the hair follicles. Subsequently, a drop of superglue (UHU GmbH & Co. KG, Brühl, Germany) was placed on the surface of the treated skin and covered with a glass slide under slight pressure. After polymerization (approximately 2 min), the cyanoacrylate was strongly linked with the upper layers of the stratum corneum, the hair shafts and the content of the follicular infundibula and was removed with one quick movement. After removal, the cyanoacrylate skin surface biopsies were checked microscopically to make sure that the dye was only located in the casts of the hair follicles.

Afterwards, the samples were punched to a constant size of 15 mm in diameter and were extracted in ethanol (Uvasol, Merck, Darmstadt, Germany) using ultrasound (Sonorex Super RK102H, Bandelin Electronic, Berlin, Germany) and centrifugation (at 4000 rpm for 10 min at 20 °C, Centrifuge MR1812, Jouan GmbH, Unterhaching, Germany). Then, the solvents were analyzed in the fluorescence spectrometer LS50B (Perkin-Elmer Instruments GmbH, Überlingen, Germany). The fluorescence of the dye was excited in the maximum of the absorption band at 450 nm; the fluorescence signal was detected in the spectral region from 520 to 650 nm. The intensity of the fluorescence signal was used as a measure for the concentration of the dye penetrated into the hair follicles.

2.6. Statistical analysis

Statistical analysis was performed with the software program SPSS® 12.0. Based on non-significant results ($p > 0.05$) of the Kolomogorov–Smirnov test, mean values and standard deviations were calculated using the software program Microsoft® Excel 2003.

In the case of study design A, the Wilcoxon test was utilized to analyze the penetration depth of the fluorescent dye in particle and non-particle form and with and without massage appliance affording a significance $p < 0.05$.

3. Results

3.1. Study design A

In the case of study design A, the follicular penetration depth of the topically applied fluorescein in particle and

non-particle form was determined by analyzing biopsies of porcine skin. In Fig. 1, the penetration of the fluorescent dye in the particle and non-particle form into the hair follicles of porcine skin, after the application of massage, is demonstrated by the superposition of a transmission and fluorescent image. The distribution of the fluorescent dye in the hair follicles is depicted in white. Fig. 1A shows the penetration into the hair follicles of the fluorescent dye in the particle form, whilst Fig. 1B demonstrates the penetration of the fluorescent dye in the non-particle form. Significant differences can be observed: the particles penetrate much deeper into the hair follicles than the non-particle form if massage is applied.

Fig. 2 represents the mean values and standard deviations of the follicular penetration depths of the fluorescent dye in particle and non-particle form. The results were obtained at an average on 50 hair follicles. The penetration depth was significantly deeper ($p < 0.05$), if the fluorescent dye had been applied in the particle form.

On the skin of six further pig ears, the same two formulations were gently applied to the skin without massage. Typical results can be seen in Fig. 3, where the superposition of fluorescent and transmission images are presented. The penetration depth of the two formulations is nearly identical ($p > 0.05$). The average penetration depth determined for the non-particle formulation was 300.74 ± 65.37 μm, for the particle formulation 297.45 ± 71.6 μm (Fig. 4). For both formulations, the penetration depth was significantly lower for non-massage appliance than in the case of a massage being applied ($p < 0.05$).

3.2. Study design B

In the case of study design B, the concentration of the fluorescent dye, applied in particle and non-particle form by means of massage, penetrated into the hair follicle infundibula of the calf region of volunteers, was analyzed

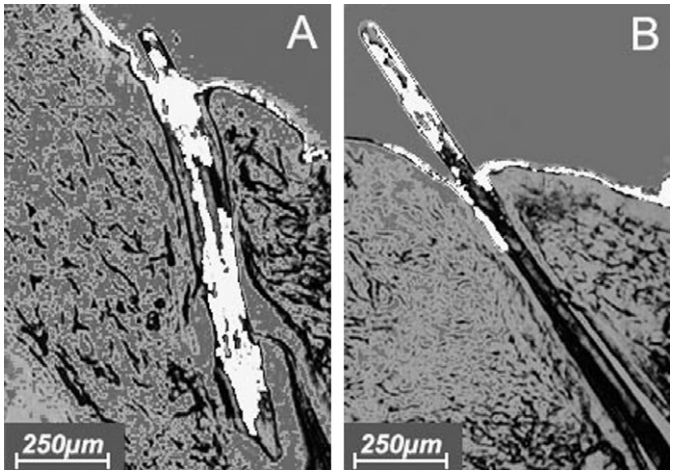

Fig. 1. Superposition of a transmission and fluorescent image, demonstrating the in vitro penetration of the dye-containing formulation into the hair follicles of porcine skin after application of a massage. (A) Dye in particle form. (B) Dye in non-particle form.

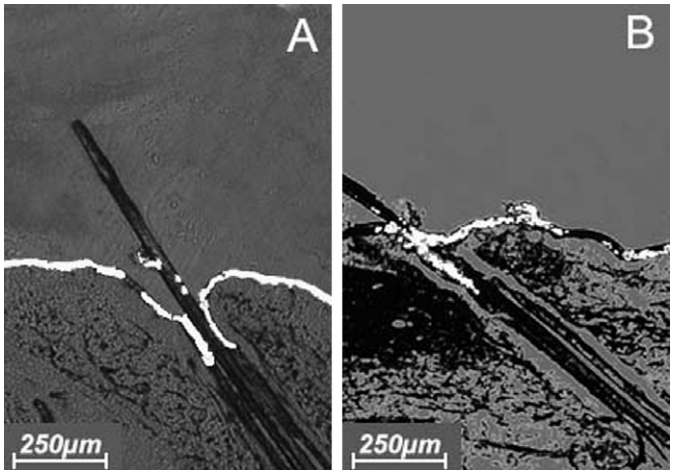

Fig. 3. Superposition of a transmission and fluorescent image, demonstrating the in vitro penetration of the dye-containing formulation into the hair follicles of porcine skin without massage. (A) Dye in particle form. (B) Dye in non-particle form.

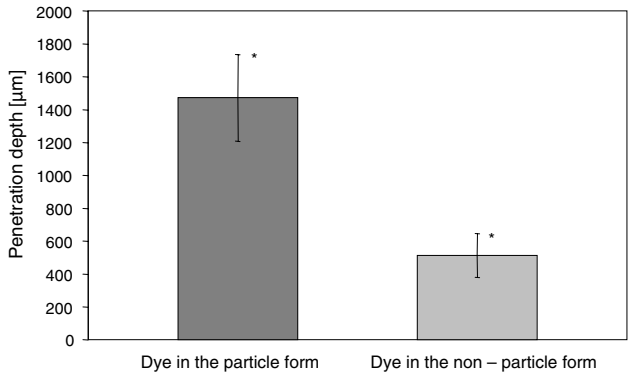

Fig. 2. Average penetration depth of the particle and non-particle containing formulations after massage appliance (porcine skin). Significant differences ($p < 0.05$) are indicated with *.

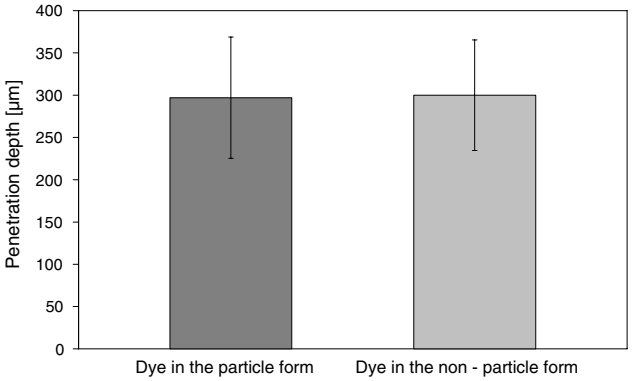

Fig. 4. Average penetration depth of the particle and non-particle containing formulations without massage appliance (porcine skin). Significant differences ($p < 0.05$) are indicated with *.

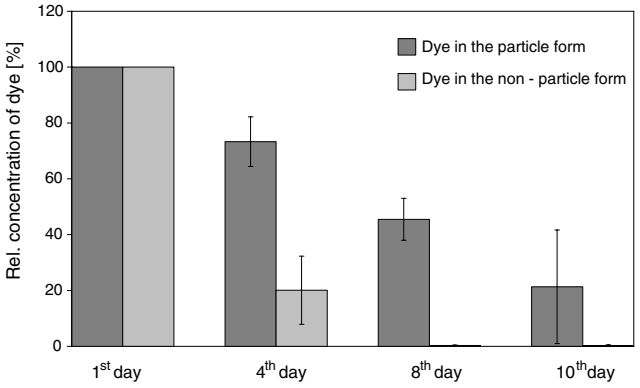

Fig. 5. Semi-quantitative determination of the fluorescent dye in the hair follicle infundibula by cyanoacrylate skin surface biopsy at different time points after application (calves of six volunteers, mean values and standard deviations).

quantitatively utilizing differential stripping. The concentration of the fluorescent dye was analyzed at different time points after application. The obtained results are summarized in Fig. 5.

As the absolute amounts of fluorescein detected after one day in the hair follicles of different volunteers varied by a factor of 3, the concentration values determined after one day were standardized to 100% for better comparison of the mean values and standard deviations. The concentration of the dye decreased in time. If the skin had been treated with the formulation containing the fluorescent dye in the non-particle form, the dye could be detected only up to 4 days in the hair follicles. In contrast, the fluorescent dye in the particle form could still be detected 10 days after application.

4. Discussion

Follicular penetration is an important and promising pathway for selective dermatotherapy. To optimize penetration and storage of topically applied molecules, innovative formulations are conceived. These approaches are mainly focused on nano or microsphere technology.

In the present study, we were able to demonstrate the superiority of particles versus non-particle formulations, not only for penetration but also for storage behavior. Interestingly, penetration of particle-containing formulations was enhanced by mechanical massage, reaching significant deeper penetration depths than without massage. However, without any mechanical manipulation on the skin surface, no significant differences between the two formulations were observed.

Regarding the storage behavior of human hair follicles, striking results could be obtained: the nanoparticles remained in the hair follicles much longer than the non-particle substances.

These results appear to be surprising, as it could be expected that the small amounts of non-particle substances, with their relatively small size, penetrate better into the small hair follicles than the much larger particles. The results obtained show the opposite effect, but only in the case of a massage being applied. Possibly, apart from fluid mixing, this effect can be explained by the structure of the hairs and the hair follicles, which are very similar in porcine and human skin. The stratum corneum extends deeply into the hair follicles. From the structure analysis of hair surface and hair follicles, it is known that the cuticle produced by keratinocyte desquamation forms a structured surface, which can be approximated by a zigzag relief [24]. This relief is determined by the thickness of the keratin cells, which is between 500 and 800 nm. If the hairs are moved by massage, the cuticle cells may act as a geared pump. Particles, comparable in size to the surface structure of the hairs and hair follicles, are probably pushed into the follicles by means of the pump movement of the hairs.

These findings are in agreement with the results obtained by Toll et al. [14], the microparticles with a diameter of 750 nm penetrated better into the hair follicles of excised human skin than larger particles, when a massage had been applied.

Under in vivo conditions, this assumed pump mechanism also occurs without a massage, on account of the continuous movement of the body, which is able to stimulate the pump mechanism of the terminal and vellus hairs. This in vivo movement is less than in the case of a massage, but it occurs continuously.

The in vivo storage experiments demonstrated that the penetration into the hair follicles is a fast process (1 h), in comparison to the release of the nanoparticles out of the follicles, which continues for some days. The penetration process into the hair follicles is determined by the concentration gradient of the topically applied substances. If the reservoir of the skin surface and the stratum corneum, as the source of the penetration into the hair follicles, is depleted by textile contact and desquamation, the sebum production seems to be mainly responsible for the penetration of the substances out of the hair follicles. The non-particle substances are quickly moved out by the sebum production, probably, because they are not inhibited by the surface structures of the follicles and the hairs, whereas, the movement of the particles out of the hair follicles is assumed to be retarded by the surface structure. Taking into consideration the experiments concerning the follicle penetration of TiO_2 microparticles [16], it can be expected that all particles at a size of >100 nm, which penetrate into the hair follicles, will also be moved out after some time on account of the sebum production not having reached the living cells.

5. Conclusions

Nanoparticles are well suited to penetrate efficiently into the hair follicles, reaching deeper functional structures, where they can be stored for some days. In the case of non-particle substances, such a long-term effect cannot be

observed, either in the hair follicles or in the stratum corneum. In principle, the stratum corneum is not suited as a long-term reservoir of topically applied substances, as these substances are mainly located on the skin surface or in the upper-cell layers after topical application [13]. These upper-cell layers are later continuously scuffed by desquamation. Therefore, the hair follicles are the only long-term reservoir for topically applied substances; consequently, they are important targets for drug delivery, as they are surrounded by a close network of blood capillaries and dendritic cells (Langerhans cells). Additionally, the hair follicles contain stem cells. Selecting the correct size of particles as drug carriers, thus, an efficient selective drug delivery and storage of topically applied substances into hair follicles is possible, which is important for selective dermatotherapy.

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