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Research paper

Increasing the percutaneous absorption and follicular penetration of retinal by topical application of proretinal nanoparticles

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ABSTRACT

Topical retinoids are frequently applied for therapeutic and cosmeceutical reasons although their bioavailability is low due to their chemical and photochemical instability. Moreover, skin irritation is a common side effect. Therefore, proretinal nanoparticles (PRN) as a novel formulation of topical retinoids, which are based on chitosan grafted with retinal through reversible linkage, were developed and their skin penetration behavior was studied. As nanoparticles preferably penetrate into the hair follicles, the follicular penetration depths of PRN at different time points were investigated. Moreover, the release capacity of the nanoparticulate system was studied using fluorescein as a model drug. Additionally, the concentration of retinal in the stratum corneum and in the hair follicles was quantified after application in particulate and non-particulate form. The results showed that the nanocarriers reached the infundibular area of the hair follicles, irrespective of the incubation time. The nanoparticles were able to release their model drug within the hair follicle. The retinal concentration delivered to the stratum corneum and the hair follicles was significantly higher when retinal was applied in the particulate form. In conclusion, the presented proretinal nanoparticle system may help to overcome the main problems of topical retinoid therapy, which are skin irritation, chemical and photochemical instability and low bioavailability, thus improving the topical retinoid therapy.

1. Introduction

Vitamin A and its derivatives (retinoids) are known as crucial natural regulators in skin proliferation and differentiation. In order to reduce the side effects related to systemic administration, such as teratogenicity, skin and mucous membrane dryness [1] which limit the use of retinoids in therapeutic purposes, topical application of retinoids is often the preferred method of administration for dermatological or cosmeceutical indications.

Retinaldehyde (retinal) is one of the natural intermediate precursors of retinoic acid, which exerts the biological retinoid effects [2]. Unfortunately, topically applied retinal is still irritative to human skin [3] and moreover, chemically and photochemically unstable. Topically applied retinal can induce a retinoid dermatitis [4]. This inflammation is induced by an overload of non-physiological amounts of exogenous retinoic acid in the skin [5]. Therefore, the aim is to eliminate this dose-related side effect and the instability of the chemical by developing a topical nanoparticulate controlled-release drug delivery system, which releases retinal continuously to prevent an excessive amount of retinal on the skin immediately after application. Polymer-based nanoparticulate drug delivery systems have already been considered and evaluated as topical carriers for different drugs [6]. Especially chitosan-based nanoparticulate drug delivery systems have been used as transdermal drug carriers [7]. They are assumed to provide several advantages in transdermal drug delivery via their mucoadhesive, permeation enhancement and controlled-release properties by the degradation of the polymer, which may enhance the drug bioavailability [8,9].

Next to dose-related side effects, also the penetration of topically applied retinal itself represents a challenge. In general, most topically applied drugs provide extremely limited bioavailability rates, which are often below 1%. The bioavailability for retinoids is also less than 1%

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after single topical application [10,11]. The low bioavailability of most topically applied drugs is due to the strong barrier properties of the skin and mainly the stratum corneum, the outermost layer of the skin. Three potential penetration pathways, in general, provide access to the skin: the intercellular penetration pathway [12], the follicular penetration pathway and transcellular penetration pathway [13]. Topically applied substances can try to overcome the skin barrier via the intercellular penetration pathway, which is located within the lipid layers that are surrounding the corneocytes of the stratum corneum or they can - at least theoretically - pass the skin barrier by intracellular penetration, which, however, is probably of minor importance. In addition, the hair follicles represent an interesting entry port into the skin, which is especially of importance for particulate substances. Previous studies could demonstrate that nanocarriers penetrate very efficiently into the hair follicles, which was hypothesized to be due to a mechanical transport mechanism induced by hair movement. Due to rhythmic movement of the hair in the hair follicle, the nanocarriers can be transported deeply into the hair follicle comparable to a ratchet mechanism. This effect was shown to be dependent, inter alia, on the size of the nanocarriers and on the frequency of hair movement [14].

Although nanocarriers show an effective follicular penetration, they are not able to overcome the stratum corneum directly due to their increased size. Already in 2000, the 500-Dalton rule had been established which clarifies that substances, which have a molecular weight more than 500 Dalton, are not able to penetrate via the intercellular pathway [15]. Nevertheless, there are several studies that report an increased penetration of topically applied substances delivered by particulate systems, although the particles are not able to penetrate themselves. Here, the concentration gradient and other, partially unclarified mechanisms seem to play the major roles [16,17].

By grafting retinal onto chitosan polymer, a biocompatible and bioabsorbable polymer derived from the natural chitin polymer, and inducing self-assembly of the grafted polymers, proretinal nanoparticles showed sustained release of retinal *in vitro* [18]. Abilities of the proretinal nanoparticles to produce physiological effects without skin irritation side effects had already been demonstrated both, in an animal model and also in human volunteers [18]. Nevertheless, the journey of the particles on skin, together with their location are unknown. Therefore, the aim of the present study was to investigate and quantify the skin and hair follicle penetration behavior of topically applied proretinal nanoparticles and the release of retinal from this system or of a model drug, respectively, in order to improve topical retinoid therapy in the future. It was hypothesized that proretinal nanoparticles penetrate very effectively into the hair follicles and might be able to increase the bioavailability of retinal in the skin.

2. Materials and methods

2.1. Materials

2.1.1. Nanoparticles

PRN: PRN was prepared as previously described [18]. Briefly, chitosan (CS, molecular weight of ~40,000–50,000 Da, Taming Enterprise, Samut Sakhon, Thailand) was dissolved in 0.1% acetic acid, and the pH of the obtained solution was adjusted to 5.9 using NaOH. The final solution contained 45 mg CS in 19.0 mL solution. Then retinal (15 mg in 1.0 mL of ethanol) was slowly added dropwise into the CS suspension at 5 °C in the dark, under ultrasonic (40 kHz) and N_2 atmosphere. After 2 h, the suspension was dialyzed against water under N_2 atmosphere and lightproof condition to obtain PRN suspension in water. The suspension was then freeze-dried. The size of PRN was 240.1 ± 29 nm.

Rhodamine labelled-PRN: 5(6)-Carboxytetramethylrhodamine (6.34 mg) was dissolved in dimethyformamide (DMF, 0.5 mL) at 0 °C under N_2 atmosphere. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 8.45 mg, Sigma Aldrich) was added to the solution and stirred for 30 min at 0 °C. Then N-hydroxysuccinimide (NHS, 3.5 mg, Sigma Aldrich) was added, followed with PRN suspension (120 mg in 20 mL water) and the mixture was stirred for another 4 h. The suspension was then dialyzed against water under N_2 and lightproof condition. Suspension in the dialysis bag was then freeze-dried.

Rho-Flu-Chitosan nanoparticles: On ice, 5(6)-carboxytetramethylrhodamine (4.0 mg) was dissolved in DMF (0.3 mL) under N_2 atmosphere. EDCI (6.0 mg) was added to the solution and stirred for 30 min. Then NHS (3.5 mg) was added, followed by N-succinylchitosan suspension (120 mg in 20 mL water) and the mixture was stirred for another 4 h before being dialyzed against water. Then the obtained suspension was stirred with the activated 5-carboxyfluorescein solution (prepared by dissolving 5-carboxyfluorescein (4.0 mg in 0.3 mL DMSO) and mixed with EDCI (6.0 mg) and NHS (3.5 mg) on ice) for 4 h and then dialyzed against water. The obtained suspension was freeze-dried.

RAL: Moreover, all-trans-retinal (RAL) (Sigma Aldrich, St. Louis, USA) was prepared in the solution as the same retinoid concentration corresponding to PRN and served as control.

2.2. Methods

2.2.1. Ex vivo skin preparation

The studies were performed *ex vivo* on porcine ear skin. Porcine ear skin is as an appropriate model for human skin [19] and very suitable for investigating the follicular penetration process as hair follicle size and density are well comparable. Other than excised human skin, porcine ear skin remains fixed to the cartilage during the experiments. Thus, any contraction of the skin and the hair follicles can be excluded [20]. Fresh pig ears (6-month-old German domestic pig) with no abnormal external appearances and no skin lesions were obtained from a local slaughter house in Niederlehme, Germany. The protocol for this study adhered to the ethical principles of the Veterinary Board of Control, Dahme-Spreewald. The porcine ears were cleaned and rinsed with cold water, dried with paper towels and fixed on a polystyrene board. The topical substances were applied to the well-demarcated skin areas using a silicon barrier (Marabu Window Color, Marabu GmbH, Bietigheim-Bissingen, Germany) to prevent any lateral spreading of the applied formulations from the designated skin areas. 20 µL per cm^2 of each formulation was applied to the skin areas, distributed homogeneously with 2 min of 50 Hz massage appliance (Novafon Pro soundwave appliance, Weinstadt, Germany) and incubated following the indicated penetration times following each application protocol at room temperature in a moisture chamber. Each experiment was performed on 6 independent pig ears. Due to the light sensitivity of retinoids, all experiments were carried out in a darkened room.

2.2.2. Application of topically applied substances

2.2.2.1. Experiment A: Determination of the follicular penetration depths of PRN. In experiment A of the study, the follicular penetration depths of PRN were investigated using 5(6)-carboxytetramethylrhodamine labelled-PRN for visualization by confocal laser scanning microscopy. Therefore, two skin areas of 2×3 cm^2 for each pig ear were demarcated as described above. One skin area was treated with PRN, the other skin area remained untreated and served as control. After the topical application and an incubation time of 2 h, skin biopsies were excised.

2.2.2.2. Experiment B: Determination of the time dependency of the follicular penetration depth and the release of the 5-carboxyfluorescein from the 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles. In experiment B of the study, 4 areas of 2 x 3 cm^2 were prepared as described above. The chitosan nanoparticles double-labelled with 5(6)-carboxytetramethylrhodamine and 5-carboxyfluorescein were applied to three of the marked areas. The fourth area remained untreated and served as control. Each of the three treated skin areas was designated to have a different penetration time of

2, 4 or 24 h, respectively.

2.2.2.3. Experiment C: Modified differential stripping technique to estimate the amount of retinal in the stratum corneum and the hair follicle after topical application of RAL and PRN. In experiment C of the study, three areas of 4 × 3 cm^2 were marked. PRN and RAL were topically applied and one skin area remained untreated and served as control. The incubation was determined to be 4 h according to the deepest follicular penetration depths in experiment B and according to the previous in vitro study determining the release efficacy of free retinal from chitosan nanoparticles, which was approximately 60% at this time point [18].

2.2.3. Analytical methods

2.2.3.1. Investigation of follicular penetration depths (experiment A and B). In experiment A and B, skin samples were processed after the different incubation times as follows: full thickness skin biopsies were cut into probes of 5 × 5 mm in size using a surgical blade. The subcutaneous fat tissue was removed and the probe was immediately fixed with cryospray (Solidofix-cryospray, Carl Roth, Karlsruhe, Germany), snapped in liquid nitrogen and kept in −20 °C. Subsequently, cryosections of hair follicles of 10 µm thickness were prepared using a cryostat (Microm Cryo-Star HM 560, Microm International GmbH, Walldorf, Germany). At least 10 hair follicles per investigated skin area were prepared.

2.2.3.2. Confocal laser scanning microscopy. The hair follicle sections were examined with confocal laser scanning microscopy (CLSM700 Zeiss, Oberkochen, Germany) without additional tissue processing. Laser excitation wavelengths of 488 and 555 nm were used to scan the hair follicle sections. Untreated skin samples were used as controls to avoid any autofluorescence measurement.

The CLSM provided dual-colored images of the fluorescent signals of 5-carboxyfluorescein ($\lambda_{ex/em}$ = 488/518 nm) and 5(6)-carboxytetramethylrhodamine ($\lambda_{ex/em}$ = 555/580 nm), respectively. The images were studied using 100x magnification. Once the images were obtained, follicular penetration depths were measured in micron for each formulation using ZEN 2012 software program (Carl Zeiss, Oberkochen, Germany). The release properties of 5-carboxyfluorescein dye from the chitosan nanoparticles in experiment B were also calculated as a percentage of the number of hair follicles that had deeper follicular penetration depths of 5-carboxyfluorescein than 5(6)-carboxytetramethyl rhodamine at each time point against all hair follicles of that time point.

2.2.3.3. Modified differential stripping protocol (experiment C). In experiment C, the modified differential stripping protocol according to Knorr and colleagues [21] was used to estimate the amount of retinal penetrating into the stratum corneum and into the hair follicles after topical application of RAL and RPN. Based on the assumption that most of the topical applied retinal is located on the skin surface and in the stratum corneum, the stratum corneum was removed by 70 tape strips. Subsequently, split skin of 600 µm in thickness was removed with an electronic dermatome (Acculan 3Ti Dermatome, Aesculap, B Braun, Tuttlingen, Germany). As tape stripping did not show any penetration of retinal into the inferior part of the stratum corneum, it was assumed that retinal extracted from the remaining split skin can only originate from the hair follicles.

The tape stripping procedure was performed as described by Weigmann and colleagues in 1999. For the described experiment, adhesive tapes of a width of 18 mm (TESA film No.5529, Beiersdorf, Hamburg, Germany) were used. After incubation of the topically applied substances, tape stripping was started. Seventy consecutive tapes were collected from each skin area. Immediately after tape stripping, the split skin was removed.

2.2.3.4. UV/Vis spectroscopy and extraction protocol. At first, the transmission spectrum was determined for each removed tape strip by UV/Vis spectroscopy (Perkin Elmer Lambda 650 S, Uberlingen, Germany) with an empty tape in the reference beam. The absorption measured at 600 nm was used as measure for the mass of the corneocyte aggregates placed on the individual tapes [22]. Subsequently, all tape strips, and the tiny pieces of dermatomized skin were extracted in ethanol. Therefore, the tapes were cut to a size of 1.5 × 3 cm^2. Each single tape strip of No. 1–10 and the dermatomized skin were placed in a single test tube, which was filled with the amount of 3.14 mL ethanol (Ethanol UVASOL, Merck, Darmstadt, Germany) as extraction solvent. The tapes 11–70 were placed, each 5 consecutively together, in a test tube avoiding any overlapping and adhering to the test tube and other tapes. Then, all tubes were ultrasonicated for 10 min (Sonorex Super RK102H, Bandelin Electronic, Berlin, Germany) and centrifugated at 4,000 rpm for 10 min at 20 °C (Hettich® Universal 320/320R centrifuge, Sigma Aldrich, St. Louis, USA). After the extraction, the absorption spectra of retinal were recorded on the UV–visible spectrometer at 25 °C in the range of 250–500 nm using a quartz cuvette with 10 mm path length (Quartz Suprasil, Hellma Analytics,). The band maxima of the retinal is at 380 nm. The concentration of free retinal in the extracted tapes and the dermatomized skin was calculated using the standard reference curve, which had been prepared of retinal standards. The results obtained from the PRN and retinal were compared with the untreated skin areas as control.

2.2.4. Statistical analysis

To analyze the data, unpaired *t*-test for comparison of two pairs of samples and Kruskal Wallis One-way ANOVA were used to investigate the differences between the groups (GraphPad Software, San Diego, California, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Experiment A: Determination of the follicular penetration depths of PRN

The follicular penetration depth of 5(6)-carboxytetramethylrhodamine-labelled PRN was determined by confocal laser scanning microscopy. The red fluorescent signal emitted by the topically applied PRN was detected in the hair follicles and also on the skin surface. In total, 60 hair follicles from 6 independent skin samples were analyzed, the mean follicular penetration depth was 411 ± 60 µm (max = 725; min = 209) (Fig. 1A). No red fluorescent signal was detected in untreated skin areas (Fig. 1B).

3.2. Experiment B: Determination of the time dependency of the follicular penetration depth and the release of the 5-carboxyfluorescein from 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles

The CLSM images of the hair follicle cross sections after topical application of 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles loaded with 5-carboxyfluorescein after different incubation times are depicted in Fig. 2. The fluorescence of 5(6)-carboxytetramethylrhodamine could be visualized at laser line 555 in red and the fluorescence of 5-carboxyfluorescein at laser line 488 in green. In the right column, a merge of both fluorescence signals is demonstrated. The untreated skin showed no fluorescent signal. The corresponding penetration depths of the 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles, of the 5-carboxyfluorescein and of the PRN are summarized in Fig. 3. Both figures demonstrate that 2 h after topical application, the follicular penetration of 5-carboxyfluorescein (445 ± 21 µm) was deeper than that of 5(6)-carboxytetramethylrhodamine (408 ± 25 µm). Therefore, it can be assumed that 5-carboxyfluorescein has been successfully released from the 5(6)-carboxytetramethylrhodamine-labelled nanoparticles. After 4 h of incubation time, the deepest follicular penetration of 5(6)-carboxytetramethylrhodamine (474 ± 31 µm) and of 5-carboxyfluorescein

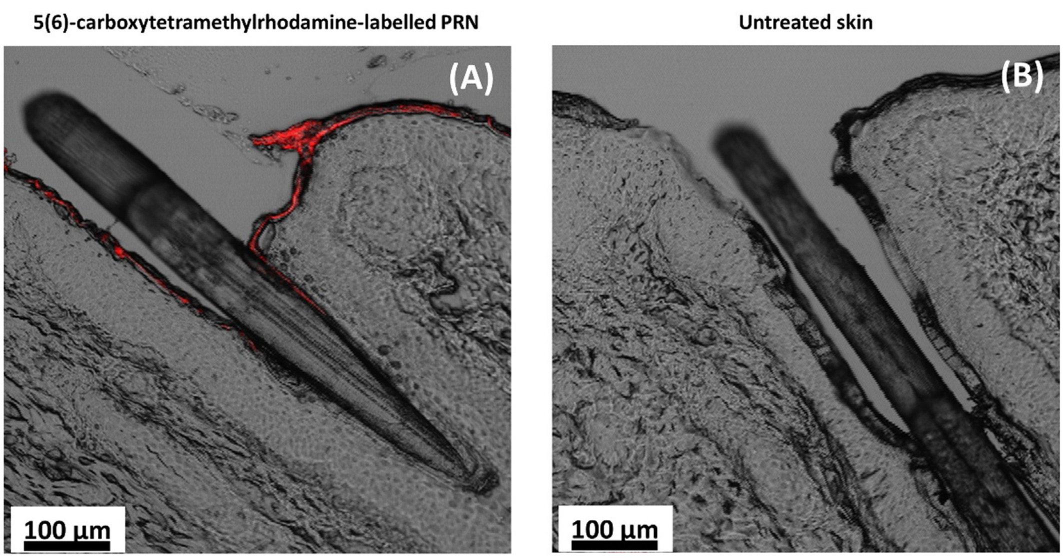

Fig. 1. Representative hair follicle cross sections showing the follicular penetration of 5(6)-carboxytetramethylrhodamine-labelled PRN (A) and untreated skin as control (B). The distribution of the red fluorescent signals reflects the PRN distribution in the hair follicle after 2 h.

(509 ± 34 µm) was detected. After an incubation time of 24 h, 5(6)-carboxytetramethylrhodamine (369 ± 28 µm) and 5-carboxyfluorescein (413 ± 28 µm) showed the lowest follicular penetration depths. The follicular penetration depths of 5-carboxyfluorescein were significantly deeper than those of 5(6)-carboxytetramethylrhodamine at every time point for the majority of the hair follicles (2 h: 80%, 4 h: 70% and 24 h: 87%) ($p < 0.05$).

3.3. *Experiment C Modified differential stripping protocol*

After topical application of PRN and retinal, the concentration of retinal was quantified in the stratum corneum and in dermatomized skin as shown in table 1 and Fig. 4. As the tape stripping revealed only very low or absent concentrations of retinal in the middle and lower stratum corneum, respectively, the modified differential stripping technique was applied analogously to a previous study of Knorr and colleagues in 2013. This technique is based on the assumption that if no substance is found on the tape strips removed from the lower stratum corneum (as could be demonstrated for PRN and retinal, see Fig. 4E and F), the concentration found in the residual epidermis and dermis highly probably originates from the hair follicles. Therefore, the concentrations of retinal extracted from the dermatomized skin are referred to hereinafter as follicular content.

The results revealed that the retinal concentration was higher in the PRN than in the retinal-treated group, which was statistically significant for tape 1 and the follicular content ($p < 0.05$) (Fig. 4A–C). Although the concentration of retinal in tapes 2–10 and tapes 11–70 of the PRN-treated group was not significantly different to the retinal group, there is still a clear tendency for a higher concentration in the PRN group. After 70 tape strips, the cumulative concentration of retinal of PRN group was 3-fold higher than in the retinal group (Fig. 4D).

The penetration profile of PRN in Fig. 4E demonstrates that retinal is located approximately in the upper 75% of the horny layer. In contrast, the retinal from RAL is located only in the upper 25% of the horny layer (Fig. 4F).

4. Discussion

The usefulness of retinoids is widely recognized in dermatology for the therapy of acne and other retinoid responsive disorders, but also as an anti-aging cosmeceutical. Due to its susceptibility, the development of topical retinoid preparations requires improvement in stability and controlled release properties. Therefore, the utilization of particulate drug delivery systems seems to be a promising approach as nanoparticle systems can provide a better chemical and physical stability of drugs and moreover, offer a sustained release. Unfortunately, nanoparticles are rarely able to overcome an intact skin barrier directly, but they have been described to penetrate very efficiently into the hair follicles and to improve skin absorption of actives by maintaining a concentration gradient and by other partly still unclarified mechanisms [16,17].

Previous studies clearly demonstrated that the follicular penetration mechanism is mainly mechanically driven and comparable to a ratchet [14]. Due to the movement of the hair, whereby movement direction and frequency also play significant roles, the nanoparticles are transported deeply into the hair follicle. This process is facilitated if nanoparticle size and the thickness of the cuticula cells, which cover the hairs, are comparable in size. In an earlier study it was shown that, by selecting a specific particle size, different target sites within the hair follicle can be reached. For nanoparticles with a diameter of 643 nm, the follicular penetration depth was significantly deeper than for nanoparticles of smaller or larger sizes. Whereas the 643 nm nanoparticles reached follicular penetration depths of more than 1000 µm, smaller or larger particles remained only in the infundibulum. The authors could reproduce their findings for different particle types [23]. In the present study, nanoparticles of a size of about 240 nm were utilized. The deepest follicular penetration depth for PRN was around 470 µm after an incubation time of 4 h, meaning a penetration down to the inferior infundibulum part of the hair follicle. When comparing the data of the present study with data obtained for the PLGA particles with a comparable size [24], the 230 nm PLGA particles penetrated approximately 800 µm into the hair follicle. The detected differences could be due to an aggregation effect of the particles used in the present study, which can affect the penetration behavior [25]. Nevertheless, it can be stated that the target site of interest for retinal in the therapy of acne, which is the infundibulum, can be successfully reached in order to affect the main problems of acne, which are inflammation and abnormal keratinization [26]. If deeper areas of the hair follicle have to be targeted in further clinical indications, probably other vehicles have to be selected to avoid the aggregation effect and to enhance the follicular penetration. Actually, the slightly different follicular penetration depths can also be due to different vehicle formulations as has been reported by Patzelt et al [23]. The PRN were prepared as an aqueous suspension, which might support the aggregation effect [25]. Patzelt et al. also reported that the same type of particles penetrated slightly deeper into

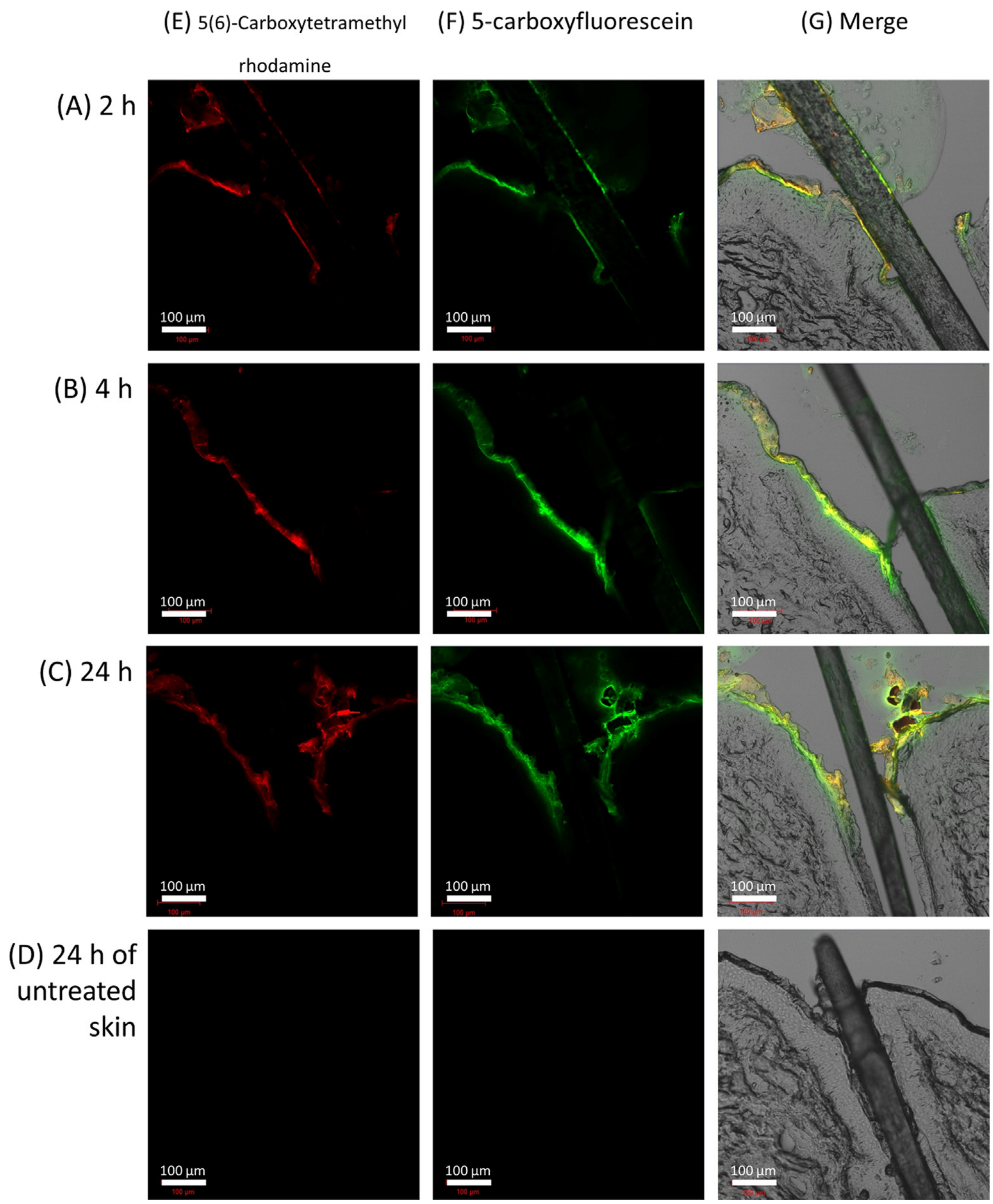

Fig. 2. CLSM images of hair follicle cross sections prepared at different time points ((A) 2 h (B) 4 h (C) 24 h) after topical application of 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles loaded with 5-carboxyfluorescein. Column E presents the distribution of 5(6)-carboxytetramethylrhodamine in the hair follicles, column F the distribution of 5-carboxyfluorescein. Column G is the merge of both fluorescent signals and the transmission mode. The last row represents hair follicle cross sections of untreated skin. Here, no fluorescent signal was detectable.

the hair follicles when applied in gel formulation than in aqueous suspension [23].

Double fluorescent-labelled chitosan nanoparticles were used to investigate the time dependency of the follicular penetration depth and the potential release of the model drug fluorescein. Although many studies have succeeded in encapsulating retinoids into nanocarriers [27,28], especially the follicular penetration of these nanocarriers at different time points has not been investigated so far to our knowledge.

For the investigation of the potential release, retinal was replaced by 5-carboxyfluorescein for the visualization of the release by confocal laser scanning microscopy. Although 5-carboxyfluorescein is not able to replace retinal completely as the chemical bond to the chitosan nanocarriers was changed from imine to ester bond for fluorescein, both nanocarriers (PRN and double-labelled chitosan nanocarriers) at least provided the same follicular penetration depth at 2 h. Therefore, it can be concluded that the follicular penetration behavior of both particle types is comparable.

Therefore, the fluorescent signal of 5(6)-carboxytetramethylrhodamine was used to study the penetration behavior of the nanocarrier and the fluorescent signal of 5-carboxyfluorescein was used to study the release properties of the nanocarriers. Due to the solubility of chitosan, chitosan nanoparticles possess pH-dependent drug release [29]. The skin pH could control the grafted active substance sustainably releasing from the chitosan nanoparticles. The sustain release character of the chitosan nanoparticles at the skin pH was hypothesized to deliver the grafted active substances on chitosan nanoparticles after single application [18]. Although the ester linkage between 5-carboxyfluorescein and chitosan is not as labile as the imine linkage between retinal and chitosan, ester bond hydrolysis is expected in hair follicles due to the lipase presence in the sebaceous glands and in the external root sheath of the hair follicle [30,31]. Faster release of 5-carboxyfluorescein as compared to that of the 5(6)-carboxytetramethylrhodamine is likely the result of a more hydrophobicity of the latter comparing to that of the former. Therefore, here the release of 5-

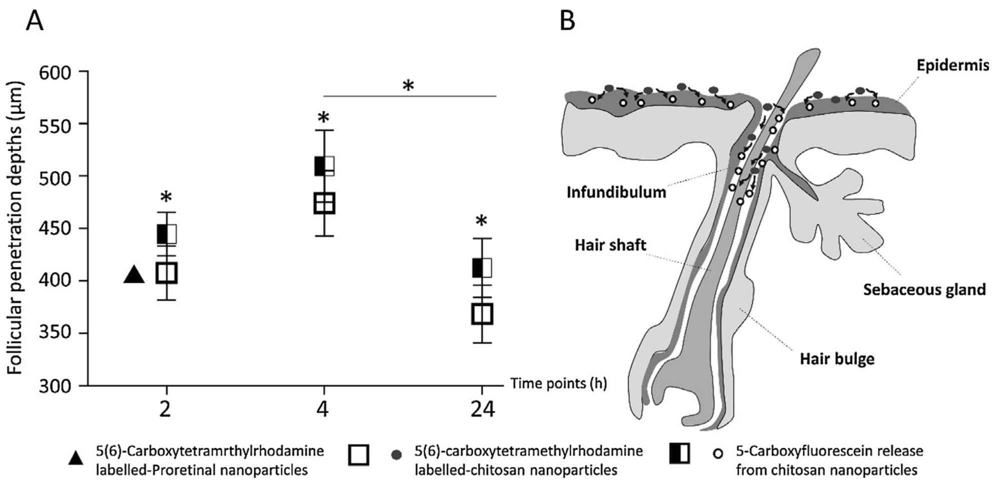

Fig. 3. (A) Presentation of the mean follicular penetration depths in µm of 5(6)-carboxytetramethylrhodamine and 5-carboxyfluorescein, at different time points. The black triangle represents the follicular penetration depth of 5(6)-carboxytetramethylrhodamine-labelled PRN after 2 h of incubation. The box plot graph shows the mean follicular penetration depth of 5(6)-carboxytetramethylrhodamine at different time points (which was linked to the chitosan nanoparticles). Due to the significantly deeper follicular penetration depths of the model drug 5-carboxyfluorescein (*$p < 0.05$), it can be assumed that 5-carboxyfluorescein has been released from the 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles. (B) Schematic illustration of a hair follicle and follicular penetration depths for 5-carboxyfluorescein dye as a drug model release (clear dots), 5(6)-carboxytetramethylrhodamine as the indicator for penetrated particles (black dots) and 5(6)-carboxytetramethylrhodamine-labelled chitosan nanoparticles 2 h after topical application. After the investigated time points, chitosan nanoparticles and PRN were found mainly in the infundibulum of hair follicle.

carboxyfluorescein was expected to be representative of drug release from the penetrated particles in hair follicles. The follicular penetration depths of 5(6)-carboxytetramethylrhodamine and 5-carboxyfluorescein were significantly different at all time points, whereby 5-carboxyfluorescein could be detected approximately 50 µm deeper inside the hair follicle than 5(6)-carboxytetramethylrhodamine. Therefore, it can be assumed that the model drug fluorescein was transported into the hair follicle by the nanocarrier, then it was released from the nanocarrier within the infundibulum of the hair follicle, as expected, and subsequently, penetrated deeper into the hair follicle independently from the nanocarrier.

The follicular penetration depths of the chitosan nanoparticles were found to be not time dependent. There was no significant difference in the follicular penetration depths after 2 h and 4 h, meaning that the maximum penetration depth can already be reached at early time points. Based on the in silico data [14], it can even be assumed that the maximum follicular penetration depth in ex vivo skin models can be reached already within a couple of minutes during the massage application as the results suggest that the follicular penetration process has to be mechanically stimulated. As previous studies [18], however, demonstrated that more than 50% of the release occurs after 4 h, this incubation time was selected. Surprisingly, the follicular penetration depth was shown to be significantly reduced after 24 h. This effect is probably due to a reduction of the fluorescence signal of 5(6)-carboxytetramethylrhodamine and 5-carboxyfluorescein over time as a retrograde penetration cannot be expected in an ex vivo skin model. For the in vivo situation, it is known that the particles are transported out of the hair follicle by sebum flow and hair growth, which are lacking in ex vivo skin [32].

Additionally, the intercellular and follicular penetration of retinal was investigated for retinal either released from a conventional RAL formulation or from PRN. Most topically applied substances only show a very limited skin penetration due to the strong barrier function of the skin. The results of the study showed that the cumulative concentration of retinal in the stratum corneum was 3-fold higher when retinal was delivered by PRN than by a conventional RAL formulation. Moreover, the penetration of retinal was significantly deeper when applied as PRN. Retinal released from conventional RAL was only detected in the upper 10 tape strips, whereas retinal from PRN was also detected in the lower part of the SC. The penetration profiles clearly demonstrate these effects. It can be supposed that due to the good encapsulation efficacy, retinal can be continuously delivered from the PRN reservoir located on the skin surface and upper stratum corneum layers, whereas retinal from conventional RAL is easily degrading due to its chemical and photochemical instability.

According to the Fick's law explaining the passive transport of substances through the skin [33], the flux of a substance is positively correlated to the concentration gradient. Due to the increased concentration of retinal on the skin surface when applied as PRN, a high concentration gradient exists resulting in a high flux of retinal into the stratum corneum. These results are in agreement with [34] which explained that nanoparticles would rather enhance the drug diffusion through the skin barrier and increase the drug gradient than penetrating through the healthy stratum corneum. Moreover, as already reported in other studies, it has to be taken into consideration that the amount of retinal recovered from the middle and lower part of the stratum corneum could already originate from the hair follicle openings [35], which are also removed by the tape stripping procedure. This assumption can be supported by the fact that especially retinal from PRN is localized at higher concentrations in the lower stratum corneum.

Table1
The summary of recovered retinal amount found in PRN and retinal-treated groups in each tape stripping compartment and follicular content. Data were represented as the mean values and the standard deviations of 6 replicated experiments. ł$p < 0.05$.

Treatment groups	Tape1	Tape2-10	Tape11-70	Follicular content	total
PRN					
Concentration of recovered retinal ($\mu g/cm^2$)	14.5 ± 7.7ł	5.3 ± 2.0	1.5 ± 1.7	2.6 ± 1.2ł	23.8 ± 8.4
Percentage of recovered retinal (%)	16.1 ± 8.6	5.9 ± 2.2	1.7 ± 1.9	2.9 ± 1.3	26.4 ± 9.3
Percentage of relative amount of recovered retinal (%)	59.5 ± 19.0	23.5 ± 9.3	6.4 ± 8.7	11.2 ± 6.0	100
Retinal					
Concentration of recovered retinal ($\mu g/cm^2$)	2.9 ± 1.4	3.5 ± 2.0	0.4 ± 0.5	0.2 ± 0.2	7.0 ± 2.5
Percentage of recovered retinal (%)	3.2 ± 1.6	3.9 ± 2.2	0.5 ± 0.5	0.2 ± 0.3	7.7 ± 2.7
Percentage of relative amount of recovered retinal (%)	43.4 ± 15.1	50.2 ± 15.6	5.5 ± 4.7	2.5 ± 2.9	100

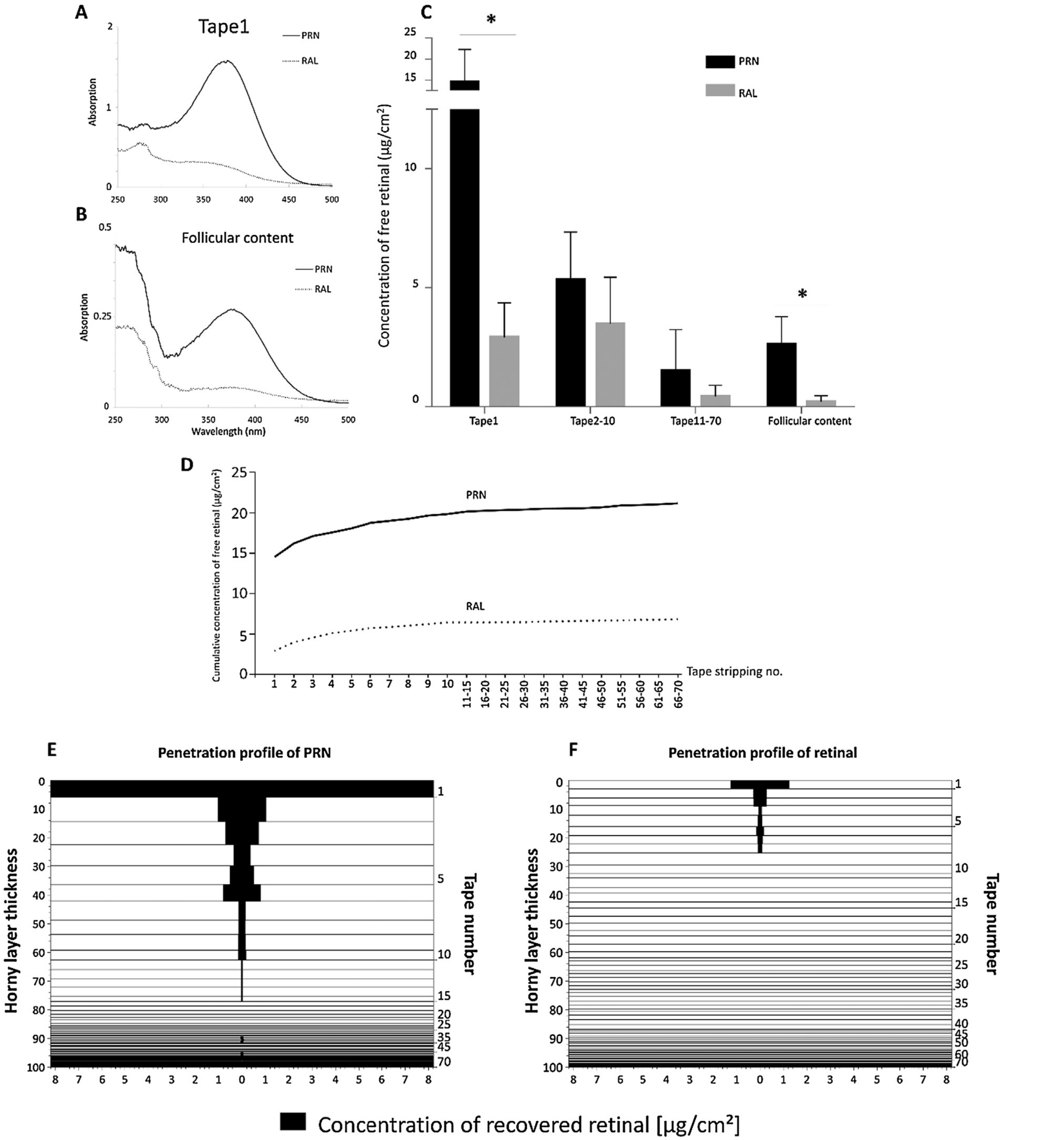

Fig. 4. UV-absorption spectra of recovered retinal (λ380nm) from PRN (———) and RAL (·····) in the region of 250–500 nm (A) tape1 (B) follicular content. (C) Comparison of the concentration of retinal in PRN and RAL-treated groups (µg/cm^2) for tape 1, tape 2–10, tape 11–70 and the follicular content after 4 h of incubation (* $p < 0.05$). (D) Cumulative concentration of retinal (µg/cm^2) after topical application of PRN and RAL in the stratum corneum. (E-F) Distribution of retinal in (E) PRN- (black fill) and (F) RAL-treated skin (black fill) inside the stratum corneum.

The better penetration of retinal from PRN application observed here agrees well to the more pronounced in vivo biological effect observed for the PRN as compared to the RAL reported earlier [18].

In the present study, this assumption was the basis for the method to quantify the follicular content. It was suggested that unless retinal is available in the lower stratum corneum, t it will likewise not be detectable in the rest of the epidermis but only in the hair follicles. This assumption is also according to the well-established method of differential stripping that is used to quantify the follicular content [36]. As the removal of the follicular content was not possible by cyanoacrylate skin surface biopsies in the porcine ear skin model as suggested by the differential stripping method, subsequently to the removal of 70 tape strips, split skin was removed, extracted and quantified to determine the follicular content. The follicular content was around 5% of the applied retinal, which is in the same range reported by other studies, which used the unmodified differential stripping technique [36].

5. Conclusion

The findings of the present study provide a novel nanoparticle-based system, which is able to increase the stratum corneum and hair follicle concentration of retinal significantly. This system might be able to improve the therapy of retinoid-responsive and hair follicle-associated skin diseases such as acne. Moreover, the utilized model drug was able to reach the lower infundibulum, which is surrounded by antigen-presenting cells [37]. If the released retinal is able to translocate transfollicularly and independently from the nanocarriers, this might be beneficial as retinoids can also effect the development of immune cells [38] and possess anti-inflammatory properties. Although further investigations are necessary to clarify the required doses and dose intervals in clinical settings, the suggested system may help to overcome the main problems of topical retinoid therapy, which are skin irritation, chemical and photochemical instability and low bioavailability.

Declaration of interest

None.

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