Penetration of topically applied nanocarriers into the hair follicles of dog and rat dorsal skin and porcine ear skin

Fanny Knorr*, Alexa Patzelt*, Maxim E. Darvin*, Claus-Michael Lehr†, Ulrich Schäfer†, Achim D. Gruber‡, Anja Ostrowski‡ and Jürgen Lademann*

*Center of Experimental and Applied Cutaneous Physiology (CCP), Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany
†Department of Biopharmaceutics and Pharmaceutical Technology, Saarland University, University Campus, 66123 Saarbrücken, Germany
‡Department of Veterinary Medicine, Institute of Veterinary Pathology, Freie Universität Berlin, Robert-von-Ostertag-Strasse 15, 14163 Berlin, Germany

Correspondence: Alexa Patzelt, Center of Experimental and Applied Cutaneous Physiology (CCP), Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany. E-mail: alexa.patzelt@charite.de

Background – In humans, topically applied nanocarriers penetrate effectively into the hair follicles where they can be exploited for the localized and targeted treatment of skin disorders.

Objective – The objective of the present study was to examine the applicability of particle-based systems for follicular drug delivery in companion animals and livestock, which have a large follicular reservoir.

Animals – Skin samples from 10 beagle dogs, 14 Wistar rats and four ears from freshly slaughtered cross-bred pigs were used.

Methods – Fluoresceinamine labelled poly (L-lactide-co-glycolide) nanocarriers (256 or 430 nm) were applied on the different skin samples. After penetration, skin biopsies were removed and cryohistological cross sections prepared and investigated with regard to the follicular penetration depths (in μm ± standard deviation) of the nanocarriers using confocal laser scanning microscopy.

Results – In canine, rat and porcine hair follicles, the smaller nanoparticles were detected at mean follicular penetration depths of 630.16 ± 135.75 μm, 253.55 ± 47.36 μm and 653.40 ± 94.71 μm, respectively. The larger particles were observed at average follicular depths of 604.79 ± 132.42 μm; 262.87 ± 55.25 μm and 786.81 ± 121.73 μm, respectively, in canine, rat and porcine hair follicles. Statistically significant differences (P < 0.05) in the mean follicular penetration depths of the differently sized nanocarriers could be determined for the canine and porcine skin samples.

Conclusion – The mean follicular penetration depths of the differently sized nanocarriers were mostly significantly different between the different species, which might be due to different species-specific follicular dimensions. This issue needs to be addressed specifically in further studies.

Introduction

Nonparticulate substances administered to the skin generally are transported across the horny layer by passive diffusion via transcellular, intercellular1,2 or follicular routes.3,4 Drug-loaded nanocarriers applied to intact or modestly compromised skin generally cannot invade the stratum corneum beyond the superficial layers;5,6 instead, they remain on the skin surface and accumulate in the hair follicles7–9 and sebaceous glands10 as demonstrated in Figure 1. Studies in laboratory animals and humans suggest that the hair follicles may be exploited for the selective targeting of differentiated structures along the follicular duct, including the infundibula, sebaceous glands, bulge regions and hair matrix cells, in the localized treatment of skin diseases and hair follicle related disorders, as well as for access to the systemic vasculature.11–13 One study14 proposed that topically applied nanocarriers preferentially move along the cleft in the cuticular hair lock to deeper regions within the hair follicles, if the size of the nanocarriers corresponds to the dimensions of the cuticular cells, inferring that by modification of the particle sizes, specific compartments or cell populations within the follicular canals can be targeted selectively.13

In the follicular ducts particle-based systems are shielded from desquamation and other environmental insults, enabling drug retention and long-term drug release for up to 10 days in humans,8 until they are cleared by hair growth, sebum production15 or degradation, allowing for their prolonged retention and the rapid or sustained drug release by decomposition or diffusion of the released compounds to the target sites.16 Current

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research is aimed at identifying endogenous as well as exogenous trigger mechanisms which induce the targeted release of the active agents at the respective target sites. Although the epithelium in the uppermost parts of the follicular infundibulum are nearly impermeable, the corneocytes in the lower parts of the infundibular epithelium represent a more incomplete barrier. Released drugs may thus potentially traverse into the viable skin.

Although drug-loaded nanocarriers are finding widespread use in human drug delivery, with indications including cancer, infection and analgesia, most of these preparations are still prohibitively expensive for use in small animal and livestock veterinary practices. However, as particle-based delivery systems generally lower systemic toxicity and drug concentrations in nontarget tissues while increasing drug concentrations at the intended sites of action, and have a reduced clearance compared to parent compounds, they require reduced doses in comparison to free drugs. This is of particular relevance for veterinarians because it may allow the use of costly medications whose application was previously precluded by the expense of dosing and furthermore may also reduce drug levels in livestock carcasses.

Although several nanoparticle systems have been evaluated for drug and vaccine delivery, commercial particle-based pharmaceuticals for the treatment of skin disorders are not yet widely available, in spite of the obvious abundance of hair follicles and the comparatively large follicular reservoir in companion and farm animals. Painless, simple and cost-effective therapy regimens are essential for patient and patient-owner compliance and play a key role in successful patient convalescence. Drug-loaded nanocarriers that can be administered topically and in fewer intervals for treating skin-associated diseases are therefore expected to facilitate animal care and result in reduced animal handling and economic savings.

In the present study, the follicular penetration of topically applied fluoresceinamine-labelled poly(L-lactide-co-glycolide) (FA-PLGA) nanocarriers (256 or 430 nm) into excised dorsal dog and rat skin and porcine ear skin was investigated in the context of follicular targeting. Target parameters of this pilot study were the follicular penetration depths of the different species and the detection of differences in follicular penetration depths between the different carriers and between the species.

### Material and methods

#### Test formulations

Fluorescent biodegradable and biocompatible nanoparticles comprising fluoresceinamine-labelled poly(L-lactide-co-glycolide) (FA-PLGA) and sized 256 nm (FA-PLGA256) and 430 nm (FA-PLGA430) were synthesized according to a previously reported method. The particle size was analysed using a Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). The nanoparticles were compounded with 1.5% hydroxyethylcellulose gel at a concentration of 0.1%, yielding two test formulations.

#### Skin samples

All experiments were approved by the Veterinary Board of Control (Veterinäramt Treptow-Köpenick, Berlin). Full thickness skin samples excised from the dorsum of 10 dogs and 14 rats were obtained from control animals belonging to and sacrificed by Bayer Schering Pharma AG (Berlin, Germany). Beagle dogs aged 14 months had been euthanized prior to excision of the skin using intravenously injected pentobarbital (Narcoren®, Merial; Halbergmoos, Germany). Wistar rats aged 8–12 weeks were anaesthetized with chloroform and exsanguinated via the jugular veins. Skin samples were transported and prepared for experimentation within 1 h following the death of the animals. Four ears from freshly slaughtered 7–8-month-old cross-bred pigs (German landrace, German large white and Durow), which had been removed prior to steam sterilization, were provided by Gut Hesterberg (Neuruppin-Lichtenberg, Germany). These were available within 24 h after slaughter. All skin samples were stored under cooling conditions until the start of the experiments.

Porcine ear skin is often used in follicular penetration studies because its follicular reservoir capacity corresponds to that of viable human skin. In contrast to ex vivo human skin, it maintains a constant follicular reservoir because the skin is fixed on an underlying cartilage and does not contract upon removal of the ears from the cadavers. The breed, age and keeping conditions of the animals from which specimens were derived remained the same for all individuals of one species. Scarred, injured or diseased skin was excluded from the study. The pig ears were cleaned with cold tap water and dabbed dry using paper towels. The fur of dog and rat skin samples and the bristles of porcine ears were clipped to a length of 1–2 mm. All samples were immobilized on polystyrene boards with the dorsal sides facing upwards. Using a permanent marker, two test areas of 4 × 4 cm were marked on each canine and porcine specimen and only one test area of 4 × 4 cm was marked on each rat skin sample due to the smaller sample sizes.

#### Administration

The test formulations (32 mg) were applied homogeneously to the corresponding test regions and massaged into the skin with a handheld massage device (Petrit® Electric PC60 Massagerät; Elektronikfabrik; Burgau, Germany) for 3 min using standardized pressure (60–80 g, Laborwaage Kern® 440-S1, Kern & Sohn GmbH; Balingen-Frommern, Germany). This was followed by an incubation time of 30 min. The two test areas marked on the dog and porcine skin samples were each treated with one test formulation, respectively. Due to the small size of the samples excised from rats only one test formulation was applied to each test area per specimen. FA-PLGA256 was applied to eight specimens, whereas FA-PLGA430 was applied to six.

#### Skin biopsies

Five full-thickness punch biopsies with a diameter of 8 mm were sampled from each test region. The biopsies taken from the porcine ears were separated from the underlying cartilage using a scalpel. The subcutaneous fat was removed from all biopsies. All skin samples were flattened between two glass slides to facilitate later preparation and fixed with cryospray (Cryospray Instant Freezing Spray, Bio Optica®, Milan, Italy). The samples were shock frozen in liquid nitrogen and stored at −20°C until further use.

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Cryohistological cross sections
The frozen biopsies were embedded in Neg-50™ (Richard-Allan Scientific®, Kalamazoo, MI, USA), a water-soluble medium for frozen sections, and cut using a cryostat (Microm Cryo-Star HM 560, MICROM International® GmbH; Walldorf, Germany). Sections, 10 μm thick, were cut from the rat skin samples, whereas the dog and porcine skin samples were cut into 14–18-μm-thick sections on account of the high density of hair follicles. The sections were mounted on glass slides which were stored at −20° C after preparation.

Confocal laser scanning microscopy
A confocal laser scanning microscope (LSM 410, Zeiss; Berlin, Germany) supplied by the Department of Chemistry of the Humboldt-Universität Berlin (Germany) was used to examine the cross sections in the transmission and fluorescence modes of the microscope. Digital images and measurements of the penetration depths were obtained using the software LSM 410 Invert Basic, v.3.98 (Zeiss). The fluorescent probes were excited using an argon ion laser with a wavelength of 488 nm. As skin exhibits autofluorescence between the wavelengths of 520 and 560 nm, emission signals were detected with the FT 589 dichroic beam splitter and long pass filter LP 590 to exclude any autofluorescence effects. The depths of fluorescence detection were determined from the skin surface to the deepest maximum signal in the hair follicle. The results were reported as mean values [±standard deviation (SD)].

Statistics
For statistical analysis, the software programme IBM SPSS Statistics 22 (IBM Corporation; Endicott, NY, USA) was utilized. For all statistical tests, the accepted level of significance was 5% (P ≤ 0.05). Results obtained for dog and porcine skin were evaluated using an unpaired Student’s t-test. The null hypothesis of equal variance was accepted on the basis of Levene’s test. For rat skin, a modified t-test for independent variables, which does not assume equality of variances, was implemented.

Results
Using confocal laser scanning microscopy, the fluorescence emitted by the topically applied nanocarriers was detected in cryohistological cross sections and could be observed only on the skin surfaces and in the follicular ducts (Figure 2a,b). No fluorescence signal was detected in the epidermis.

In 362 follicular cross sections of excised dog skin treated with FA-PLGA256, fluorescence was visualized at a mean depth of 630.16 (SD = 135.75; Min = 313.80; Max = 1349.00) μm. In 414 follicular cross sections of skin treated with FA-PLGA430, fluorescence was found at a mean depth of 604.79 (SD = 132.42; Min = 243.10; Max = 987.20) μm. These mean follicular penetration depths differed significantly (P = 0.009), as was determined using an unpaired Student’s t-test.

In the rat specimens, fluorescence was detected at a mean penetration depth of 253.55 (SD = 47.36; Min = 154.00; Max = 481.00) μm in 188 follicular cross sections for FA-PLGA256 and at an average penetration depth of 262.87 (SD = 55.25; Min = 146.40; Max = 384.70) μm in 155 follicular cross sections for FA-PLGA430. A modified t-test for independent variables that does not assume equality of variances was used to establish that the mean penetration depths did not differ significantly (P = 0.098).

In 62 follicular cross sections of porcine ear skin treated with FA-PLGA256, fluorescence was detected at a mean depth of 653.40 (SD = 94.71; Min = 492.00; Max = 848.00) μm. For skin treated with FA-PLGA430, 59 follicular cross sections yielded a mean penetration depth of 786.81 (SD = 121.73; Min = 508.00; Max = 1142.00) μm. An unpaired Student’s t-test indicated that the mean penetration depths for FA-PLGA256 and FA-PLGA430 differed significantly (P < 0.0005).

Differences between the mean follicular penetration depths for FA-PLGA256 were statistically significant between dog and rat (P < 0.0005) as well as porcine and rat skin (P < 0.0005), but were not statistically significant between dog and porcine skin (P = 0.099). For FA-PLGA430, the mean follicular penetration depths were statistically significant between all species (all P < 0.0005). The results are illustrated in Figure 3.
Discussion

The investigation of the penetration depths of two different particle sizes in dog, rat and porcine hair follicles mainly revealed significant differences between the different species. Given the known morphometry of dog, rat and porcine hair follicles, the dimensions of the follicular openings and infundibula may play a role in determining the follicular penetration depth of nanoparticles. By massaging the nanocarrier formulations into the skin samples during topical application (i.e. by exerting vibrational pressure using the hand-held massage device), the nanocarriers were pressed into the follicular orifices, filling the infundibulum and pushed into the follicular canal. In saturated infundibulum of larger volumes, the likelihood is increased that the particles can traverse to greater depths. According to a previous study, the follicular orifices are widest in porcine skin (200 μm), followed by the dog (155 μm) and rat skin (38 μm), respectively. The infundibulum of the hair follicles extend more than 500 μm into the skin of the pig, whereby in the dog they reach the infundibula of the hair follicles extend more than 500 μm. Maximum (extreme) outliers: data points that are greater than Q1 – 3 * IQR or Q3 + 3 * IQR. Minor outliers: data points that are greater than Q1 – 1.5 * IQR or Q3 + 1.5 * IQR and are not maximum outliers.

Interestingly, the significant influence of particle size known from previous investigations for porcine skin and again demonstrated in the present study for porcine skin, could not be shown for rat skin and seemed to be rather negligible for dog skin. There may have been an effect of the species-specific follicular dimensions, but in order to clarify this in detail, further investigations with other particle sizes and a greater number of animals will be required. Additionally, it might be of clinical interest to investigate the influence of different anatomic sites on the penetration effect in order to assess the optimum application site.

In contrast to applications in humans, substantial evidence regarding the role of the hair follicles as target structures for drug delivery using nanoparticles in dermatologically diseased pets and livestock is scarce. In the present study, topically applied fluoresceinamine-labelled PLGA nanocarriers (256 or 430 nm) were chosen because this particle type and size range already have been frequently used in equivalent studies and are thus suitable for comparison. The mean follicular penetration depths of the differently sized nanocarriers were significantly different between the different species, which might be due to different species specific follicular dimensions. This issue should be addressed in further studies.

The research conducted in the present study on the follicular penetration of particulates, highlights the potential for hair follicle targeting in animals. Potential applications may include the treatment of skin diseases and hair follicle-associated disorders, as well as the stimulation of systemic effects via the adjacent circulatory system or stem or immune cells.

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References

Résumé

Contexte – Chez l’homme, les nanovecteurs topiques pénètrent efficacement dans les follicules pileux où ils peuvent être utilisés en traitement localisé et ciblé des troubles cutanés.

Objectif – L’objectif de cette étude était d’examiner l’applicabilité des systèmes de particules pour la libération de médicaments dans le follicule pileux chez les animaux de compagnie et d’élevage, qui ont des réservoirs folliculaires larges.

Sujets – Des échantillons cutanés de 10 chiens beagles, 14 rats Wistar et de quatre oreilles de porcs croisés récemment abattus.

Méthodes – Les nanovecteurs de fluorescéinaminé (L-lactide-co-glycolide) (256 ou 430 nm) ont été appliqués sur différents échantillons cutanés. Après pénétration, les biopsies cutanées ont été retirées et des coupes cryohistologiques ont été réalisées et analysées pour la profondeur de pénétration folliculaire des nanovecteurs (en μm ± déviation standard) par microscopie confocal à balayage laser.

Résultats – Pour les follicules pileux de chien, de rat et de porc, les plus petites nanoparticles ont été détectées à une profondeur de pénétration folliculaire moyenne respective de 630.16 ± 135.75 μm, 253.55 ± 47.36 μm et 653.40 ± 94.71 μm. Les particules les plus larges ont été observées à des profondeurs folliculaires moyennes respectives de 604.79 ± 132.42 μm; 262.87 ± 55.25 μm et 798.61 ± 121.73 μm pour les chiens, rats et porcs. Des différences statistiques significatives (P < 0.05) de la profondeur de pénétration folliculaire moyenne des différentes tailles de nanovecteurs ont pu être déterminées pour les échantillons de peau canine et porcine.

Conclusion – Les profondeurs de pénétration folliculaire moyenne des différentes tailles de nanovecteurs étaient significativement différentes entre les différents espèces ce qui pourraient être dû aux dimensions folliculaires différentes spécifiques d’espèces. Cette question doit être traitée spécifiquement dans d’autres études.
Resumen

Introducción – en humanos, la aplicación tópica de nanopartículas penetra de forma efectiva en los folículos pilosos y pueden ser utilizados para el tratamiento localizado y dirigido de las enfermedades de la piel.

Objetivo – el objetivo del presente estudio fue examinar la aplicabilidad de sistemas basados en nanopartículas para la liberación de fármacos a nivel follicular en animales de compañía y ganado, los cuales tienen un reservorio grande follicular.

Animales – se utilizaron muestras de piel de 10 perros de raza Beagle, 14 ratas Wistar, y cuatro orejas de cerdos cruzados recientemente sacrificados.

Métodos – nanopartículas con poli (L-lactida-co-glicolido) marcadas con fluoresceinamina (256 y 430 nm) fueron aplicadas en las diferentes muestras de piel. Tras la penetración las biopsias de piel fueron retiradas, se prepararon criocciones histológicas y se investigaron con respecto a la profundidad de penetración follicular (en micrómetros ± desviación estándar) de las nanopartículas utilizando microscopía confocal de láser.

Resultados – en los foliculos pilosos de perros, ratas y cerdos las nanopartículas más pequeñas fueron detectadas a una media de penetración follicular de 630,16 ± 135,75 µm, 253,55 ± 47,36 µm y 653,40 ± 94,71 µm, respectivamente. Las partículas de mayor tamaño se observan a profundidades medias de 604,79 ± 132,42 µm, 262,87 ± 55,25 µm y 786,81 ± 121,73 µm, respectivamente en foliculos pilosos de perros, ratas y cerdos. Se determinaron diferencias estadísticamente significativas (P < 0,05) en la profundidad de penetración media follicular de los diferentes tamaños de nanopartículas para las muestras caninas y de cerdo.

Conclusión e importancia clínica – las profundidades medias de penetración follicular de los diferentes tamaños de nanopartículas fueron significativamente diferentes entre las distintas especies, lo cual puede ser debido a las diferentes dimensiones de los foliculos entre las especies. Este hallazgo necesita ser investigado específicamente con otros estudios.

Zusammenfassung

Hintergrund – Beim Menschen penetrieren topisch aufgetragene Nanocarrier effektiv in die Haarfollikel, wo sie für die lokализierte und gezielte Behandlung von Hauterkrankungen genutzt werden können.

Ziel – Das Ziel dieser Studie war eine Untersuchung der Applizierbarkeit von Partikel-haltigen Systemen zur Wirkstofflieferung in die Follikel bei Haus- und Nutztieren, die ein großes Folikel-reservoir aufweisen.

Tiere – Hautproben von 10 Beagles, 14 Wistar Ratten und vier Ohren von frisch geschlachteten Schweinemischlingen wurden verwendet.


Ergebnisse – In den Haarfollikeln von Hund, Ratte bzw Schwein wurden kleinere Nanopartikel bei einer durchschnittlichen follikulären Penetrationstiefe von 630,16 ± 135,75 µm, 253,55 ± 47,36 µm bzw 653,40 ± 94,71 µm gefunden. Die größeren Partikel wurden bei einer durchschnittlichen follikulären Tiefe von 604,79 ± 132,42 µm, 262,87 ± 55,25 µm bzw 786,81 ± 121,73 µm bei Haarfollikeln von Hund, Ratte bzw Schwein gefunden. Statistisch signifikante Unterschiede (P<0,05) bei der durchschnittlichen follikulären Penetrationstiefe der verschiedenen großen Nanocarrier konnte für die Hautproben der Hunde und der Schweine festgestellt werden.

Schlussfolgerung – Die durchschnittliche Penetrationstiefe der verschiedenen großen Nanocarrier waren hauptsächlich zwischen den verschiedenen Spezies statistisch signifikant unterschiedlich, was auf unterschiedliche Spezies-spezifische folliculäre Dimensionen zurückzuführen sein könnte. Diese Tatsache sollte in zukünftigen Studien speziell untersucht werden.
められた。サイズの異なるナノキャリアの平均毛包浸透深度における統計学的な有意差(P < 0.05)はイヌとブタの皮膚材料で判明した。

結論 — サイズの異なるナノキャリアの平均毛包浸透深度は異なる動物種間で、大部分に有意差が存在したが、それは異なる動物種特異的毛包サイズに起因しているかもしれない。この内容は今後の研究において、具体的に取り組む必要がある。

摘要

背景 — 在人类，局部应用纳米载体可有效渗入到毛囊并在其中产生作用，进而对皮肤病进行靶向治疗。

目的 — 当前研究的目的为检测将药物输入毛囊的粒子系统，在大容量毛囊的伴侣动物和家畜身上的使用。

動物 — 皮肤样本取自10只比格犬、14只维斯塔鼠和四只新屠宰的杂交猪的耳朵。

方法 — 将氨基荧光素标记的聚合物(羟乙酸)纳米载体(256或430纳米)应用于不同的皮肤样本。渗透作用后，皮肤活检、制备组织细胞学交叉切片，并使用共聚焦激光扫描显微镜进行纳米载体毛囊深度扫描调查(单位是1m ± 标准偏差)。

結果 — 在犬、鼠和猪的毛囊中，平均毛囊渗透深度分别为630.16 ± 135.75 μm、253.55 ± 47.36 μm和653.40 ± 94.71 μm时，可发现较大的纳米粒子。较大的粒子可见于平均深度分别为604.79 ± 132.42 μm、262.87 ± 55.25 μm和786.81 ± 121.73 μm的毛囊。不同大小纳米载体的平均毛囊渗透深度在统计学上有显著差异(P < 0.05)。这些差异可鉴别犬和猪的皮肤样本。

結論 — 不同种属间、不同大小纳米载体的平均毛囊渗透深度有显著不同，可能是因为不同种属的毛囊大小各异。这个问题在未来需要进一步研究。