Nanocarriers for optimizing the balance between interfollicular permeation and follicular uptake of topically applied Clobetasol to minimize adverse effects

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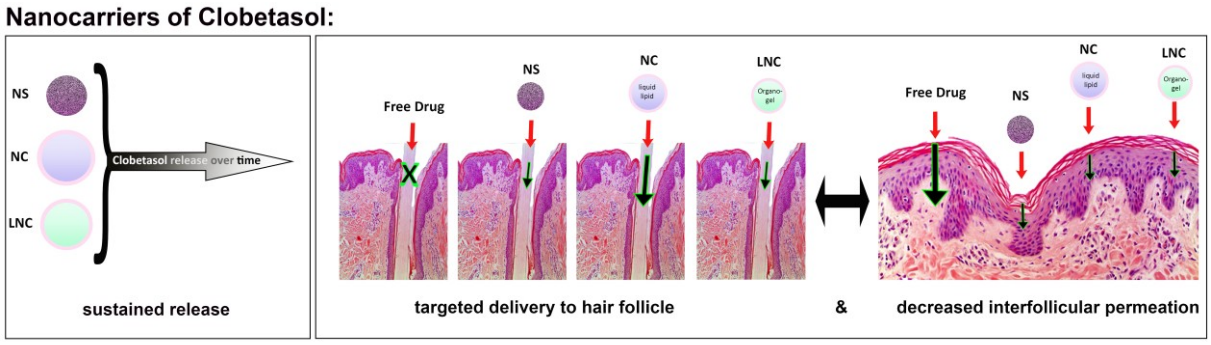
Abstract
The treatment of various hair disorders has become a central focus of good dermatologic patient care as it affects men and women all over the world. For many inflammatory-based scalp diseases, glucocorticoids are an essential part of treatment, even though they are known to cause systemic as well as local adverse effects when applied topically. Therefore, efficient targeting and avoidance of these side effects are of utmost importance. Optimizing the balance between drug release, interfollicular permeation, and follicular uptake may allow minimizing these adverse events and simultaneously improve drug delivery, given that one succeeds in targeting a sustained release formulation to the hair follicle. To test this hypothesis, three types of polymeric nanocarriers (nanospheres, nanocapsules, lipid-core nanocapsules) for the potent glucocorticoid Clobetasol propionate (CP) were prepared. They all exhibited a sustained release of drug, as was desired. The particles were formulated as a dispersion and hydrogel and (partially) labeled with Rhodamin B for quantification purposes. Follicular uptake was investigated using the Differential Stripping method and was found highest for nanocapsules in dispersion after application of massage. Moreover, the active ingredient (CP) as well as the nanocarrier (Rhodamin B labeled polymer) recovered in the hair follicle were measured simultaneously, revealing an equivalent uptake of both. In contrast, only negligible amounts of CP could be detected in the hair follicle when applied as free drug in solution or hydrogel, regardless of any massage. Skin permeation experiments using heat-separated human epidermis mounted in Franz Diffusion cells revealed equivalent reduced transdermal permeability for all nanocarriers in comparison to application of the free drug. Combining these results, nanocapsules formulated as an aqueous dispersion and applied by massage appeared to be a good candidate to maximize follicular targeting and minimize drug penetration into the interfollicular epidermis. We conclude that such nanotechnology-based formulations provide a viable strategy for more efficient

drug delivery to the hair follicle. Moreover, they present a way to minimize adverse effects of potent glucocorticoids by releasing the drug in a controlled manner and simultaneously decreasing interfollicular permeation, offering an advantage over conventional formulations for inflammatory-based skin/scalp diseases.

Graphical Abstract



Abbreviations

AA: Alopecia areata
CCT: capric/caprylic triglyceride
DLS: Dynamic light scattering
HG: Hydrogel
LLOQ: Lower limit of quantification
LNC : Lipid-core nanocapsules
NS: Nanospheres
PDI: Polydispersity index
SC: Stratum corneum
ANOVA: Analysis of variance
CP: Clobetasol propionate
FFA: Frontal, fiborsing alopecia
HHSE: Human heat-separated epidermis
LPP: Lichen planopilaris
NC: Nanocapsules
PCL: Poly(Ɛ-caprolactone)
SM: Sorbitan monostearate

1. Introduction

The identification and treatment of various hair disorders has become an important niche in the field of dermatology because it affects men and women of all ages all around the world. A proper diagnosis and management of these types of diseases is crucial, since they oftentimes lead to (permanent) hair loss that can add considerable socio-psychological stress to the patient [1]. However, specifically inflammatory-caused scalp diseases are often difficult to treat locally as the epidermal barrier is neither disrupted, nor more permeable. In these conditions the inflammation is either located deep around the hair roots or in the upper parts around the stem-cell containing isthmus and/or infundibular region of the hair follicle [2]. For example, Alopecia areata (AA) is a non-scarring condition which manifests itself in recurrent patchy or even complete inflammatory-caused hair loss of the scalp (alopecia totalis) or the entire body (alopecia universalis) [2]. It afflicts up to 1% of all dermatologic patients, which correspond to a life-time risk of up to 2% of the general population, children and adults [3]. Besides this high prevalence condition, there exists a plethora of scarring alopecias, like the Lichen planopilaris (LPP) or frontal, fibrosing alopecia (FFA), which can also cause permanent scalp hair loss resulting in significant patient discomfort. While in AA the inflammation occurs mainly at the lower portion of the hair follicle, for LPP and FFA the inflammation predominantly involves the upper follicular region [4].

Clobetasol propionate (CP), a potent glucocorticoid, has been used in many different clinical trials and was shown to be effective for various inflammatory-caused scalp diseases, e.g. AA, LPP, FFA, and

scalp psoriasis [4-9]. However, the described therapies are tedious for the patient as they entail application of an ointment to the desired site of action under occlusion with a plastic film, or application of a shampoo, cream, or foam several times a day for efficient treatment, all of which resulting in decreased patient compliance. Improved targeting and delivery to this compartment are therefore of utmost importance from a clinical perspective. Moreover, the topical treatment is the first and most attractive choice to manage these types of diseases because systemic side effects are reduced when compared to oral or parenteral administration [10]. Nonetheless, some major drawbacks of topically applied glucocorticoids are the high occurrence of local adverse effects due to high dermal absorption. Cutaneous atrophy, one of the most challenging local side effects associated with glucocorticoids, is characterized by a reduction in the viable epidermis and *stratum corneum* (SC) thickness, decreased number of keratinocytes, changes in the organization of collagen and elastin fibers, elimination of fatty tissue, and loss of mast cells [11, 12]. Yet, the degree of this adverse event observed in patients is directly related to factors like skin site ("thicker" on scalp or forearm vs. "thinner" on eyelid), age, potency, and use of occlusion [13]. A way to minimize the risk of developing this adverse event would be to limit exposure of the skin to high concentrations by releasing the drug in a controlled manner. From previous works of our group and others it is known that polymeric nanoparticles can act as controlled drug release systems [14-18]. Hence, a sustained release of drug and avoidance of a burst effect would protect the epidermal epithelial regenerative cells from high drug concentrations, resulting in a reduced risk of developing local skin atrophy and enhanced regeneration. Thus, a formulation with a sustained-release effect and/or targeted follicular delivery of glucocorticosteroids could be a new strategy to improve patient compliance and simultaneously minimize local adverse effects. In 2007, Lademann et al. reported that poly(lactic-co-glycolic acid) nanoparticles can accumulate in porcine hair follicles and be used to target drugs to this skin annex [19]. They demonstrated a depot effect of nanoparticles in the hair follicle for up to 10 days, while nanoparticles on the skin surface were only detectable for up to 24 h. Consequently, besides the already mentioned specific scalp diseases, typical high-prevalence, barrier-weakening inflammatory skin disorders, e.g. eczema, or psoriasis vulgaris of the scalp/arms/thighs, could also benefit from an application that for one accumulates in the hair shaft of vellus and/or terminal hair follicles, and also penetrates into the upper epidermal layers to exhibit a sustained release of drug over time there.

Based on the aforementioned arguments, we hypothesize that a balance between interfollicular and follicular delivery of CP could be optimized in two ways by encapsulating the drug into nanocarriers: i) by achieving a sustained release of drug and reducing interfollicular permeation plus related adverse events, and/or ii) by targeting the hair follicle. To test this hypothesis, three different types of poly(Ɛ-caprolactone) (PCL) nanoparticles [nanospheres (NS), nanocapsules (NC), lipid-core nanocapsules (LNC)] were prepared and CP release was evaluated. All particles were formulated as an aqueous dispersion and as a Carbopol® hydrogel (HG). Follicular uptake of the different types of nanoparticles and formulations was quantified in order to determine differences in the magnitude of delivery. Furthermore, the influence of massage on follicular uptake was evaluated. Lastly experiments with excised human skin were conducted to assess skin permeation under conditions where the follicular absorption pathway is supposed to be minimal [20].

2. Materials and Methods

2.1. Materials

Poly(ε-caprolactone) (80,000 MW) and sorbitan monostearate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caprylic/capric triglyceride mixture was delivered from Alpha Quimica (Porto Alegre, Brazil). Polysorbate 80 and polyethylene glycol 400 were supplied by Oxiteno (Pasadena, TX,

USA) and Alpha Quimica (Porto Alegre, Brazil), respectively. Clobetasol propionate was a gift from Neo Quimica (Goias, Brazil). Dialysis bags (Spectra Por 7, 10 Kd, Spectrum Laboratories, USA) were purchased from Bioagency (SaoPaulo, Brazil) and acetone from Nuclear (Sao Paulo, Brazil). Carbopol® Ultrez 10 was purchased from Ginama (Valencia, Spain). Human skin was kindly donated by the Hospital 9 de Octubre, Valencia, Spain, after previous signed consent of patients. Pig ears were obtained from Emil Faerber GmbH & Co. KG Zweibruecken (Zweibruecken, Germany). Methanol, acetonitrile and ethanol solvents were of HPLC quality and purchased by Scharlau (Barcelona, Spain). HPLC water was obtained by MilliQ-purification.

2.2. Preparation of Nanoparticles

Drug-free and CP-loaded NS, NC, and LNC were prepared by the nanoprecipitation-solvent evaporation technique described by Fessi et al [21], which has been successfully applied to PCL [16, 17]. This polymer was chosen because it has been approved by the Food and Drug Administration (FDA) for drug delivery specific applications and has prior been used for nanoparticle preparation [17, 22-24]. Concentrations of components of the three nanocarriers are given in Table 1.

Table 1: Concentrations of components of nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) in aqueous dispersion

	Type of Particle	PCL unlabeled (mg/ml)	PCL labeled (mg/ml)	SM (mg/ml)	CCT (mg/ml)	Polysorbate 80 (mg/ml)	CP (mg/ml)
10% labeled PCL, CP-loaded	NS	9	1	3.8	-	7.7	0.5
	NC	9	1	-	16.0	7.7	0.5
	LNC	9	1	3.8	16.0	7.7	0.5
100% labeled PCL, drug-free	NS	-	10	3.8	-	7.7	-
	NC	-	10	-	16.0	7.7	-
	LNC	-	10	3.8	16.0	7.7	-
100% unlabeled PCL, CP-loaded	NC	10	-	-	16.0	7.7	0.5
100% labeled PCL, CP-loaded	NC	-	10	-	16.0	7.7	0.5

CCT, capric/caprylic triglyceride; CP, Clobetasol propionate; PCL, poly(ε-caprolactone); SM, sorbitan monostearate; propionate

For the preparation of NS the organic phase contained only PCL and sorbitan monostearate. For NC capric/caprylic triglycerides (CCT) was added as a liquid lipid core, and LNC contained PCL, CCT and the lipophilic surfactant sorbitan monostearate dispersed in the core forming an organogel [25]. In contrast to NS (matricial system), both NC and LNC gave rise to a core and shell structure. Specifics to the preparation methods for each particle can be found in S1: Preparation of nanoparticles by the nanoprecipitation-solvent evaporation technique of Supplementary Material.

2.3. Preparation of Hydrogels

Carbopol® hydrogels (HGs) were prepared by dispersing Carbopol® Ultrez 10 NF at 0.5 % in the nanoparticle dispersions (drug-free and drug-loaded NS, NC and LNC). The components were mixed for 5 min to ensure complete dispersion and the final concentration yielded 0.05% (w/w) of the drug. To obtain a proper semisolid formulation for application on the skin, triethanolamine (0.2%, w/w) was added in order to neutralize the dispersion. For each type of nanoparticle, a HG was also produced containing drug-free particles to exclude changes in particle size and polydispersity brought about by encapsulation of drug. Additionally, a control HG containing non-encapsulated CP dissolved in water/ethanol (50:50) was prepared. All HG were prepared in triplicate, protected from light and stored at room temperature (25ºC).

2.4. Particle Characterization

2.4.1.Determination of Size and ζ -Potential

A Zetasizer Nanoseries (Malvern Instruments, Worcestershire, UK) was used to measure size, polydispersity index (PDI) and ζ-potential of all three types of particles. All dispersion-samples as well as HG-samples were diluted 1:500 in ultra-pure water for dynamic light scattering (DLS) analysis and 1:500 in 10 mM NaCl for determination of the ζ-potential. Three replicates of each were measured at room temperature. Results are presented as mean ± standard deviation.

2.4.2.Stability Study and pH Determination

To assure stability of the formulations over a period of time, dispersions in water (0.5 mg/ml) were monitored for up to 3 months after preparation in terms of particle size, PDI, ζ-potential, and pH. For the pH determination a calibrated potentiometer (MPA-210 Model, MS-Tecnopon, Sao Paulo, Brazil) was used. pH values were measured by the direct immersion of the electrode in the undiluted dispersion or after previously dispersing an aliquot of the HGs in ultrapure water at 10% (w/v). The samples were stored in amber glass flasks at room temperature.

2.4.3.Drug Content and Encapsulation Efficiency

Drug content was analyzed following a slightly modified version of the method described by Fontana *et al.* [26], dissolving 1 ml of each dispersion or 1 g of each HG in 25 ml acetonitrile instead of methanol. To determine the encapsulation efficiency, 1 ml of each dispersion was centrifuged (Ultrafree-MC 10,000 MW, Millipore) at 12,000 rpm for 5 min. Next the ultrafiltrate was analyzed for CP content and the drug entrapped was determined by calculating the difference between the total drug and the ultrafiltrate concentrations. The encapsulation efficiency was then obtained by dividing the measured drug entrapped by the total drug content. All measurements were made in triplicate and samples were analyzed by means of HPLC [26].

2.4.4. Drug Release Study

In vitro drug release tests were performed using the dialysis bag method with water/polysorbate 80/PEG 400 (60:0.5:40 v/v) as the receptor medium [15, 26, 27]. According to previous studies, polysorbate 80 and PEG 400, at the concentrations described above, were added in order to maintain sink conditions during the experiments. Moreover, this receptor medium was chosen for comparison purposes due to previously published release data [26]. Stability of CP in the release medium was previously determined using HPLC and no degradation of CP was observed for up to 72 hours. Briefly, 1 ml of each dispersion (NS, NC, and LNC containing 0.5 mg/ml CP) was added into the dialysis bag (Spectra Por 7, 10 Kd) which was then placed into 250 ml Erlenmeyer flasks containing 200 ml of the receptor medium. 1 ml samples of this medium were withdrawn after 2, 4, 6, 8, 24, 30, 48, 54 and 72 hours and replaced by fresh medium. The concentration of the samples was determined using HPLC [26]. In order to compare the drug release behavior of the nanoparticles with the non-encapsulated drug, an ethanolic solution (50% V) of CP with a concentration of 0.5 mg/ml was prepared.

2.5. Follicular Uptake Study based on the Differential Stripping Method

Follicular uptake of NS, NC, and LNC (in dispersion and in Carbopol HG) on porcine ear skin was assessed quantitatively based on the Differential Stripping method [28]. Depending on the formulation, either 20 µl of the nanoparticle dispersion, or 50 mg of the nanoparticle HG were applied to a predetermined area, followed by a three minute massage or not (depending on experimental setup), and incubated for one hour before starting the stripping process. As a control, dissolved drug in ethanol (50%) and in HG were tested to determine whether a targeted delivery to the hair follicle is achieved. The Differential Stripping method is the most straightforward technique to determine

follicular uptake quantitatively, as has recently been described by Raber et al [29]. For detailed instructions see S2: Differential Stripping Method in Supplementary Material.

2.5.1. Follicular Uptake of Drug-free Particles

In order to first determine whether the type of nanocarrier, ore more specifically the molecular architecture of the particle, influences the penetration into the hair follicle, follicular uptake of drug-free NS, NC, and LNC was determined. It was measured based on the fluorescence of the Rhodamin-B-labeled polymer PCL (λexc = 560 nm, λem = 662 nm) [30] (for more details see Supplementary Material S3.1: Plate Reader). Preliminary experiments demonstrated that the amount penetrating into the hair follicle was not quantifiable when nanocarriers were prepared using only 10% of the labeled PCL and 90% unlabeled. Thus, all nanocarriers used for follicular uptake studies quantified via fluorescence were prepared using 100% labeled PCL. Composition of all particles used can be found in Table 1. Besides the influence of the type of particle and type of formulation on the extent of follicular penetration, the effect of a three minute massage (applied pressure ~ 2N; for more details refer to Supplementary Material S2: Differential Stripping Method) after administration of the formulation was evaluated as well. For each formulation two pig ears were used, each containing three areas of formulation and one blank (n=6 for each).

2.5.2. Follicular Uptake of Clobetasol-loaded Nanocapsules

After first uptake results of drug-free particles into the hair follicle, NC in dispersion were chosen as the most promising nanocarrier for this application. Next, to evaluate the delivery of CP to the hair follicle, NC loaded with 0.5 mg/ml CP were applied onto porcine ear skin followed by a three minute massage and uptake was quantified based on drug content rather than polymer using LC-MS-MS (for details on LC-MS-MS methodology see Supplementary Material S3.2: LC-MS-MS).

2.5.3.Follicular Uptake of Clobetasol and Polymer Simultaneously

As we were interested in co-tracking polymer and drug, NC were prepared using 100% labeled PCL and simultaneously loaded with CP in order to measure follicular uptake using both quantification methods for the same samples. In comparison to previous NC used, a smaller particle size was obtained for these experiments (see Supplementary Material, Section S1: Preparation of nanoparticles by the nanoprecipitation-solvent evaporation technique).

2.5.4.Recovery

Recovery was calculated for each experiment using a mass-balance and based on the guidelines of the Scientific Committee on Consumer Safety (SCCS). Only data that fit the limits of 85-115% were chosen [31].

2.6. Skin Permeation Study

To evaluate the fate of substance not taken up by the hair follicle and therefore available for dermal absorption, skin permeability was assessed using static Franz diffusion cells and human heat-separated epidermis (HHSE). HHSE was used because follicular delivery is considered to be minimal in this model due to swelling of the SC, the outermost layer of the epidermis, as well as closure of the hair follicles after excision [20]. Details on methodology can be found in S4: Skin Permeation Study of Supplementary Materials. Samples were taken from the receptor compartment of the Franz diffusion cells after predetermined time intervals and subsequently analyzed for CP content using HPLC. Cumulative amounts of CP in the receptor compartment were measured and experimental data was fitted by a non-linear least-squares routine to an analytical solution of Fick's second law of diffusion (see Supplementary Material S4.1 & S4.2).

2.7. Statistical Analysis

Results are expressed as arithmetic mean ± standard deviation or geometric mean (geometric standard deviation). Significant differences were investigated applying one-way analysis of variance (ANOVA) and two-way ANOVA to follicular uptake data obtained for all three types of particles and formulations, and Student's t-test (two-sided, unpaired, with Welch's correction, $p < 0.5$) to determine intra-individual variability of permeability data *in vitro* [32]. All tests were calculated using the software SigmaPlot version 12.5, from Systat Software, Inc., San Jose California USA.

3. Results and Discussion

3.1. Size Distribution, ζ-Potential, and pH

The nanoparticle dispersions gave rise to particle sizes ranging between ~100 - 260 nm (see Table 2 for exact values). NC and LNC revealed a slightly larger size (except for the smaller NCs, see Supplementary Materials Section S1) when compared to NS. This can be explained by the presence of the oil phase leading to a core and shell structure instead of a matrix system [33]. All dispersions presented a PDI between 0.06 and 0.19, displaying a homogeneous size distribution.

Table 2: Size distribution, PDI values, ζ-Potential, drug content, and encapsulation efficiency of nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) in aqueous dispersion; mean ± standard deviation.

	Type of Particle	DLS (nm)	PDI	ζ-Potential (mV)	CP content (mg/ml)	Encapsulation Efficiency (%)
10% labeled PCL CP-loaded	NS	176 ± 07	0.12 ± 0.04	-12.17 ± 1.11	0.48 ± 0.02	98.46 ± 1.0
	NC	218 ± 02	0.18 ± 0.01	-10.38 ± 0.69	0.50 ± 0.01	98.25 ± 0.8
	LNC	222 ± 14	0.19 ± 0.06	-12.03 ± 1.12	0.51 ± 0.01	97.92 ± 0.3
100% labeled PCL drug-free	NS	128 ± 02	0.17 ± 0.02	-13.59 ± 0.73	-	-
	NC	257 ± 11	0.15 ± 0.01	-14.42 ± 1.09	-	-
	LNC	197 ± 04	0.14 ± 0.01	-13.29 ± 1.27	-	-
100% unlabeled PCL, CP-loaded	NC	221 ± 07	0.06 ± 0.03	-12.21± 0.73	0.50 ± 0.03	98.45 ± 0.4
100% labeled PCL CP-loaded	NC	106 ± 01	0.08 ± 0.01	-13.22 ± 0.34	0.51 ± 0.01	98.76 ± 0.7

CP, Clobetasol propionate; DLS, dynamic light scattering; PCL, poly(ε-caprolactone); PDI, polydispersity index.

The ζ-Potential of all suspensions was negative (between ca. -10.0 mV to -14.5 mV). The slight negative ζ-potential can be explained by the end-terminal carboxylic acid groups present in PCL coated by polysorbate 80 micelles as has previously been reported [34, 35]. The actual stability, which prevents coalescence, is obtained due to steric effects of the surfactant between the two phases [36]. pH studies revealed pH values suitable for skin application (between pH 5.1-6.1). Overall formulations were stable concerning particle size, PDI, ζ-Potential, and pH over a period of three months (see Table S1 in Section S5: Stability Study Results of Supplementary Material).

3.2. Hydrogel Characterization: pH, Particle Size and Drug Content

The results obtained for the HG characterization are summarized in Table 3. The pH values of the Carbopol® HG were between 5.6 and 6.7, making all suitable for topical administration [37]. The presence of the drug did not affect the pH of the HG, regardless of the drug carrier ($p > 0.05$ for each formulation). Particle sizes were still in the same range as in the original dispersion, and drug content was close to the expected concentration of 0.5 mg/g for all formulations. Rheological properties of the gels were evaluated and appropriate for topical application (data not shown).

Table 3: Hydrogel (HG) Characterization; mean ± standard deviation

	Type of Particle	pH	Size (nm)	CP content (mg/g)
10% labeled PCL CP-loaded	NS-HG	5.61 ± 0.22	183 ± 17	0.51 ± 0.01
	NC-HG	6.05 ± 0.11	219 ± 15	0.51 ± 0.01
	LNC-HG	5.90 ± 0.12	230 ± 18	0.52 ± 0.03
	CP-HG	5.87 ± 0.18	-	-
100% labeled drug-free	NS-HG	5.92 ± 0.31	185 ± 2.05	-
	NC-HG	6.73 ± 0.57	206 ± 2.61	-
	LNC-HG	6.03 ± 0.42	143 ± 1.11	-

CP, Clobetasol propionate; LNC, lipid-core nanocapsules; NC, nanocapsules; NS, nanospheres; PCL, poly(ε-caprolactone)

3.3. Drug Content and Encapsulation Efficiency

The drug content of all types of nanocarriers was approximately 0.5 mg/ml, and the encapsulation efficiency close to 100% for all three types of particles (for exact values see Table 2).

3.4. Drug Release Study

The release profiles of the three types of nanoparticles in dispersion (NS, NC, LNC) vs. free drug were assessed using the dialysis bag method (see Section 2.4.4). Results are shown in Figure 1.

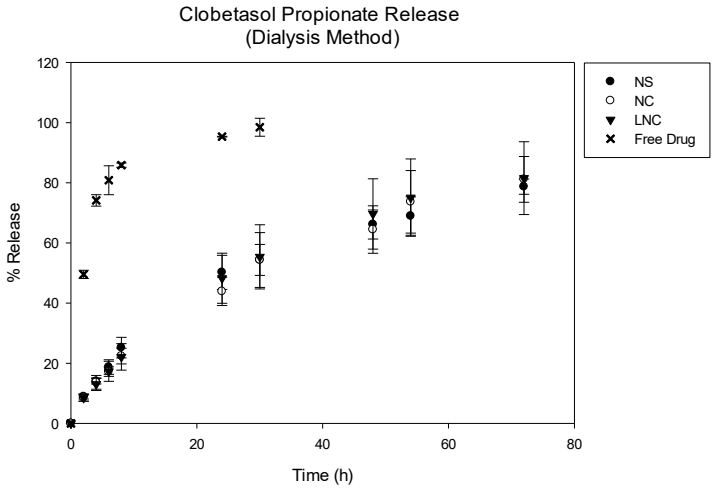


Figure 1: Release profiles of Clobetasol-loaded nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) vs. free drug (mean ± standard deviation).

No significant differences were detected between the release profiles of NS, NC, or LNC ($p>0.05$). However, all three CP-loaded nanocarriers exhibited a sustained release effect of drug, as was desired. After 8 h, only ~ 45 % of the encapsulated drug was released for all three carriers, and even at the end of a 72 hour period the mean amount of released CP was still below 80%. Free drug, in contrast, exhibited a fast release profile in which ~80 % of the drug was detected after only 5h.

3.5. Follicular Uptake Study

For all particles in both formulations, quantification limits were below 0.2 % of the total amount applied for all matrices. Thus, the goal to quantify < 1.0 %, set due to previous experience in follicular uptake studies [29], was attained for all particles in dispersion as well as in HG.

3.5.1.Follicular Uptake of Drug-free Particles

Results for the follicular uptake studies of drug-free particles can be seen in Figure 2. Figure 2(a) presents the recovery (w/w %) in the hair follicle of the total amount applied when no massage was performed. For this experimental setup, NC exhibited a significantly higher uptake into the hair

follicle when formulated as an aqueous dispersion or HG, as opposed to NS and LNC (two-way ANOVA, $p<0.05$) (also see Table S2 of Section S6: Follicular Uptake Study Results in Supplementary Material for exact values). However, no significant difference could be determined between the two types of dosage forms when no massage was performed.

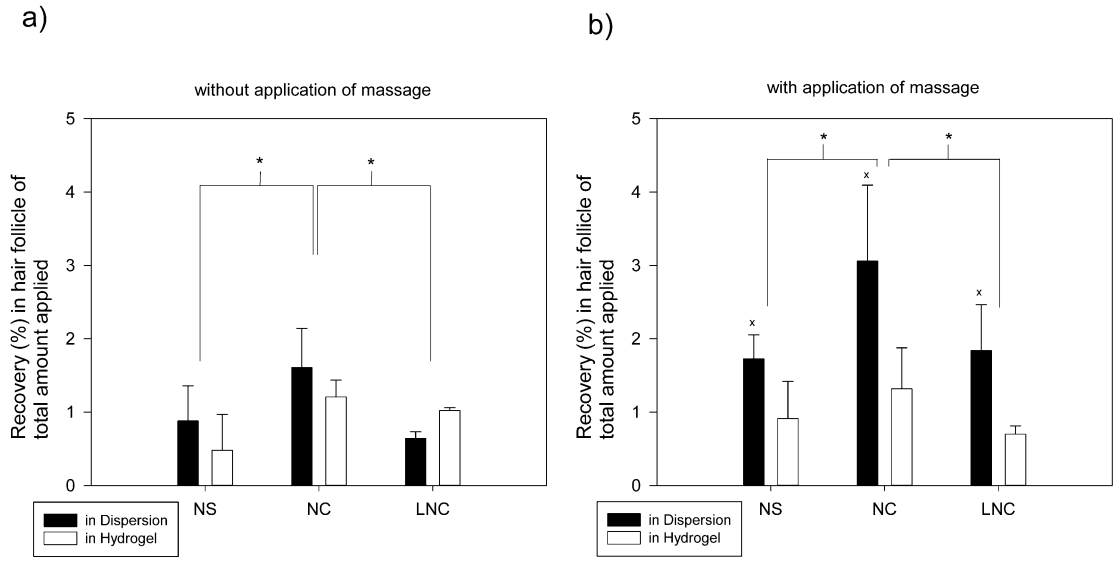


Figure 2: Recovery (%) in hair follicles of total amount applied of drug-free particles nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) [each formulated as a dispersion or hydrogel] plus free Clobetasol in an ethanolic solution or hydrogel without application of massage (a) and after application of massage (b); * represents statistical significance ($p<0.05$) between types of particles (NC vs. NS and NC vs. LNC), ^{x} represents statistical significance ($p<0.05$) between formulation (in dispersion vs. in hydrogel).

Figure 2(b) displays the results for the same experimental set-up, except here the formulation was massaged onto the porcine ear skin for three minutes prior to the one hour incubation. A similar trend, yet a generally higher uptake into the hair follicle was seen after this additional treatment. This positive effect of the applied massage upon follicular penetration has already been explained in literature before, where the authors claim that the process of moving the hair by massaging the skin acts like a 'gear pump', transporting the particles into the hair follicle, i.e. into the hair shaft, by mimicking the natural movement of the hair together with the scaly structure of the hair cuticula [19]. Similar as in (a), NC provided a significantly higher follicular recovery as opposed to NS and LNC (two-way ANOVA, $p<0.05$) after application of the massage when formulated as both, an aqueous dispersion and HG. The differences in the supramolecular structure between the three nanocarriers may be the reason for this higher accumulation. Due to the core and shell structure of NC one can assume that they are more flexible than a rigid matrix system like the NS. Moreover, a previous study on NC and LNC revealed that LNC exhibit a stiffness almost twice as high as NC based on the calculated Young's modulus (MPa) from atomic force microscopy force-displacement curves [34]. Therefore it might be speculated that an enhanced uptake into the hair follicle may be a results of using more flexible particles. Moreover, in (b) a statistically significant reduced follicular penetration was observed for all three types of particles when they were formulated as a HG, as opposed to particles suspended in liquid (two-way ANOVA, $p < 0.05$). This may be due to viscosity differences. Hence, it may be more difficult for the particles to leave the matrix during the incubation time when the vehicle is more viscous.

3.5.2. Follicular Uptake of Clobetasol-loaded Nanocapsules and controls

To confirm that the detected amount of polymer is representative of the CP content measured in the hair follicle, the uptake of similarly sized drug-free NC and CP-loaded NC were compared. As shown in Table 4, a very good correlation was found for the amount of polymer and drug. Moreover, the smaller NCs (size of 106 nm) were taken up similarly as the larger NC in previous experiments. For

these particles quantitative data on follicular uptake of both, the active ingredient (CP) and the nanocarrier itself (100% labeled PCL), was measured for the same sample set and revealed no significant difference after applying the two quantification methods ($p>0.05$; see Table 4: CP-loaded NC, 100% labeled). Of note, the small range in particle size investigated in this study may be responsible that no size-dependent effect could be observed in comparison to the data presented previously by Lademann *et al.* [38]. To elucidate this topic further, a larger range in particle size should be investigated in the future.

Table 4: Type of formulation and recovery (w/w %) in hair follicle of total amount applied for differently formulated and quantified NC in dispersion; mean ± standard deviation

Type	PCL	Size (nm)	Quantification Method	Recovery (%) in hair follicle of total amount applied
drug-free NC	100% labeled	257	Fluorescence	3.06 ± 1.03
CP-loaded NC	100% unlabeled	221	LC-MS	3.29 ± 0.53
CP-loaded NC	100% labeled	106	Fluorescence	2.98 ± 0.65
			LC-MS	3.09 ± 0.17

CP, Clobetasol propionate; NC, nanocapsules; PCL, poly(ε-caprolactone)

As for the controls, the amount detected in the hair follicle of free CP in an ethanolic solution and free CP in HG, with and without the application of massage, was below the lower limit of quantification (LLOQ) for both experimental setups and thus negligible [19, 29, 39]. This confirms that by means of encapsulation a more targeted delivery to the hair follicle was achieved.

3.5.3. Recovery

Mass balance was calculated for each above described experiment and the total recovery (w/w %) for all applied formulations ranged between 86-110% of the total amount applied, fitting well the limits of recovery of 85-115% provided by the SCCS [31].

3.6. Skin Permeation Study

To investigate the degree of non-follicular skin permeation, *in vitro* Franz Diffusion cell studies with excised human skin were conducted assuming that the follicular pathway is negligible [20, 40]. Figure 3 displays the results for the cumulative amounts of CP which have permeated through the HHSE after time (t). For exact amounts of permeated CP at t = 24h as well as flux data please refer to Supplementary Material, Table S3 & S4 in Section S7: Skin Permeation Study Results. Figure 3(a) shows the comparison of permeated drug between free CP in solution/HG vs. permeated drug of the three nanoparticle dispersions (NS, NC, LNC), and Figure 3 (b) displays the permeated amount of drug for free CP in solution/HG vs. permeated drug of three nanoparticle HGs (NS, NC, LNC). In both, Figure 3(a) and (b), it is clear to see that the incorporation of free CP into a HG decreases the cumulative amount of CP permeated into the receptor compartment, hence the rate of permeation, about 50% with respect to free CP in solution ($p < 0.05$). Similarly, among the nanocarriers a statistically significant lower cumulative amount, and thus decreased permeation, was also observed when increasing the viscosity of the medium ($p < 0.05$). However, no significant differences were detected between NS, NC, or LNC when they were formulated the same, meaning that all particles in dispersion revealed similar permeation profiles, and all particles in HG, too ($p > 0.05$). These results are in good agreement with the release profiles of CP from the three nanocarriers, in which we also observed no differences between the particles. When comparing the permeation of CP from the three nanoparticles in dispersion to non-encapsulated CP in solution, a clear and significant reduction was seen by encapsulating the drug, as was desired. Compared to previous works on rat skin permeability of CP formulated in form of microemulsion, microemulsion-gel, and a marketed formulation, human skin permeability for CP was two to four times lower when compared to the ethanolic solution [41]. In

the previously mentioned report, the authors observed greater CP permeability when CP was formulated in a microemulsion compared to the marketed formulation, but a significant decrease of permeation after increasing the viscosity of the medium. This effect was also observed in this study. One thing to keep in mind is that all results described here were obtained under *in vitro* conditions and may not be 100% predictive of the *in vivo* situation, because results under these conditions are known to generally overestimate the observed effect [42]. This is most likely a result of the non-existing clearance *in vitro* due to lack of blood flow, as well as the ongoing desquamation process in living tissue. The general trend observed, however, is representative and to be expected under real-life conditions. In that sense the observed decreased interfollicular permeation of CP, achieved by means of encapsulating the drug into nanocarriers, would be beneficial in two ways. For one, less free drug is available to permeate the skin, enter the blood circulation, and cause adverse events; and secondly, the particles which do penetrate into the skin reside in the upper epidermal layers (especially SC) where they continuously release the drug over time.

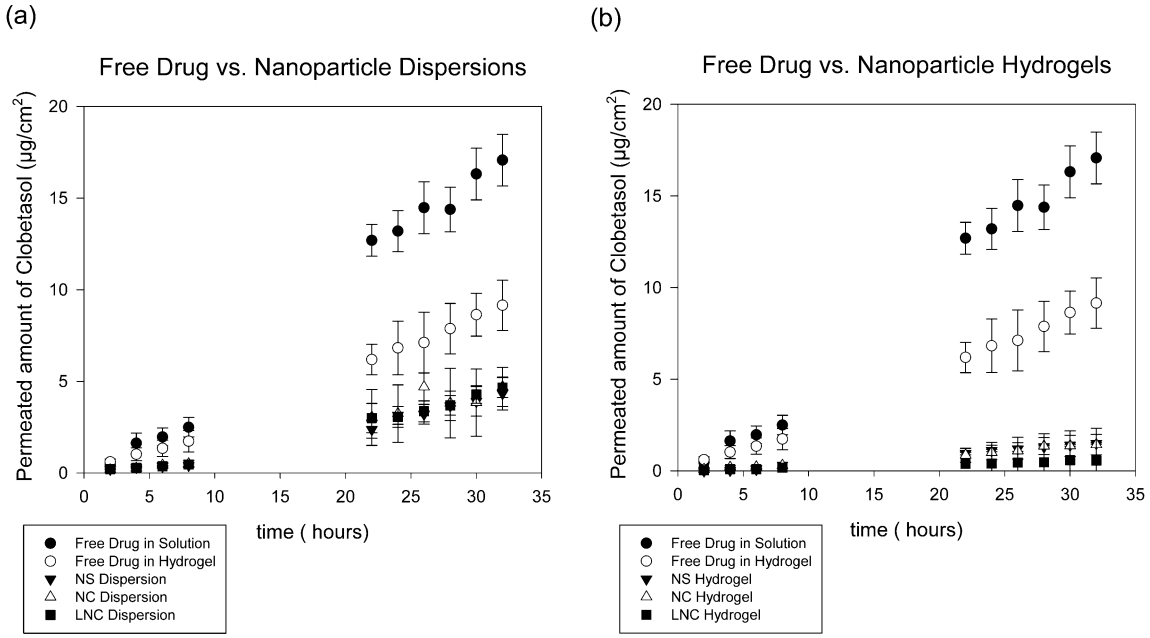


Figure 3: Clobetasol permeability through human heat-separated epidermis (HHSE) from ethanolic solution, Clobetasol-Hydrogel and nanoparticle [nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC)] dispersions (a); Clobetasol permeability through HHSE from ethanolic solution, Clobetasol-Hydrogel, and nanoparticle (NS, NC, and LNC) Hydrogels (b) (mean ± standard deviation).

3.6 Potential of Nanocarriers from a Clinical Perspective

Application of these nanocarriers, especially the NC in dispersion, could potentially revolutionize therapy for AA, LPP, FFA, or other inflammatory-based scalp diseases, as the data herein describes an improved delivery to the hair follicle of up to 4% in comparison to the negligible amount achieved with conventional formulations (here free drug in solution or hydrogel). Moreover, all follicular uptake studies described were performed on the porcine ear skin model since these experiments on excised human skin are not feasible as excision results in immediate contraction and subsequent closure of the hair follicles [20]. In previous years our groups was able to establish an excellent correlation regarding the amount of hair follicles available, as well as the quantitative follicular uptake of nanoparticles between pig ear tissue *in vitro* and human forearm skin *in vivo* [29]. However, it is noteworthy that the amount of terminal hairs available for this type of therapy on the average human scalp is much higher than what is seen on porcine ear skin (124-200 hairs per cm^2 on human occipital scalp vs. 11-25 cm^2 on porcine ear skin) [43-45]. Therefore, an even higher follicular recovery and potential for a depot effect on the scalp is to be expected *in vivo,* only further corroborating the potential of this type of application for the above mentioned conditions**.** Besides these inflammatory-based scalp diseases, barrier-weakening inflammatory skin disorders (e.g. eczema or psoriasis

vulgaris) could also benefit from this application. In these conditions the particles are able to penetrate into deeper epidermal layers since the limiting barrier is impaired, yet still exhibit the sustained release effect of drug over time.
Generally, dosing frequency should be re-evaluated and unnecessary exposure of all glucocorticoids to healthy skin should be kept to a minimum. Hence, formulations should only be applied to diseased skin/region and excess formulation should always be wiped off.

4. Conclusion

In conclusion, CP could be successfully formulated into three different kinds of PCL-based nanoparticles. All three nanocarriers demonstrated sustained-release characteristics, as was intended. Regarding their potential to target hair follicles, all three types of nanocarriers achieve a more targeted delivery to the hair follicle than free drug in solution or free drug in HG. A reoccurring difference between the types of nanocarriers could be observed, with NC yielding the highest recovery of up to 4% in comparison to NS and LNC. This emphasizes the influence of the nanoparticle structure on the degree of penetration into the hair follicle. Application of massage even amplified this effect. A formulation-dependent trend was also shown, as particles in an aqueous dispersion were up taken better than in HG. For the skin permeation experiments, the particles were able to control the drug permeability in a proper way regardless their structure. In contrast to follicular uptake studies, no statistically significant difference among the type of particle within the group of formulation was observed, even if the chemical properties were clearly different. However, a formulation-dependent trend was seen here as well, as the particles suspended in HG permeated significantly less than particles in dispersion. Our follicular uptake and skin permeation data corroborate the initial hypothesis that it should be possible to maximize follicular uptake, and simultaneously decrease penetration and release of drug into the interfollicular epidermis, depending on the type of vehicle and/or carrier used, as well as the desired therapy. Such nanotechnology-based formulations could be an advantage especially for more effective treatment of the previously mentioned inflammatory-based scalp diseases (e.g. scalp psoriasis, AA, LPP, and FFA), yet also for more common skin diseases like eczema and psoriasis. The information obtained from the present *in vitro* study appears as a most-useful basis for the translation of CP nanocarriers into future clinical trials.

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