Supplementary Material for manuscript:

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

S1: Preparation of nanoparticles by the nanoprecipitation-solvent evaporation technique

The LNC dispersions were prepared by mixing either a Rhodamin B-labeled PCL conjugate (previously described by Poletto et. al [1]) and/or unlabeled PCL, sorbitan monostearate, CCT and CP (in the case of the drug-loaded particles) dissolved in acetone. This organic solution was injected into an aqueous phase containing polysorbate 80 under moderate stirring. NS were prepared omitting the presence of the oil phase and for the preparation of NC, no sorbitan monostearate was used. For NC prepared with 100% labeled polymer and loaded with CP the stirring speed was increased during the addition of the organic phase into the aqueous phase, resulting in a smaller particle size. Acetone was removed using a rotational evaporator and the formulation was concentrated to 25 ml at 40ºC under reduced pressure. In the case of the drug-loaded particles, the final drug concentration was 0.5 mg/ml of CP. All formulations were made in triplicate, stored at room temperature (25º C) in amber glass flasks to protect the fluorescent dye from light degradation.

S2. Differential Stripping Method

The formulation (20 µl of nanoparticle dispersions or 50 mg of nanoparticle hydrogel) is first applied onto a predetermined area on the skin followed by a three minute massage or not, depending on the experimental setup. Massage is performed by using a gloved forefinger and massaging in a circular motion (60 rpm) for three minutes with applied pressure (~ 2 Newton). To minimize deviation, all experiments were performed by one well-trained person. After a one hour incubation period, ten subsequent tape strips (Tesa® adhesive tape, Tesa AG, Hamburg, Germany) are taken, used to clean the skin surface and remove the SC layer by layer, followed by the application of two cyanoacrylate skin surface biopsies enabling the removal of the whole follicular content [2]. The tapes and cyanoacrylate biopsies are pressed onto the skin using a roller in order to stretch the skin surface and assure reaching the skins wrinkles and furrows [3]. For quantification and mass-balance purposes the tape strips, cyanoacrylate biopsies, as well as all application devices and skin rest are placed in acetonitrile for extraction, centrifuged at 20°C using 12000 rpm for five minutes and analyzed for fluorescence or CP content.

S3. Quantification Methods

S3.1. Plate Reader

The analysis and quantification for follicular uptake of the particles prepared using labeled PCL was done via fluorescence measurements (λexc = 560 nm, λem = 662 nm) using a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA, USA). The lower limit of quantification (LLOQ), done in order to determine the analytical limitations of the quantification method [4], was calculated based on the fluorescence of the particles and the background fluorescence of the different types of matrices (tape strips, application devices, cyanoacrylate biopsies). The LLOQ was previously determined for each type of matrix.

S3.2. LC-MS-MS

LC-MS-MS analysis and quantification for follicular uptake of the CP-loaded NC was carried out using a TSQ Quantum Access Max (Thermo Fisher Scientific, Waltham, MA, USA) tandem quadrupole mass spectrometer coupled to an Accela UHPLC system consisting of a quaternary mixing pump with a built-in

solvent degassing system, thermostated autosampler and column oven. An Accucore RP-MS column (150mm x 2.1mm, 2.6um, Thermo Fisher Scientific, Waltham, MA, USA) was implemented, which was set at 30°C during the experimental run. The system was operated by the standard software Xcalibur. A method described by Nam et al. was used with slight modification [5].The LC system was run isocratically for 5 minutes at 400 µl/min using acetonitrile + 0.1% formic acid and water + 0.1% formic acid (65:35). Heated-electrospray ionization (H-ESI) in positive mode was used. The optimized H-ESI conditions were: capillary voltage of 4500 V, vaporizer temperature of 500 °C, ion transfer tube temperature of 350 °C. Nitrogen was used as sheath and auxiliary gas and the settings were of 40 and 5 (arbitrary units), respectively. Quantitation was performed operating in selective reaction monitoring (SRM) mode. Observed ions were as follows (values are given for mother ion [m/z]; collision energy [V]; product ion [m/z]; scan time [s]; scan width [m/z]: 467; 12; 355; 0.2; 0.02. The method was validated over a concentration range of 3.0-200.0 ng/ml (samples were diluted prior to measuring accordingly) with a correlation coefficient of $r^2 = 0.9995$.

S4. Skin Permeation Study

The skin used for all permeation experiments was obtained from the Hospital 9 de Octubre, Valencia, after written consent was obtained from the patients. Epidermis was separated from the dermis using the method described by Kligmann et al. in 1963 [6]. The acceptor compartments of the Franz cells were filled with 12.1 ml of an ethanol/phosphate buffer solution mixture (50:50) to ensure sink conditions, and placed into a heated water bath to achieve a temperature of 32ºC (physiologic temperature of the SC). The prepared epidermis was placed onto a Whatman® filter, acting here as a supporting membrane. Afterwards, the epidermis and the filter were placed on top of the acceptor compartment to separate it from the donor. Previous experiments demonstrated that this membrane did not limit permeability of the substance (data not shown). After 30 min, 1 ml of the LNC, NC and NS dispersions and the CP ethanolic solution were pipetted onto the donor compartment. Carbopol® HGs were weighed (0.5 g) and applied directly onto the donor compartment. The gels were evenly spread onto the membrane surface to cover the complete diffusion area. Both compartments were fixed together with a clamp. The donor compartment was sealed with Parafilm® and the water bath containing all Franz-diffusion cells was covered with aluminum foil to avoid light degradation of the drug. Samples were taken after predetermined time intervals and the removed volume was replaced by clean buffer solution. Samples were analyzed using HPLC [7]. Replicates ≥ 3.

S4.1. Calculation of steady-state flux J_{SS}

Experimental data was fitted by a non-linear least-squares routine to an analytical solution of Fick's second law of diffusion (Eq. 1) [8, 9]. For fitting of permeation data and statistical evaluation R 3.0.3 was used [10].

$$M(t) = A \cdot P_1 \cdot \left[P_2 \cdot t - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(-P_2 n^2 \pi^2 t\right) \right] \quad \textbf{(1)}$$

Here, M(t) denotes the accumulated mass of substance that can be found in the acceptor compartment crossing the area of application A after time t. The free parameters P1 and P2 were fitted to experimental data, with $P1=K \bullet h \bullet C_0$ and $P2=D/h^2$ [11]. D denotes the apparent diffusion coefficient, K the vehicle/barrier partition coefficient and h the length of the apparent diffusion path. C_0 is the average initial concentration of drug in the donor compartment at t=0. Using parameters P1 and P2 from fittings a steady-state flux J_{SS} could be calculated using equation

$$J_{ss} = P_1 \cdot P_2 \quad \textbf{(2)}$$

S4.2. Calculation of average steady-state flux and test for significant differences

There is evidence that the intra-individual differences in permeability ($k_p = J_{SS}/C_0$) follow a log-normal distribution (see [12] for an overview). In this context, the geometric mean of J_{SS} was computed to present a measure of central tendency. Variability was expressed by calculating the geometric standard deviation.

Significant differences were investigated applying student's t-test (two-sided, unpaired, with Welch's correction, $p < 0.1$) to address the intra-individual variability of permeability in-vitro [12]) on the log-transformed J_{SS} values.

S3. Stability Study Results

Table S1: Physiochemical characteristics of nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) dispersions using 10% polymer tracked for 3 months; mean ± standard deviation (size measured using DLS analysis). PDI, polydispersity index.

Type of Particle	Month	Size (nm)	PDI	ζ -Potential (mV)	pH
NS	0	158 ± 2	0.09 ± 0.02	-14.40 ± 1.75	6.12 ± 0.23
NS	1	169 ± 1	0.12 ± 0.03	-11.90 ± 1.59	5.86 ± 0.13
NS	2	167 ± 9	0.14 ± 0.05	-11.47 ± 1.84	5.53 ± 0.26
NS	3	173 ± 7	0.14 ± 0.03	-13.49 ± 1.09	5.50 ± 0.14
NC	0	195 ± 4	0.08 ± 0.02	- 12.10 ± 1.93	5.99 ± 0.23
NC	1	218 ± 3	0.16 ± 0.02	-10.30 ± 1.71	5.92 ± 0.19
NC	2	208 ± 4	0.13 ± 0.03	-13.63 ± 0.65	5.42 ± 0.12
NC	3	223 ± 10	0.14 ± 0.04	-13.39 ± 2.57	5.25 ± 0.13
LNC	0	193 ± 3	0.08 ± 0.04	-10.60 ± 3.43	6.11 ± 0.21
LNC	1	217 ± 3	0.15 ± 0.03	-12.46 ± 2.52	5.90 ± 0.11
LNC	2	209 ± 3	0.16 ± 0.02	-14.31 ± 1.91	5.92 ± 0.19
LNC	3	216 ± 10	0.16 ± 0.04	-12.11 ± 1.64	5.11 ± 0.10

S6. Follicular Uptake Study Results

The table below demonstrates the specific amounts of labeled PCL (for unloaded particles) and Clobetasol (for controls) that have been recovered in the hair follicle in relation to the whole amount applied. The data was obtained using the Differential Stripping Method.

Table S2: Recovery in hair follicle (w/w %) of total amount applied for the three different types of particles, with and without application of massage, and controls (CP in solution vs. CP in hydrogel). Note: the amounts detected for the controls were below the limit of quantification and thus negligible. Results presented as mean ± standard deviation. CP, Clobetasol propionate

		Recovery (%) of labeled polymer in hair follicle without massage		Recovery (%) of labeled polymer in hair follicle with massage	
		Aqueous Dispersion	**Hydrogel**	**Aqueous Dispersion**	**Hydrogel**
NS	drug free	0.88 ± 0.48	0.48 ± 0.49	1.73 ± 0.33 ^{x}	0.91 ± 0.51
NC	drug free	1.61 ± 0.54*	1.21 ± 0.23*	3.06 ± 1.03 ^{x,}*	1.32 ± 0.56*
LNC	drug free	0.64 ± 0.09	1.02 ± 0.04	1.84 ± 0.63 ^{x}	0.70 ± 0.11
		Recovery (%) of CP in hair follicle without massage		**Recovery (%) of CP in hair follicle with massage**	
CP in ethanolic solution		< LLOQ		< LLOQ	
CP in hydrogel		< LLOQ		< LLOQ	

*represents statistically significant difference between types of particles (NC vs. NS and NC vs. LNC NC vs. controls) $p<0.05$

^{x} represents statistical significant difference between formulation (in dispersion vs. in hydrogel) $p<0.05$

S7. Skin Permeation Study Results

The table below demonstrates the cumulative amount of Clobetasol that have permeated through human heat-separated epidermis (HHSE) at t = 24 h. The data was obtained using Franz Diffusion cells and formulations tested were: free Clobetasol in solution and hydrogel, as well as Clobetasol loaded in nanospheres, nanocapsules, and lipid-core nanocapsules, each formulated as dispersion and hydrogel.

Table S3: Mean cumulative amounts of Clobetasol permeated (µg/cm^2) for nanospheres (NS), nanocapsules (NC), and lipid-core nanocapsules (LNC) in dispersion as well as in hydrogel. Results presented as arithmetic mean ± standard deviation.

	Cumulative amount of Clobetasol permeated (µg/cm^2) at t = 24 h							
Sample Time (hour)	**Free Drug Solution**	**Free Drug Hydrogel**	**NS Dispersion**	**NC Dispersion**	**LNC Dispersion**	**NS Hydrogel**	**NC Hydrogel**	**LNC Hydrogel**
24	13.20 ± 1.13	6.82 ± 1.46	3.14 ± 0.48	3.33 ± 1.57	3.06 ± 0.58	1.10 ± 0.06	1.00 ± 0.39	1.16 ± 0.26

Table S4 displays the results for J_{SS} obtained using a non-linear Fitting procedure and calculated as described in Eq. 1 and 2. Figure 3 in the main manuscript depicts the permeation profiles accordingly. The calculated fluxes (J_{SS}) indicate the amount of CP crossing the membrane per time unit (at a steady state). Similarly as for the cumulative amounts permeated, calculating the J_{SS} values of the different nanoparticles revealed no statistical significant difference within this group for each formulation. These results are in accordance with the release profiles of CP from the different nanocarriers in which also no differences were observed. Comparing J_{SS} values of the three nanoparticles in dispersion to non-encapsulated CP in solution, a clear and significant decrease in permeation was seen by encapsulating the drug, as was desired.

Table S4: Mean J_{SS} values (µg/cm^2/h) for NS, NC and LNC in dispersion as well as in HG presented as geometric mean (geometric standard deviation)

	J_{SS} (µg/cm²/h)			
Formulation	**NS**	**NC**	**LNC**	**Free drug**
Dispersion	0.174 (1.152)	0.128 (1.859)	0.189 (1.226)	-
Hydrogel	0.057 (1.039)	0.072 (1.513)	0.073 (1.255)	-
Free drug in solution	-	-	-	0.593 (1.136)
Free drug dissolved in hydrogel	-	-	-	0.292 (1.191)

Incorporation of free CP into a hydrogel decreased the flux and rate of permeation about 50%, with respect to free CP in solution. Similarly, among the nanocarriers a statistically significant lower flux and thus decreased permeation was also observed when increasing the viscosity of the medium. In summary, similarities between flux data and skin permeation profiles clearly reflect the equivalence of the permeability profiles of the nano-sized formulations.

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