Journal of Controlled Release 179 (2014) 25–32

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Quantification of nanoparticle uptake into hair follicles in pig ear and human forearm

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ARTICLE INFO

Article history:
Received 29 October 2013
Accepted 20 January 2014
Available online 29 January 2014

Keywords:
Nanoparticle
Hair follicle
Pig ear skin
In vitro–in vivo correlation
Phospholipid coating

ABSTRACT

Drug delivery via the hair follicle (HF) especially with nanoparticles (NP) recently gained attention due to a depot effect and facilitated absorption conditions within the lower HF. With the prospect of transdermal drug delivery, it is of interest to optimize the follicular uptake of NP. In this study, a method was developed to quantify NP uptake into HF and applied *in vitro* in a pig ear model and *in vivo* in human volunteers. The influence of NP material on HF uptake was investigated using fluorescence-labeled NP based on poly(D,L-lactide-co-glycolide) (PLGA). All NP had similar hydrodynamic sizes (163–170 nm) but different surface modifications: (i) plain PLGA, (ii) chitosan-coated PLGA (Chit.-PLGA), and (iii) Chit.-PLGA coated with different phospholipids (PL) (DPPC (100), DPPC:Chol (85:15), and DPPC:DOTAP (92:8). Differential stripping was performed, including complete mass balance. The samples were extracted for fluorescence quantification. An effect of the PL coating on follicular uptake was observed as DPPC (100) and DPPC:DOTAP (92:8) penetrated into HF to a higher extent than the other tested NP. The effect was observed both in the pig ear model as well as in human volunteers, although it was statistically significant only in the *in vitro* model. An excellent *in vitro–in vivo* correlation (IVIVC, $r^2 = 0.987$) between both models was demonstrated, further supporting the suitability of the pig ear model as a surrogate for the *in vivo* situation in humans for quantifying NP uptake into HF. These findings may help to optimize NP for targeting the HF and to improve transdermal delivery.

1. Introduction

Recently, hair follicles (HF) gained attention as an alternative pathway for dermal absorption, which often facilitates permeation. Despite a relatively low surface coverage of approximately 0.1% [1], HF contribute significantly to the absorption of small molecules, especially those with low to intermediate octanol–water partition coefficients [2] and, importantly, also to the absorption of (sub)micron particles [3]. In fact, for drug delivery approaches, the HF represent interesting target sites. Due to the slow clearance rate by sebum flow and hair growth, nanoparticles (NP) build a depot inside the HF where they are protected from contact with clothing and are not easily washed off [4]. Also, the stratum corneum (SC) is absent in the lower third of the HF, which facilitates the absorption of substances. Thus, transfollicular delivery has been investigated in the context of needle-free vaccination [5], delivery strategies for macromolecules [6,7], NP-based sunscreens [8], and risk assessment applications [9]. Moreover, for HF-related disorders such as alopecia [10,11] or acne [12], the HF itself is the target site.

To improve therapeutic drug delivery via the HF, it is of interest to optimize NP in a way that achieves the highest possible uptake into the HF. It is well established that NP invade deeper into HF than solutions [13] and that invasion depth depends on particle size. From the cross sections of terminal HF on pig ear, an optimum particle size of approximately 645 nm was identified, which seems to be independent of particle composition [3]. Therefore, a mechanical effect for follicular entry has been postulated [3]. Besides the penetration depth, the amount of NP delivered into the HF is of interest as it allows, for example, comparing the efficacy of therapeutic treatments by comparing the delivered dose of an encapsulated active and optimizing the targeting efficiency of NP into the HF. The differential stripping technique should be suitable to quantify follicular uptake. This technique relies on, first, sampling the SC by tape stripping to evaluate the portion of analyte deposited on the skin surface

Abbreviations: NP, nanoparticle; HF, hair follicle; PLGA, poly(D,L-lactide-co-glycolide); Chit, chitosan; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium; DPPC, dipalmitoylphosphatidylcholine; IVIVC, *in vitro–in vivo* correlation.

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http://dx.doi.org/10.1016/j.jconrel.2014.01.018

and penetrated into the SC and separately sampling the HF content by a cyanoacrylate (superglue) biopsy. Differential stripping has been applied before *in vitro* on the pig ear model as well as in human volunteers to qualitatively and semi-quantitatively investigate the follicular uptake of fluorescent solutions as well as a number of model NP or sunscreens [14,15]. This was done by relating the amount of fluorescent NP recovered from the HF to the amount remaining on the skin surface. However, by demonstrating mass balance and by coupling differential stripping with a sufficiently sensitive and robust analytical method (e.g., fluorescence spectrometry, HPLC-UV/MS, and scintillation counting depending on NP cargo or label), it should be possible also to use the method for quantifying NP distribution after topical application, and particularly for quantifying NP uptake into HF.

There are only two studies investigating the influence of particle size, surface charge, material, and shape on the uptake across the inter-follicular epidermis. However, follicular targeting was not addressed in particular. Labouta et al. [16] found greater skin penetration of gold NP with a hydrophobic surface. Lee et al. [17] found negatively charged gold nano-rods to be superior compared to positively charged nano-rods. It is currently not known whether these results are also relevant to uptake into the HF. Furthermore, it has been shown before that the penetration of superfine particles is quite different from NP a few 100 nm in size or microparticles [24]. It is therefore uncertain whether the results for superfine particles can be extended to larger particles. In this study, we developed a method to quantify NP uptake into HF and applied it *in vitro* in a pig ear model and *in vivo* in human volunteers. This method allows investigating the uptake of NP into HF systematically. The HF is filled with sebum and, therefore, provides a mainly lipophilic environment [18]. Therefore, we hypothesized that lipid coating may facilitate NP uptake into HF. To exclude size effects, we used NP of similar hydrodynamic sizes (163–170 nm) but different surface modifications. As it is known that phospholipids (PL) are part of the sebum, we investigated systematically quantitative HF deposition with fluorescence-labeled NP based on poly(D,L-lactide-co-glycolide) (PLGA): (i) plain PLGA, (ii) chitosan-coated PLGA (Chit.-PLGA), and (iii) Chit.-PLGA coated with different phospholipids (PL) (DPPC (100), DPPC:Chol (85:15), and DPPC:DOTAP (92:8)). Differential stripping was performed, including complete mass balance. The samples were extracted for fluorescence quantification. An effect of the PL coating on follicular uptake was observed as plain DPPC and DPPC:DOTAP (92:8) penetrated into HF to a higher extent than the other tested NP. The effect was observed both *in vitro* in a pig ear skin model as well as *in vivo* in human volunteers, although it was statistically significant only in the *in vitro* model. An excellent *in vitro–in vivo* correlation (IVIVC, $r^2 = 0.987$) between both models was demonstrated, further supporting the suitability of the pig ear model as a surrogate for the *in vivo* situation in humans to study NP uptake into HF quantitatively.

2. Experimental methods

2.1. Chemicals and methods

We purchased PLGA (Resomer RG 50:50H; inherent viscosity 0.31 dl/g) from Boehringer Ingelheim GmbH & Co. KG, Ingelheim, Germany; ultrapure chitosan chloride salt (Protasan® 8 UP CL113) from FMC BioPolymer AS, Oslo, Norway; polyvinyl alcohol Mowiol® 4–88 (PVA) from Kuraray Specialties Europe GmbH, Frankfurt, Germany; Tesa film from TESA SE, Hamburg, Germany; and nano-screen-MAG NP from chemicell GmbH, Berlin, Germany. All other solvents and chemicals used were of the highest purity available. Deionized water (dd water, Milli Q Plus System, Millipore, Bedford, MA, USA) was used throughout.

2.2. Pig ear skin and human volunteers

Pig ears were obtained from a local abattoir from freshly slaughtered pigs removed before scalding. Only ears with immaculate skin surface were used. Ears were stored at 2 °C–8 °C for a maximum of 2 days. Treatment occurred on the outer auricle.

Eleven volunteers (Caucasian, five females, six males, skin types II–IV) with no history of skin disease participated in the study, which was approved by the "Ärztekammer des Saarlandes" ethical committee and in adherence with the Declaration of Helsinki principles. All volunteers signed informed consent. Treatment occurred on the outer forearm due to the larger number of hair follicles compared to the inner forearm. Subjects were asked not to use any cosmetics on the application site on the day of the experiment.

2.3. Nanoparticles

For visualization, nano-screen-MAG nanoparticles (chemicell GmbH, Berlin, Germany) were used. The particles have a hydrodynamic diameter of 100 nm and are fluorescently labeled. They are composed of a solid magnetite core and covered with a chitosan matrix. The nano-screen-MAG serves as a model NP as they are visible on the skin by eye due to their dark brown color as well as by fluorescence and environmental scanning electron microscopy ESEM microscopy.

PLGA-NP were prepared by a double emulsion method described by Mittal et al. [5]. Chit.-PLGA NP were prepared by the emulsion–diffusion–evaporation technique described by Schaefer et al. [19]. For PL coating, Chit.-PLGA NP were incubated with large, unilamellar vesicles consisting of DPPC, a mixture of DPPC:cholesterol (85:15), or DPPC:DOTAP (92:8), respectively. Via ultrasonification, the lipids formed self-organized PL bilayers on the NP surface [19]. PLGA was covalently labeled with fluoresceinamine (FITC-PLGA) according to Weiss et al. [20] to prevent the label from leaking from the NP and to avoid free dye diffusion. The cleavage of FITC-PLGA by esterases in the skin is not relevant within the timeframe of the experiment [20].

PLGA NP were stored as freeze-dried powder and re-suspended in dd water to obtain the desired concentration prior to use. Chit.-PLGA NP and PL-coated Chit.-PLGA NP were stored as stable suspension at 4 °C–8 °C for a maximum of 14 days after manufacturing. Immediately before each experiment, the hydrodynamic diameter, size distribution, and zeta potential were determined using a Zetasizer Nano-ZS (Malvern, Malvern UK).

2.4. Application protocol and sampling

The same standardized application and sampling protocol was used for pig ears *in vitro* and human volunteers. The ears and the outer forearm were wiped with cold water, blotted dry with tissue, and allowed to dry completely. Hairs were shortened with scissors, and incubation sites of 1.767 cm^2 were marked with water resistant marker. PLGA NP were tested on 4 ears with 2 application areas each and 6 volunteers with 3 application sites each; plain and PL-coated Chit.-PLGA NP were tested on 3 ears with 2 application sites each and 5 volunteers with 1 application sites each. On each ear and in each volunteer, one additional area received blank formulation (dd water). Due to space limitations, not all particles can be tested in the same volunteers. The results are nonetheless comparable between particles due to subtracting blanks obtained for each individual. Fifteen microliters of PLGA NP suspension was applied and massaged for 3 min with a gloved forefinger. Due to constraints from manufacturing, the concentration of plain and PL-coated Chit.-PLGA NP was much lower than for PLGA NP. In order to obtain reasonable quantification limits, a higher volume of 30 μl of NP suspension was applied, and no massage was performed to avoid removal of formulation. Instead, the suspension was carefully distributed with a pipette tip. The formulation remained on the skin of the volunteers for 1 h without coverage. Pig ears were kept in an incubator at 32 °C for an equal time period. The temperature of 32 °C is equivalent to the human skin surface temperature and is recommended for the performance of *in vitro* skin