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Penetration of normal, damaged and diseased skin — An *in vitro* study on dendritic core–multishell nanotransporters

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ABSTRACT

A growing intended or accidental exposure to nanoparticles asks for the elucidation of potential toxicity linked to the penetration of normal and lesional skin.
We studied the skin penetration of dye-tagged dendritic core–multishell (CMS) nanotransporters and of Nile red loaded CMS nanotransporters using fluorescence microscopy. Normal and stripped human skin *ex vivo* as well as normal reconstructed human skin and *in vitro* skin disease models served as test platforms. Nile red was delivered rapidly into the viable epidermis and dermis of normal skin, whereas the highly flexible CMS nanotransporters remained solely in the stratum corneum after 6 h but penetrated into deeper skin layers after 24 h exposure. Fluorescence lifetime imaging microscopy proved a stable dye-tag and revealed striking nanotransporter–skin interactions. The viable layers of stripped skin were penetrated more efficiently by dye-tagged CMS nanotransporters and the cargo compared to normal skin. Normal reconstructed human skin reflected the penetration of Nile red and CMS nanotransporters in human skin and both, the non-hyperkeratotic non-melanoma skin cancer and hyperkeratotic peeling skin disease models come along with altered absorption in the skin diseases.

1. Introduction

A local approach is the favored treatment option in skin diseases as it bears low risk of systemic adverse effects, yet drug access to viable skin is only a few percent. Aiming to overcome the stratum corneum barrier more efficiently a wide variety of nanoparticles such as liposomes, lipid nanoparticles, dendritic carriers, and microemulsions have been developed and tested (for review see: [1,2]). However, today only very few drugs loaded onto such carrier systems have been introduced into the pharmaceutical market world-wide which is often due to limited stability and for safety reasons. Safety is also a matter of concern with respect to the increasing use of nanoparticles in consumer products [3,4] including cosmetics. Moreover, occupational exposure provokes concern [5].

The magnitude of skin absorption of nanoparticles is the subject of ongoing research. Yet, the results are controversial. Some nanoparticles appear to pass the intact stratum corneum and reach the viable epidermis, whereas others obviously fail to access viable skin [6–8]. Very small particles with sizes ≤30 nm might penetrate into deeper skin layers *via* the intercellular route or aqueous pores of the skin [4], although, again, controversial results are being published [7]. The Scientific Committee on Consumer Products (SCCP) reviewed the likelihood of cutaneous absorption of nanoparticles [9] and summarized it as follows: There is some evidence for penetration into deeper skin layers of nanoparticles with sizes ≤10 nm. Nanoparticles ≥20 nm in size do not penetrate into viable skin layers in normal skin.

Despite increasing research efforts almost nothing is known about the cutaneous absorption of nanoparticles or loaded drugs in diseased skin. Skin diseases, however, most likely influence the cutaneous absorption, especially diseases which are associated with either damages of the outermost barrier (scratching, wounding, inflammation) or those linked to disturbed epidermal differentiation resulting in a thickened stratum

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corneum; *e.g.* various forms of ichthyosis and frequent in non-melanoma skin cancer. In order to reflect a disturbed barrier function, current approaches make use of stripped skin. Yet, lesional skin may be more accurately reflected by *in vitro* models mimicking rare or common skin diseases such as constructs reflecting autosomal recessive generalized peeling skin disease (PSD) [10], autosomal recessive congenital ichthyosis [11] and non-melanoma skin cancer (NMSC) [12]. The flexible dendrimer-type carrier, core–multishell (CMS) nanotransporters, can enhance skin penetration of loaded agents, are devoid of cytotoxicity in keratinocytes [13–15] and thus may offer the horizon for the treatment of severe and recalcitrant skin diseases, such as ichthyoses and recurrent NMSC [16,17].

Here, we compared the penetration of a loaded model dye and dye-tagged CMS nanotransporters, respectively, in normal human skin *ex vivo* as well as following the almost complete removal of the stratum corneum by stripping. The penetration of nanotransporters and loaded cargo in diseased skin was investigated using normal reconstructed human skin (RHS) and models of the hyperkeratotic PSD and NMSC, respectively.

2. Materials and methods

2.1. Particle preparation and characterization

CMS nanotransporters ($PG_{10000}(NH_2)_{0.7}(C_{18}mPEG_6)_{1.0}$; GPC: Mw 74,000 g/mol; Mn 92,000 g/mol) were synthesized and loaded with Nile red (0.004%; ABCR, Karlsruhe, Germany) as described previously [18]. Nile red loading was checked by UV/vis measurement (entrapment efficiency 83%). Alternatively, CMS nanotransporters were tagged by the fluorescent dye indocarbocyanine (ICC; Fig. 1A). Here, an amide coupling was chosen in order to achieve high linkage stability. For the dye-tagged CMS nanotransporters (CMS–ICC nanotransporters), hyperbranched polyglycerol amine was dissolved in methanol, ICC-N-hydroxysuccinimide ester (absorbance: 550 nm, fluorescence: 580 nm; mivenion, Berlin, Germany) was added and the mixture was stirred at room temperature for 6 h. Subsequently, 1-(2,5-dioxopyrrolidin-1-yl)-18-methoxy-poly(ethylene glycol)yl octadecanedioate was added dropwise and the mixture was stirred for 18 h. Methanol was evaporated and the crude residue was dissolved in water. After purification using size-exclusion chromatography the product was freeze-dried for storage upon use (yield: 95%; GPC: Mw 66,000 g/mol; Mn 91,000 g/mol). Size and polydispersity index were measured using photon correlation spectroscopy (PCS, Malvern Zetasizer ZS, Malvern Instruments, Malvern, UK) revealing nanoparticle unimers of 16 nm in size (PDI 0.2). The unloaded CMS nanotransporter and CMS–ICC nanotransporter unimers form aggregates up to 140–160 nm (PDI 0.34) depending on the polymer concentration [18]. Aggregates of Nile red loaded CMS nanotransporters are up to 200–240 nm in size (PDI 0.18, can vary with the amount of Nile red) [19]. These aggregates are stable under normal conditions. However, when experiencing high shear stress disaggregation into unimers was observed [18,19].

Furthermore, the particles' surface was analyzed by atomic force microscopy (AFM) to gain information about their flexibility/rigidity. The AFM measurements have been performed using a Nanoscope MultiMode 8 (Veeco, now Bruker AXS, Karlsruhe). The microscope was operated in the tapping and soft-tapping mode using silicone probes PPP-NCL-R (NanoAndMore GmbH, Wetzlar) with a length of 225 μm and width of 38 μm and a tip radius of <7 nm at resonance frequencies of 146–236 kHz under ambient conditions. The force constant was 21–98 N/m. The cantilever was forced to oscillate near its resonance frequency. The sample was prepared by spin coating (Spin Coater SCV-2) at 33 rps for 300 s on freshly cleaved mica. All images were flattened previous to height analysis using algorithms contained in the software NanoScope 8.10. Tip convolution makes lateral dimension analysis difficult.

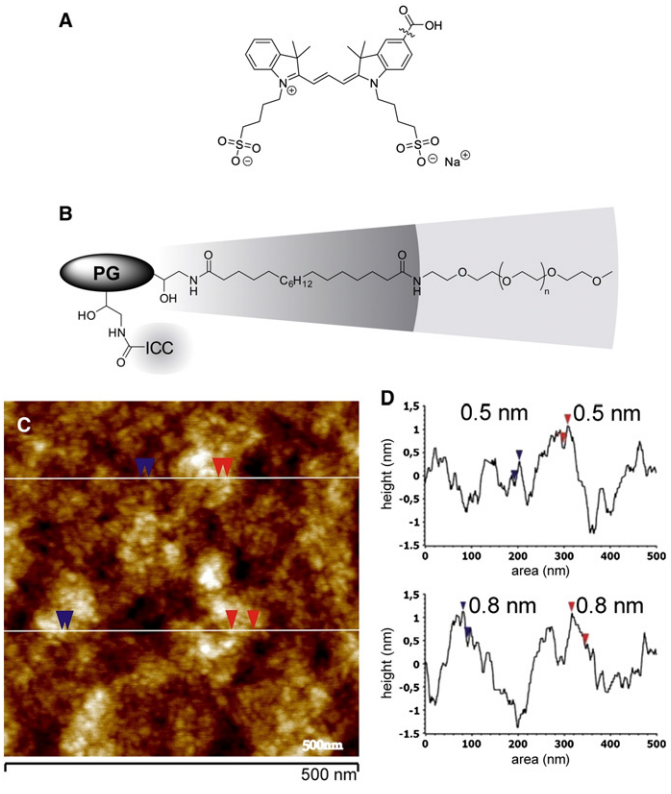

Fig. 1. Structure of CMS nanotransporters an the dye tag. A, indocarbocyanine (ICC) B, CMS–ICC nanotransporter. C, AFM height image (500 × 500 nm) and D, AFM height profile.

2.2. Human and reconstructed skin

Human skin was obtained from 6 females who underwent abdominal reduction surgery (all patients signed written informed consent). Following removal of subcutaneous fat tissue the skin was stored at −20 °C for up to 6 months until usage [20,21].

The reconstructed normal human skin (RHS) used in these studies was EpiDermFT™ (MatTek, Ashland, MA, USA). Models of NMSC [12] and generalized PSD [10] as well as the respective normal in-house constructs were grown using fibroblasts and keratinocytes from the same donors for each disease. Briefly, for the induction of PSD in RHS, keratinocytes were transfected with an established set of three siRNA addressing the corneodesmosin gene (Stealth Select RNAi, Invitrogen, Paisley, UK; [11]). Fibroblasts (passages 2–4, 2.5×10^6 cells) mixed with 2.5 mL collagen I solution were incubated in 6 well inserts at 37 °C for 2–4 h, then normal or transfected keratinocytes (passage 2, $5 \times 10^6/cm^2$ growth area) were seeded onto the dermis equivalent. The system was cultivated in keratinocyte growth medium for 24 h, raised to the air–liquid interface, and the medium was changed to a keratinocyte differentiation medium. Penetration experiments were performed 7 days after the airlift ([10]; 1-week culture). NMSC models were built according to [12]: fibroblasts (passages 1–3, 1×10^6 cells) were mixed with 4 mL collagen I solution, then normal keratinocytes (passages 2–3, 1×10^6 cells/cm^2 growth area) were seeded onto the dermis equivalent and raised five days later to the air–liquid interface. Tumor growth was induced at day 14 by seeding SCC-12 cells (passage <100, 1×10^4 cells/cm^2 growth area) onto the RHS. Penetration experiments were performed three days after the SCC co-culture (2-week culture).

For human skin and the RHS, the stratum corneum thickness was measured using histological slices. The data are summarized in Table 1.