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Research paper

Drug delivery across intact and disrupted skin barrier: Identification of cell populations interacting with penetrated thermoresponsive nanogels

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ABSTRACT

Nanoscaled soft particles, such as nanogels, can be designed to incorporate different types of compounds and release them in a controlled and triggered manner. Thermoresponsive nanogels (tNG), releasing their cargo above a defined temperature, are promising carrier systems for inflammatory skin diseases, where the temperature of diseased skin differs from that of healthy skin areas. In this study a polyglycerol-based tNG with diameter of 156 nm was investigated for penetration and release properties upon topical application on *ex vivo* human skin with intact or disrupted barrier. Furthermore, temperature-triggered effects and the internalization of tNG by skin cells upon translocation to the viable skin layers were analyzed. The investigated tNG were tagged with indodicarbocyanine and loaded with fluorescein, so that fluorescent microscopy and flow cytometry could be used to evaluate simultaneously particle penetration and release of the fluorochrome. Topically applied tNG penetrated into the SC of both intact and disrupted skin explants. Only in barrier-disrupted skin significant amounts of released fluorochrome and tNG penetrated in the epidermis and dermis 2 h after topical application. When a thermal trigger was applied by infrared radiation (30 s, 3.9 mJ/cm^2), a significantly higher penetration of tNG in the SC and release of the dye in the epidermis were detected with respect to non-triggered samples. Penetrated tNG particles were internalized by skin cells in both epidermis and dermis. Only few CD1a-positive Langerhans cells associated with tNG were found in the epidermis. However, in the dermis a significant percentage of cells associated with tNG were identified to be antigen presenting cells, i.e. HLA-DR + and CD206 + cells. Thus, tNG represent promising carrier systems for the treatment of inflammatory skin diseases, not only because of their improved penetration and controlled release properties, but also because of their ability to effectively reach dermal dendritic cells in barrier-disrupted skin.

1. Introduction

Several characteristics of inflamed skin suggest that carrier-based drug delivery systems could help improve the efficacy and selectivity of topical therapies. For instance, inflammatory skin diseases are associated with impaired skin barrier function and stratum corneum (SC) integrity [1]. Moreover, inflammatory stimuli result in dynamic regulation of epidermal tight junctions as reported for several chronic inflammatory diseases [2]. The fact that these two key compartments of the skin barrier are profoundly disturbed compared to healthy skin suggest a facilitated penetration of large molecules and nanoparticulate formulations into lesional skin, and an increased accessibility of underlying inflamed tissue for carrier-based drug delivery system. Last but not least, this also includes facilitated access to immune cells. Increased uptake capacities of antigen-presenting cells in an activated state [3] encourage the exploration of cell targeting strategies including resident antigen-presenting cells, but also inflammatory infiltrates. The uptake of nanoparticles by Langerhans cells (LCs) and keratinocytes, as the first cell types encountering penetrating compounds, has been described by various groups in mice [4,5] as well as in human skin [6,7], mostly in the context of transcutaneous vaccination approaches [8]. However, the possibil-

Abbreviations: dPG, dendritic polyglycerol; FL, fluorescein; IDCC, indodicarbocyanine; IR, infrared; LCs, Langerhans cells; MFI, mean fluorescence intensity; relMFI, relative mean fluorescence intensity; SC, stratum corneum; Tcp, cloud point temperature; tNG, thermoresponsive nanogels; tPG, thermoresponsive polyglycerol; TS, tape stripping.

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ity of particle uptake by other cell types, e.g. epidermal T-cells, or by cells in the dermal compartment is less well explored.

We recently obtained first evidence for penetration and cellular uptake of nanogels within the different skin layers [9]. Nanogels refer to aqueous dispersions of polymers which are chemically or physically cross-linked to form nanometer sized particles [10–12]. Their attractiveness as drug delivery systems originates from the high loading capacity, long-term stability, and their responsiveness to different stimuli [13]. The considerable amount of water in the swollen nanogels enables the loading of biologically active molecules by electrostatic, van der Waals, or hydrophobic interactions, and release of the therapeutic payload in a controllable fashion. Thermoresponsive nanogels (tNG) exhibit a volume phase transition in response to temperature [14]. In aqueous solutions, polymers which have a lower critical solution temperature, are hydrated below the cloud point temperature (Tcp) and cause the tNG to swell. Above the Tcp the polymer becomes insoluble in water and cause the tNG to collapse by repealing the inner solvent. The expulsion of water could be accompanied by the release of loaded hydrophilic or hydrophobic drugs upon shrinkage of the tNG. Such triggered drug release can be achieved after accumulation of the tNG in target tissues with locally elevated temperature, e.g. due to infection or inflammation, as well as during tumor hyperthermia or by artificially elevating the temperature of the region to be treated using an external thermal trigger [15].

In this study, we investigated a tNG based on dendritic polyglycerol (dPG) and linear thermoresponsive polyglycerol (tPG) consisting of poly(glycidyl methyl ether-*co*-ethyl glycidyl ether). The unique characteristics of dPG make these molecules extremely relevant for biomedical applications [16,17]. The multifunctional surface of free hydroxyl groups enable chemical modifications of highest precision to control their properties in terms of chemical reactivity, solubility, their role as macromolecular crosslinker and indodicarbocyanine (IDCC) dye labeling. tPG has a structural similarity to the biocompatible PEG and allows to tune the Tcp in a broad range of temperatures depending on the ratio of the copolymerized monomers. For these reasons, it was chosen as thermoresponsive unit in the crosslinked polymer network [18].

The penetration and release properties of tNG were investigated after topical application on human skin explants with intact and disrupted barrier. Also, the effects of an external thermal trigger on these properties was explored using barrier-disrupted skin samples. Furthermore, the internalization of tNG by cells of both, epidermis and dermis, was analyzed. Particular attention was given to skin immunocompetent cells, since these cells are prone to take-up particulate material, and they have been shown to play a central role in both initiation and maintenance of skin conditions like psoriasis and atopic dermatitis [19].

2. Material and methods

2.1. tNG synthesis and characterization

The tNGs were synthesized according to a previously reported method [18]. Briefly, for the synthesis of tPG based nanogels (tPG_tNG), dPG functionalized with (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl carbonate (dPG-$BCN_{8\%}$) (10 mg) and di-azide functionalized tPG (tPG-$(N_3)_2$) (20 mg) were mixed in 1 mL of dimethylformamide (DMF), cooled in an ice bath and injected with a syringe into 20 mL of water at 45 °C. The mixture was stirred for 3 h and the unreacted alkynes were quenched with IDCC azide. The product was purified by dialysis membrane with a molecular weight cut-off (MWCO) of 50 kDa in water, for at least two days. For the encapsulation, nanogels were let to swell in a solution of sodium fluorescein (FL) (50 wt.%). The mixture was stirred overnight under exclusion of light. Following, the tNG with encapsulated dye were separated from the free dye by washings five times with a centrifugal filtering device. tNG were characterized by ^{1}H nuclear magnetic resonance (^{1}H NMR), dynamic light scattering (DLS), and UV–Vis spectroscopy. The hydrodynamic diameter size was found to be 156.04 nm (PDI = 0.120). The Tcp was determined by temperature-dependent UV–Vis transmission measurements to be 34.0 °C. The amounts of loaded dyes were 0.099 and 0.23 wt.% for IDCC and FL, respectively.

2.2. Skin samples

Excised human skin was obtained from informed, healthy donors who underwent plastic surgery. The study was conducted with consent of the subjects, after approval by the Ethics Committee of the Charité – Universitätsmedizin Berlin and in accordance with the Declaration of Helsinki guidelines (approval EA1/135/06, renewed on July 2015). Skin was used within 3 and 24 h after surgery. Skin was stretched on a Styrofoam block and 2 cm^2 large areas, free from injuries or redness, were cleaned with phosphate buffer saline (PBS). In order to disrupt the SC barrier 50 TS were performed. This treatment has widely be used to remove part of the SC and thereby simulate the skin barrier impairment typical of certain skin conditions [20]. Thereafter, 20 μL/cm^2 of the tNG suspension (2 mg/mL, 40 μg/cm^2) or FL (0.1 μg/cm^2) were applied on skin with intact and disrupted barrier. For each experiment, a control was prepared applying 20 μL/cm^2 of a sterile saline solution (NaCl, 0.9%). To avoid samples' dehydration, the incubation took place in humidified chambers. After 2 h incubation at 37 °C or at room temperature (RT) (see next section), non penetrated material was removed from skin surface using cotton swabs. A skin area of 0.5 × 0.5 cm was cut and shock-frozen in liquid nitrogen prior to cryo-sectioning. The remaining tissue (1.5 × 1.5 cm) was further processed to isolate skin cells.

2.3. Irradiation experiments

To visualize the effects of tNG thermoresponsivity on skin penetration and drug release, an external thermal trigger was applied on skin samples with topically applied tNG. First, 1 cm^2 large areas were treated with 50 TS and tNG (40 μg/cm^2) were applied. Then, skin was irradiated for 30 s with an infrared (IR)-lamp (Philips Infrared RI 1521, Germany), with broad irradiation spectrum and a power density in skin surface of 116.8 mW/cm^2, at a distance of 40 cm from skin surface. This corresponded to a light doses of 3.9 mJ/cm^2. During this short time of irradiation the skin surface temperature reached a maximum of 40 °C, as measured by means of an IR thermometer (Basetech, Germany). Samples treated with tNG but not irradiated were prepared for comparison. Samples were then kept in the dark and incubated for 2 h at RT. Thereafter, the non penetrated material was removed, skin was cut in four blocks of 0.5 × 0.5 cm and snap-frozen for cryosectioning.

2.4. Preparation of cryosections and fluorescence microscopy

The skin blocks were placed in tissue freezing medium (Leica Microsystems, Germany). Cryosections of 6 μm thickness were prepared using a microtome (2800 Frigocut-N, Reichert-Jung, Heidelberg, Germany). Sections were observed by means of a fluorescence microscope (BX60, Olympus). Fluorescence of samples and controls were observed using filter combinations of various wavelength; BP = 545–580 nm, LP > 610 nm for IDCC and BP = 470–490 nm, LP > 550 nm for FL. Pictures (magnification of 200×) of at least 20 randomly chosen skin sections per donor and skin sample were taken using the same camera settings. The mean fluorescence intensity (MFI) of each area was calculated using the ImageJ