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On the interaction of alginate-based core-shell nanocarriers with keratinocytes *in vitro*

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ABSTRACT

Calcium alginate nanocarriers (CaANCs) were developed as a potential tool for delivery of hydrophobic active molecules such as pharmaceutical and cosmetic active ingredients. In this study, we focused on interactions between CaANCs and keratinocytes in culture and examined toxicity, internalization and drug release. Prior to cellular interactions, cryogenic transmission electron microscopy images showed that CaANCs appear as regular, spherical and dense particles, giving evidence of the surface gelation of CaANCs. Their size, around 200 nm, was stable under tested conditions (temperature, culture media, presence of serum and presence of encapsulated dye), and their toxicity on keratinocytes was very low. Flow cytometry assays showed that CaANCs are internalized into keratinocytes by endocytosis with a predominant implication of the caveolae-mediated route. Förster resonance energy transfer (FRET) demonstrated that after a 2 h contact, the release of CaANC contents in the cytoplasm of keratinocytes was almost complete. The endocytosis of CaANCs by a lysosome-free pathway, and the rapid release of their contents inside keratinocytes, will allow vectorized molecules to fully exhibit their pharmacological or cosmetic activity.

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1. Introduction

The delivery of active pharmaceutical and cosmetic ingredients (APIs and ACIs, respectively) to the skin or through the skin constitutes a local and non-traumatic route of administration. However, because the major role of the skin is to protect the inner body from external threats, it remains a true challenge for researchers to obtain good delivery efficacy. The upper layer of the skin, the *stratum corneum*, is the main actor of the skin barrier function. It is made up of a layer of keratinized dead cells that is impermeable to most active substances. Cutaneous or percutaneous routes are then limited to a range of small molecules that possess appropriate physicochemical properties and that are efficient at low concentrations [1]. Consequently, the major skin diseases are treated *via* oral administration, possibly leading to systemic side effects.

Nanocarriers (NCs) have been quickly developing in the field of dermatology because they seem able to increase the quantity of active molecules reaching the living layers of the epidermis. They can be used by themselves on damaged or normal skin [2,3] and can be used in combination with other methods that alter the *stratum corneum*, thus increasing their accumulation in the viable epidermis. Such a method might be iontophoresis [4] or dermal injections, using nanoparticle-loaded polymer microneedles [5–7]. Once accumulated in the viable layer of the epidermis, NCs will be in contact with keratinocytes, the most abundant cells in this part of the skin. At this point, NCs efficacy and toxicity will depend on their uptake by the cells or their persistence in the extracellular matrix as well as the modalities and release kinetics of the encapsulated molecule in the intracellular or extracellular media.

Core-shell nanocarriers are the most-studied nanoparticle drug delivery systems in the dermatology and cosmetics fields. In these systems, the ACI or API is dissolved in the core of the particle and is isolated from the external medium by a protective shell. The physicochemical properties of the molecule of interest has little influence on its skin distribution [8]. The nature of the NC's core depend on the polarity of the active molecule to be dissolved within it, and the shell surface must be hydrophilic to confer good stability in aqueous medium. NCs with natural polysaccharide-based shells have increasingly attracted the attention of researchers because they present several interesting properties [9,10]. Among the natural polysaccharides used to produce nanocarriers, alginates are biocompatible materials that can be easily cross-linked by addition of a divalent cation [11]. The choice of the type of alginate and of the cross-linking ion can lead to the formation

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of a more or less tight gel [11]. This property can be used to develop controlled-released systems. Additionally, alginates have shown interesting results in skin regeneration and wound healing, which makes it an “active excipient” [11,12]. As the literature about the preparation, physico-chemical characterization and efficacy of alginate-based NCs becomes abundant [9,10,13,14], we find it interesting to explore how these NCs interact with keratinocyte cells and to learn more about their mechanisms of action.

In our lab, we developed new core-shell NCs, namely calcium alginate-based nanocarriers (CaANCs), intended to help APIs or ACIs reach living parts of the skin [15]. The core is comprised of triglycerides that can dissolve high concentrations of hydrophobic active molecules, and the shell is made of calcium alginate gel. These nanocapsules were shown to be stable when dispersed in gels for skin applications up to five weeks in conditions of accelerated aging (30 °C, 65% HR), which mimics a shelf life of several months at room temperature. When prepared for a pharmaceutical purpose, they are going to penetrate the living epidermis where they will interact with keratinocytes, either because the skin barrier is altered, or because of the use of specific devices to pass through the stratum corneum, as microneedles to reach the target cells. In this study, we focused on the interactions between CaANCs and keratinocytes in culture and examined toxicity, internalization and drug release. Prior to cellular studies, CaANCs were characterized with the help of various techniques such as dynamic light scattering (DLS), zetametry and cryogenic transmission electron microscopy (cryo-TEM). To follow CaANCs in cells by means of fluorescence-based methods (confocal spectral imaging [CSI], flow cytometry), they were loaded with the fluorescent dye DiD or with the DiI-DiD association of fluorophores known to produce the Förster resonance energy transfer (FRET) effect. The uptake pathways and the intracellular distribution of CaANCs and their cargo in keratinocytes were determined, and toxicity and drug delivery optimization were investigated.

2. Materials and methods

2.1. Reagents

Sorbitan monooleate, polyoxyethylene sorbitan monooleate (polysorbate 80) and imidazolidinyl urea were purchased from Seppic, France. Sodium alginates were kindly provided by Setalg, France. Capric triglycerides (Labrafac® WL 1349) were provided by Gattefossé, France. Trehalose and poly-D-lysine were purchased from Sigma–Aldrich, France. Calcium chloride, sodium EDTA, fetal bovine serum (FBS), Dulbecco’s modified eagle medium (DMEM), antibiotics and phosphate buffered saline (PBS) for *in vitro* tests were provided by Fisher Bioblock, France and ethanol and sodium chloride by Carlo Erba, France. Alamar Blue® was purchased from Invitrogen, France. DiI and DiD fluorescent dyes were provided by Life Technology, France.

2.2. Preparation of calcium alginate-based nanocarriers

The CaANCs were prepared using ultrasound oil-in-water emulsification followed by gelation with calcium ions as described elsewhere [15]. An oily phase made of sorbitan monooleate and Labrafac® WL 1349 was incorporated into the aqueous phase made of polysorbate 80 and sodium alginate solution using an ultrasonic probe (Vibra-cell ultrasonic processor; Sonics; 20 kHz). A nanoemulsion, where sodium alginate chains surround oil droplets, was obtained. Remaining under probe sonication, a solution of calcium chloride was added to obtain stable CaANCs.

To prepare labeled CaANCs, an appropriate quantity of DiI (3.4 mg), either alone or coupled with DiD (1.1 mg DiI and 2.2 mg DiD) was dissolved in the oily phase prior to emulsification. To eliminate excess surfactants, labels or polymer residues, all formulations were dialyzed for 48 h against distilled water (25,000 MWCO membrane; Biovalley, France). In biological studies, the CaANC concentration (g/L) is expressed on a dry matter basis. These concentrations were obtained after freeze-drying the samples.

2.3. Physicochemical characterization of CaANCs

2.3.1. Cryo-transmission electron microscopy

The CaANC suspensions, as well as the intermediate sodium alginate-stabilized nanoemulsions, were observed by cryo-transmission electron microscopy (cryo-TEM). Specimens were prepared using a “cryoplunge” cryo-fixation device (Gatan, USA) in which a 10 μL microdrop of the sample was deposited on a glow-discharged holey-type carbon coated grid (Ted Pella Inc., USA). The TEM grid was then prepared by blotting the drop containing the specimen to a thin liquid layer that remained across the holes in the carbon support film. The liquid film was vitrified by rapidly plunging the grid into liquid ethane cooled by liquid nitrogen. The vitrified specimens were mounted in a Gatan 910 specimen holder that was inserted in the microscope using a CT-3500 cryotransfer system (Gatan, USA) and cooled with liquid nitrogen. The TEM images were then obtained from specimens preserved in vitreous ice and suspended across a hole in the supporting carbon substrate. The samples were observed under low-dose conditions (<10 e-/A^2) at −178 °C using a JEM 1230 ‘Cryo’ microscope (Jeol, Japan) operated at 80 kV and equipped with a LaB_6 filament. All micrographs were recorded using a Gatan 1.35 K × 1.04 K × 12-bit ES500W CCD camera. Cryo-TEM images were processed using ImageJ software[1] to enhance the contrast.

2.3.2. Size

The hydrodynamic diameter (D_H) and polydispersity index (PdI) were assessed using a dynamic light scattering (DLS) instrument (HPPS; Malvern Instruments, UK). Each sample was diluted 1:40 in ultra-pure water before measurements in triplicate at 25 °C.

2.3.3. Surface charge

Zeta potential was determined using a 1:40 dilution of the CaANC suspension using a NanoZ zetameter (Malvern Instruments, UK) in triplicate at 25 °C. The detection angle was 13°, and a 633-nm red laser was used.

2.3.4. Nanocarrier stability in cell culture media

Over the course of 7 days, changes in D_H of the CaANCs dispersed in cell culture media (1:40 dilution in DMEM with or without 10% serum) without additional dilution and stored at 37 °C were followed using DLS.

2.4. In vitro *CaANC toxicity on human HaCaT cells*

The toxicity of blank CaANCs was explored *in vitro* on HaCaT cells in culture to determine safe concentrations. HaCaT cells (human keratinocytes) were grown in DMEM enriched with 10% FBS and 1% antibiotics at 37 °C and 5% CO_2. The Alamar Blue assay was performed in 96-well plates. HaCaT cells were seeded at 3×10^3 cells per well and grown for 48 h in complete culture medium. Then, cells were treated for 72 h with various concentrations of the CaANC suspension (0.003 to 15 g dry weight/L) dispersed in culture medium. Cell viability was determined [16,17] using a multiwell-scanning spectrophotometer plate reader (ELx800; Bio-Tek, France). Optical

[1] Research Services Branch NIMH & NINDS. ImageJ is image processing and analysis software written in Java. Web site: http://rsb.info.nih.gov/ij/

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