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Enhanced topical delivery of hyaluronic acid encapsulated in liposomes: A surface-dependent phenomenon

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ABSTRACT

In the present study, we investigated the release and permeation of hyaluronic acid (HA) encapsulated in liposomes when deposited onto two surfaces: cellulose, a model widely used for investigating transport of drugs; and human skin, a natural biointerface used for transdermal drug delivery. We prepared and characterised liposomes loaded with HA and liposomes incorporating two penetration enhancers (PEs): the non-ionic surfactant Tween® 80, and Transcutol® P, a solubilising agent able to mix with polar and non-polar solvents. *In vitro* and *ex vivo* permeation assays showed that PEs indeed enhance HA-release from liposomes. Since one of the possible mechanisms postulated for the action of liposomes on skin is related to its adsorption onto the *stratum corneum* (*SC*), we used atomic force microscopy (AFM) topography and force volume (FV) analysis to investigate the structures formed after deposition of liposome formulations onto the investigated surfaces. We explored the possible relationship between the formation of planar lipid structures on the surfaces and the permeation of HA.

1. Introduction

Supported lipid bilayers (SLBs) are a type of model membrane that has received much attention in recent years [1]. SLBs can be formed from double deposition of Langmuir–Blodgett lipid monolayers or, most commonly, from the deposition of liposomes in suspension onto convenient surfaces [2]. Numerous studies have investigated the mechanisms underlying the formation of SLBs from liposomes. The most accepted mechanism involves three steps: (i) membrane deformation and flattening after encountering the surface; (ii) rupture; and (iii) transformation into bilayer disks [3]. These steps are based on investigation of liposome deposition onto inorganic surfaces (*i.e.* mica, glass or TiO_2, among others). However, less attention has been paid to the possible connection between studies investigating lipid lateral segregation, lipid–protein interactions in biomembranes, the building of functionalised surfaces to design nanobiosensors, and the use of liposomes as drug delivery systems for topical and transdermal applications. The most external layer of the skin is the epidermis, which is constituted by three main layers: the *stratum corneum* (*SC*), the granular layer and the basal layer. Thus, from a physicochemical viewpoint, the *SC* (a heterogeneous structure comprising keratinised cells, proteins and an intercellular lipid layer) may be conceived as a surface on which liposomes are deposited, and from where systemic effects are expected in some cases. It is noteworthy that while liposomes have been widely used in cosmetics and drug delivery through the skin, the physical effect on liposome integrity resulting from the interaction with the skin is not totally understood [4].

In this study, we encapsulated hyaluronic acid (HA) in liposomes and studied its release when deposited onto cellulose, a model often used for *in vitro* experiments, and onto the *SC* from human skin. HA was selected because it is involved in various biological processes, such as hydration, nutrient exchange and cell differentiation and motility [5]. HA is a hydrophilic dipolysaccharide with a very high molecular weight (200–425 kDa in this study), the structure of which contains repeating units of D-glucuronic acid and *N*-acetyl-d-glucosamine [6]. HA is used in a wide variety of medical applications, including osteoarthritis, embryo implantation and cutaneous wound healing [7]. In particular, HA and its derivatives are used to treat dermal and subcutaneous wounds of various etiologies. In recent years, the primary focus for chronic wound care has been the development of dressings to promote a moist

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environment. Advances in dressing technology, however, have not yet resulted in the development of materials that correct abnormalities in the healing cascade, with the sole exception of those containing hyaluronic acid, which specifically promotes healing [8].

As previously reported [9,10], Atomic Force Microscopy (AFM) is a non-invasive technique that is highly suitable for studying the mechanical properties of the skin under environmental conditions and investigating the effect of pharmaceutical products designed for topical applications. Among the physical magnitudes extracted from AFM working in force spectroscopy mode, the adhesive forces provide a means for understanding the mechanisms behind the effects of pharmaceutical formulations on the skin [11].

In the present study, we developed and characterised three liposomal formulations containing encapsulated HA and investigated the effect of two penetration enhancers (PEs), Tween® 80 and Transcutol® P, on the transformation of liposomes into planar lipid structures on *SC* from human skin. The liposomal formulations under study were then characterised by AFM topography and force volume (FV), by analysing the structures adopted after spreading onto different substrates.

2. Experimental

2.1. Materials

L–α phosphatidylcholine (PC) (egg yolk, 99% purity), polyoxyethylene (20) sorbitan monooleat (Tween® 80) and Hepes sodium salt, sodium chloride, sodium hydroxide, potassium monophosphate were purchased from Sigma Chemical Co (St. Louis, MO, USA). Transcutol® P was a kind gift from Gattefossé (Saint-Priest, France). Hyaluronic acid (sodium hyaluronate) from *Streptococcus equi* was purchased from Fagron Iberica (Barcelona, Spain). Acetonitrile HPLC, methanol, chloroform and ethanol were purchased from Panreac (Barcelona, Spain). All other common chemicals were ACS grade. Cellulose membrane with a molecular weight cut off at 12,000–14,000 Da was acquired from Iberlabo (Madrid, Spain). Human skin was obtained from the hospital's plastic surgery department (Hospital de Barcelona, SCIAS, Barcelona, Spain).

The experimental protocol was approved by the Bioethics Committee of the Barcelona-SCIAS Hospital (Spain) and written informed consent forms were provided by study participants.

2.2. Liposome preparation

Liposomes were prepared according to methods published elsewhere [12]. Briefly, chloroform-methanol (2:1, v/v) solution containing the appropriate amount of PC was placed in a glass balloon and dried in a rotary evaporator at room temperature protected from light. The resulting thin film was kept under high vacuum overnight to remove any traces of organic solvent. Multilamellar liposomes were obtained by redispersion of the thin film in 20 mM Hepes, 150 mM NaCl buffer, pH 7.4. Liposomes were extruded consecutively through polycarbonate membranes with a pore size of 400 nm and 100 nm by an Avanti® Mini-extruder (Avanti Polar Lipids Inc., Alabama, USA). PC concentration was determined by the Stewart assay [13]. Final concentration of HA was assessed by HPLC after disruption of liposomes with isopropanol. HA-to-PC ratios fall typically between 0.26 and 0.37 (mol/mol).

PEs were incorporated into the liposomes containing HA with the aim of partially destabilising the lipid bilayer and enhancing its transformation into lipid planar structures. Different concentrations of PEs (0.01–25%, v/v) were added to the liposomes. Based on the average particle size and polydispersity values, two formulations were prepared by adding extemporaneously the appropriate percentage of the corresponding PE to the liposomes loaded with HA (F1). Thus, 0.15% of Tween® 80 and 3.5% of Transcutol® P were added to liposomes with HA (F1) to obtain the F2 and F3 formulations, respectively.

2.3. Liposome characterisation

2.3.1. Particle size and ζ potential

The mean particle size and polydispersity of liposomes were measured by dynamic light scattering with a Zetasizer Nano S (Malvern Instruments, UK). Electrophoretic mobility indicating the effective surface electrical charge (ζ potential) was determined by a Zetasizer Nano ZS90 (Malvern Instruments, UK). The samples were diluted (50-fold) with Hepes buffer pH 7.4. Each sample was measured three times.

2.3.2. Encapsulation efficiency

The efficiency of encapsulation of HA into liposomes (EE_{HA}) was assessed by determining the free (non-encapsulated) HA (m') after separation of liposomes by Ultracel 10 centrifugal filter devices (Amicon®, Millipore, MA, USA) at 4000 × g for 40 min (Multifuge 3 L-R, Thermo Fisher Scientific, Spain). The amount of HA non encapsulated (m') was determined by HPLC.

Hence, the EE_{HA} was obtained by applying the following equation:

$$EE_{HA} = [(m_t - m')/m_t] \quad (1)$$

where m_t is the total amount of HA added to the sample.

2.4. Human skin preparation

Human skin was obtained from the abdominal region of healthy women. After being frozen to −20 °C, it was cut with a dermatome (Model GA 630, Aesculap, Tuttlingen, Germany) into 200, 300 and 400 μm-thick pieces, starting from the *SC* [14]. Human skin integrity was verified before the experiments by measuring the quantity of water that passes through the epidermal layer of the skin to the surrounding atmosphere by diffusion and evaporation processes [15], using a transepidermal water loss (TEWL) Tewlmeter TM210 (Courage & Khazaka, Koln, Germany). The skin used showed TEWL values below 10 g/m^2 h.

2.5. Permeation assays

2.5.1. Permeation of HA through cellulose

The experiments were carried out in vertical Franz-type diffusion cells (Co. Mod Franz Crown Glass. CDCF-9) with a diffusion area of 0.6 cm^2 using cellulose membranes with molecular weight cut off at 12,000–14,000 Da. Experiments were performed under sink conditions to avoid the solubility of HA interfering during *in vitro* release. Briefly, 300 μL of each formulation was placed in the donor compartment. The receptor chamber was filled with milliQ water or buffer, maintained at 32.0 ± 0.5 °C and continuously shaken by a magnetic stirrer. Samples were withdrawn from the receptor compartment at designated time intervals over 5 h and replaced with the same volume of receptor medium immediately after each sample collection. The concentration of released HA was assessed by HPLC.

To describe the kinetics of drug release from liposomes containing HA, different mathematical models were fitted to the experimental data [16]. The cumulative amounts of HA that permeated through the surfaces investigated were adjusted to the model that best fitted our data, a first order release model [17].

$$\%R_t = \%R_\infty(1 - e^{-kt}) \quad (2)$$