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A novel strategy for the treatment of chronic wounds based on the topical administration of rhEGF-loaded lipid nanoparticles: *In vitro* bioactivity and *in vivo* effectiveness in healing-impaired *db*/*db* mice

Garazi Gainza [a,b], Marta Pastor [a,b], José Javier Aguirre [c], Silvia Villullas [d], José Luis Pedraz [a,b], Rosa Maria Hernandez [a,b], Manoli Igartua [a,b,*]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country, Vitoria, Spain
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain
[c] Hospital Universitario de Álava (HUA) Txagorritxu, Vitoria 01009, Spain
[d] Biopraxis Research AIE, Miñano 01510, Spain

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ABSTRACT

Lipid nanoparticles are currently receiving increasing interest because they permit the topical administration of proteins, such as recombinant human epidermal growth factor (rhEGF), in a sustained and effective manner. Because chronic wounds have become a major healthcare burden, the topical administration of rhEGF-loaded lipid nanoparticles, namely solid lipid nanoparticles (SLN) and nanostructured lipid carries (NLC), appears to be an interesting and suitable strategy for the treatment of chronic wounds. Both rhEGF-loaded lipid nanoparticles were prepared through the emulsification-ultrasonication method; however, the NLC-rhEGF preparation did not require the use of any organic solvents. The characterisation of the nanoparticles (NP) revealed that the encapsulation efficiency (EE) of NLC-rhEGF was significantly greater than obtained with SLN-rhEGF. The *in vitro* experiments demonstrated that gamma sterilisation is a suitable process for the final sterilisation because no loss in activity was observed after the sterilisation process. In addition, the proliferation assays revealed that the bioactivity of the nanoformulations was even higher than that of free rhEGF. Finally, the effectiveness of the rhEGF-loaded lipid nanoparticles was assayed in a full-thickness wound model in *db*/*db* mice. The data demonstrated that four topical administrations of SLN-rhEGF and NLC-rhEGF significantly improved healing in terms of wound closure, restoration of the inflammatory process, and re-epithelisation grade. In addition, the data did not reveal any differences in the *in vivo* effectiveness between the different rhEGF-loaded lipid nanoparticles. Overall, these findings demonstrate the promising potential of rhEGF-loaded lipid nanoparticles, particularly NLC-rhEGF, for the promotion of faster and more effective healing and suggest their future application for the treatment of chronic wounds.

1. Introduction

Since their first description in the1990s, lipid nanoparticles, mainly solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), have become potent drug delivery systems that have attracted much attention and interest as efficient and non-toxic carriers for various active compounds [1,2]. Both lipid nanoparticles are suitable for the protection of drugs against degradation, enhance drug stability against light, oxidation, or hydrolysis, and provide the sustained release of active compounds [3]. The main difference between SLN and NLC is the use of a liquid lipid (oil) for the preparation of NLC which, as described by several authors, increases the loading capacity and reduces leakage of the encapsulated drug during storage [1,2,4]. Lipid nanoparticles are widely used through different routes of administration (such as parenteral, oral, ocular, and pulmonary administration) due to their excellent tolerability, biodegradability, and low toxicity. These nanoparticles are also suitable delivery systems for the topical treatment of skin diseases because they provide high drug concentrations in the treated skin area. In addition, systemic site effects may be reduced compared with the oral or parenteral administration routes as a result of the lower systemic drug bioavailability provided by topical administration [5,6]. Moreover, their small particle size and lipidic composition ensure close contact between the nanoparticles and the skin, the release of the encapsulated drugs in a controlled manner, and an increase in their residence time in the skin [7]. These particles also show occlusive properties that increase skin hydration and enhance drug penetration

* Corresponding author at: Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Paseo de la Universidad, 7, 01006 Vitoria-Gasteiz, Spain. Tel.: +34 945013875; fax: +34 945013040.
E-mail address: manoli.igartua@ehu.es (M. Igartua).

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[2,8,9]. Remarkably, these particles allow the administration of hydrophilic drugs on the skin, which may improve the topical treatment of skin diseases [2,10]. All of these characteristics make the use of lipid nanoparticles as drug delivery systems an interesting and suitable strategy for the treatment of chronic wounds. In fact, the incidence of chronic wounds is rising as a consequence of the ageing population, making the improvement of chronic wound treatment a major health care issue [11–13]. Chronic wounds, such as diabetic, venous, and pressure ulcers, are those that are not able to achieve anatomic and functional integrity of the injured area after six weeks of standard medical treatment [14]. These wounds are characterised by the persistence of inflammation, impaired extracellular matrix (ECM) remodelling, deficiency in growth factor and cytokine production, lack of epithelisation, and a chronically deregulated healing state that results in the wound not being cured [15]. To cope with this, many growth factors (GF), such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or transforming growth factor family (TGF), have been identified as wound healing mediators and are considered necessary for successful tissue repair [16]. Furthermore, the application of exogenous GF may accelerate healing, repair the damage tissue and reduce the risk of infection, as well as provide stimulation to induce wound healing and restore the abnormalities observed during the chronic healing process [17]. Nevertheless, the low stability of these molecules *in vivo* requires their application in a sustained and localised manner to be effective. Moreover, their undesirable effects at high systemic levels, together with the difficulties associated with the development of suitable delivery systems, limit their clinical application [18]. The early tissue engineering approaches used to improve wound healing involved the incorporation of GF into hydrogel-like formulations prepared with different biomaterials, such as chitosan, hyaluronic acid, dextran, collagen, fibrin, and gelatin [19–22]. However, these approaches gave rise to a rapid release of the active compounds, which resulted in the need to administer these formulations daily. To prolong the release profile, proteins are often encapsulated within polymeric micro and nanospheres, mainly polylactic and polyglycolic acid derivatives [23]. Unfortunately, the topical administration of polymeric micro and nanospheres was not found to show the expected success due to the rapid leakage of the proteins from the wound site, resulting in the need to administer them once a day [24,25]. To overcome these limitations and taking into account the suitability of lipid compounds for topical administration, the encapsulation of GF into lipid nanoparticles may optimise their administration in terms of dose, delivery pattern, and safety. Thus, these particles are presented as a promising alternative for chronic wound treatment.

In this study, rhEGF (recombinant human epidermal growth factor) was encapsulated into different lipid nanoparticles, i.e., SLN and NLC. *In vitro* tests were undertaken in fibroblasts and keratinocytes to determine the bioactivity of the encapsulated rhEGF and to estimate the cell uptake capability of the lipid nanoparticles. In addition, the nanoparticles were administered topically in a full-thickness excisional wound model in *db/db* mice, and their effectiveness was compared with that of several intralesional administrations of a higher dose of free rhEGF and that of a single intralesional administration of rhEGF-loaded polylactic-co-glycolic acid (PLGA) and alginate microspheres (MS-rhEGF) developed in our laboratory [26]. Healing was evaluated in terms of wound closure, recovery of the inflammatory stage, and re-epithelisation grade.

2. Materials and methods

2.1. Lipid nanoparticle preparation

SLN-rhEGF and NLC-rhEGF were prepared through the emulsification-ultrasonication-based method [27,28]. For SLN-rhEGF preparation, 10 ml of a 1% (w/v) Tween® 80 aqueous solution was mixed with 2 ml of a dichloromethane solution containing 0.1% (w/v) rhEGF (Centre for Genetic Engineering and Biotechnology, Cuba) and 5% (w/v) Precirol® ATO 5 (Gattefossé España, S.A., Spain). Immediately after mixing, the mixture was emulsified for 30 s at 50 W (Branson® 250 sonifier, CT, USA). This step produced an o/w emulsion that was then stirred for 2 h to extract the organic solvent and allow particle formation. The SLN were then collected by centrifugation at 2,500 rpm for 10 min using a 100-kDa molecular weight cut-off centrifugal filter unit (Amicon® Ultra, Millipore, Spain) and washed three times with MilliQ water. After the addition of trehalose as a cryoprotectant at a concentration of 15% (w/w) of the weighed lipid, the SLNs were freeze-dried.

For the preparation of NLC-rhEGF, 100 μl of 20 mg/ml rhEGF aqueous solution was added to a previously prepared blend composed of a warm aqueous solution of 0.67% (w/v) Poloxamer and 1.33% (w/v) Tween® 80 and a lipidic mixture containing 200 mg of melted Precirol® ATO 5 and 20 mg of Miglyol®182 that was heated at 40 ºC for 1 min. The resulting blend was emulsified for 15 s at 50 W (Branson® 250 sonifier, CT, USA) and stored for 12 h at 4 ºC to re-crystallise the lipid for NLC formation [29]. The particles were collected, washed, and lyophilised as previously described. The target loading of rhEGF in both SLN-rhEGF and NLC-rhEGF was 1% (w/w).

For the cell experiments, fluorescent lipid nanoparticles were prepared by incorporating 0.5% (w/w) Nile Red into the lipidic phase and following the method previously described.

2.2. Gamma sterilisation of lipid nanoparticles

SLN-rhEGF and NLC-rhEGF were sterilised by γ-irradiation (named SLN-rhEGF γ and NLC-rhEGF γ, respectively). The nanoparticles were placed in 5 ml glass vials and covered with dry ice to ensure a low temperature. A dose of 25 kGy from ^{60}Co was used to ensure effective sterilisation in accordance with European Pharmacopeia recommendations [30]. The effect of the sterilisation process on the nanoparticle properties and the encapsulated rhEGF was then studied.

2.3. Nanoparticle characterisation

The mean particle size (z-average) and polydispersity index (PDI) were measured by dynamic light scattering. Each assay was performed in triplicate after nanoparticle lyophilisation. The zeta potential (ζ) was determined through Laser Doppler micro-electrophoresis. All of the measurements described above were assessed using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). The particle morphology was determined through scanning electron microscopy (SEM; Jeol® JSM-35 CF) and transmission electron microscopy (TEM) after negative staining.

The nanoparticle characterization also included a physico-chemical evaluation of the pH (Crison micropH 2001), a viscosity assessment (Advanced rheometer (AR) 1000, TA Instruments), and an occlusivity test. The rheological studies involved the triplicate measurement of the viscosity of 1 ml of the nanoparticle suspension at 0.5, 1, 2.5, and 5 rpm. The procedure used for the occlusivity test was adapted from the protocol described by Souto et al. [4]. Briefly, 5 ml of water was placed in a Franz cell covered with a filter membrane (dialysis cellulose membrane, MWCO ~ 12,000, avg. flat width 33 mm, D9652, Sigma-Aldrich), and 10.6 mg/cm^2 nanoparticles were then applied to the filter surface to form a film. Cells without sample served as a reference. The samples were stored at 32 ºC for 48 h and weighted at the beginning and at the end of the experiment to obtain the water loss due to evaporation. The occlusion factor (F) was calculated using the following equation:

$$F\,(\%) = \frac{\text{Water loss without sample} - \text{Water loss with sample}}{\text{Water loss without sample}} \times 100.$$