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Nanoparticle-mediated interplay of chitosan and melatonin for improved wound epithelialisation

Filip Blažević[a], Tamara Milekić[a], Marieta Duvnjak Romić[b], Marina Juretić[a], Ivan Pepić[a], Jelena Filipović-Grčić[a], Jasmina Lovrić[a], Anita Hafner[a,*]

[a] University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Pharmaceutics, Zagreb, Croatia
[b] R&D, PLIVA Croatia Ltd., TEVA Group Member, Zagreb, Croatia

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ABSTRACT

Herein, we propose an innovative approach to improving wound healing. Our strategy is to deliver melatonin locally at the wound site by means of lecithin/chitosan nanoparticles. We used four types of chitosan that differed in terms of molecular weight and/or deacetylation degree. Melatonin encapsulation efficiency, nanoparticle size, zeta potential, biocompatibility and in vitro drug release were studied as a function of the type of chitosan used in preparation. The nanoparticles were evaluated in terms of their potential to promote wound epithelialisation via an in vitro scratch assay using a human keratinocyte (HaCaT) monolayer. The model wounds were treated with nanoparticle suspensions at a chitosan concentration of 5 $\mu g\,ml^{-1}$, which was based on preceding cell biocompatibility studies. Nanoparticles prepared with different types of chitosan showed similar effect on the keratinocyte proliferation/migration. Nanoparticle-mediated interplay of chitosan and melatonin was shown to be crucial for improved wound epithelialisation.

1. Introduction

Wound healing is a dynamic biological process that involves complex interactions among cells, the extracellular matrix and signalling molecules and results in wound closure (Muzzarelli, 2009). Chronically and acutely infected wounds represent a significant clinical problem, due to the failure of the tissue to progress through the necessary stages of healing and due to possible complications (Dreifke, Jayasuriya, & Jayasuriya, 2015; Thomas, Motlagh, Povey, & Percival, 2011).

Innovative wound dressings are designed to provide the conditions required for optimal wound healing, such as adequate wound humidity, temperature, pH, gaseous exchange and the prevention/treatment of infection. The latest trends in functional wound dressing development are based on (bio)polymers that have the potential to improve wound healing by influencing the migration and proliferation of skin cells and by exerting antimicrobial or haemostatic effects. When formulated into nanosystems, such polymers can serve as carriers for different active compounds, such as antibiotics, anti-inflammatory agents, antioxidants, antiseptics, antibodies or other bioactives that rectify both structural and physiological imbalances at the wound site (Mayet et al., 2014). In addition to delivering these active compounds, polymeric nanosystems can improve the wound healing process by moderating the microenvironmental conditions at the wound site, depending on the polymeric composition and specific physico-chemical properties of the system, such as size (and related specific surface area), surface charge, swelling ability and rheological properties (De Cicco, Porta, Sansone, Aquino, & Del Gaudio, 2014). Considering the problems related to the excessive adherence of conventional wound dressings and possible reinjury at the wound site during dressing removal, biodegradable moisture-retentive polymers represent ideal materials for innovative wound dressing development (Dreifke et al., 2015).

Chitosan, which is a cationic polysaccharide, is a biocompatible, biodegradable and nontoxic biopolymer with an inherent ability to assist with wound pathophysiology. In addition to exhibiting haemostatic and antimicrobial activities, chitosan accelerates wound healing and helps in scar prevention (Minagawa, Okamura, Shigemasa, Minami, & Okamoto, 2007; Paul & Sharma, 2004). More specifically, chitosan can serve as a non-protein matrix for three-dimensional tissue growth. Chitosan activates macrophages for tumouricidal activity and stimulates cell proliferation and tissue organisation. Gradually depolymerising to

* Corresponding author at: A. Kovacica 1, 10000 Zagreb, Croatia.
E-mail address: ahafner@pharma.hr (A. Hafner).

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N-acetyl-β-D-glucosamine, chitosan initiates fibroblast proliferation, promotes ordered collagen deposition and stimulates natural hyaluronic acid synthesis at the wound site (Paul & Sharma, 2004). A chitosan wound dressing was also reported to enhance wound re-epithelialisation in comparison to conventional wound dressings (Kiyozumi et al., 2006; Stone, Wright, Clarke, Powell, & Devaraj, 2000).

The potential of melatonin to promote wound healing has been addressed by many investigations (Lee, Jung, Oh, Yun, & Han, 2014; Ozler et al., 2010; Soybir et al., 2003). Melatonin was shown to influence different processes involved in wound healing, such as the release of inflammatory mediators, cell proliferation and migration, angiogenesis, and the accumulation of collagen and glycosaminoglycans at the wound site. The final effect of melatonin was shown to be dependent on the dose used, the time of application and the target tissue (Drobnik, 2012). The inconsistent reports concerning whether melatonin has a positive (Carossino, Lombardi, Matucci-Cerinic, Pignone, & Cagnoni, 1996; Drobnik et al., 2013) or negative effect (Bulbuller et al., 2005; Drobnik & Dabrowski, 1996) on collagen accumulation at the wound site could partially be explained by the discrepancy between melatonin's direct effect on the cells at the wound site and its indirect action via (systemic) general regulatory mechanisms (Drobnik, 2012). Therefore, it seems reasonable to develop a delivery system that is intended to administer melatonin locally, avoiding high systemic exposure. In addition, the intradermal administration of exogenous melatonin was shown to improve the quality of wound healing and scar formation (Pugazhenthi, Kapoor, Clarkson, Hall, & Appleton, 2008).

In the present study, we aimed to prepare melatonin-loaded lecithin/chitosan nanoparticles using chitosans that differ with respect to molecular weight (MW) and deacetylation degree (DD) and to evaluate the effects of those nanoparticles on wound epithelialisation in vitro in relation to melatonin content and chitosan properties. The melatonin encapsulation efficiency, size, zeta-potential, biocompatibility and in vitro drug release properties of the nanoparticles were studied as a function of the type of chitosan used for their preparation. The melatonin-loaded nanoparticles were evaluated in terms of their potential to promote wound epithelialisation via an in vitro scratch wound healing assay using a monolayer of human keratinocytes (HaCaT cells).

2. Materials and methods

2.1. Reagents and chemicals

The following materials were used as received: four types of chitosan in the form of hydrochloride salts (Protasan® UP CL 113 (C113; DD 75–90%; MW 50–150 kDa), CL 114 (C114; DD > 90%; MW 50–150 kDa), CL213 (C213; DD 75–90%; MW 150–400 kDa) and CL214 (C214; DD > 90%; MW 150–400 kDa), Novamatrix, Norway); soybean lecithin Lipoid S45 (commercial mixture of lipids, phospholipids, and fatty acids containing 45% phosphatidyl choline; a kind gift from Lipoid GmbH, Germany); and melatonin (Sigma–Aldrich Chemie GmbH, Germany). Hank's balanced salt solution (HBSS, pH 6.0) was prepared by dissolving the following substances in double-distilled water: $MgSO_4 \times 7H_2O$ (100 mg l^{-1}), KCl (400 mg l^{-1}), $NaHCO_3$ (350 mg l^{-1}), NaCl (8000 mg l^{-1}), D-glucose monohydrate (1000 mg l^{-1}) (all purchased from Kemig, Zagreb, Croatia), $CaCl_2 \times 2H_2O$ (185 mg l^{-1}) (Sigma-Aldrich), $MgCl_2 \times 6H_2O$ (100 mg l^{-1}) (Merck KGaA, Darmstadt, Germany), KH_2PO_4 (60 mg l^{-1}) (Kemika, Zagreb, Croatia), $Na_2HPO_4 \times 2H_2O$ (60 mg l^{-1}) (Fluka Chemie AG, Buchs, Switzerland), and HEPES (7150 mg l^{-1}) (Applichem, Darmstadt, Germany). All other chemicals or solvents were of analytical grade and were purchased from Kemika Zagreb, Croatia and Merck, Darmstadt, Germany.

2.2. Preparation of lecithin/chitosan NPs

The melatonin-loaded lecithin/chitosan nanoparticles were prepared as reported previously (Hafner, Lovric, Voinovich & Filipovic-Grcic, 2009). Lecithin was dissolved in 96% ethanol at a concentration of 25 mg ml^{-1}. Melatonin was dissolved in the ethanolic solution of lecithin at a concentration of 5 mg ml^{-1} to obtain a lecithin-to-melatonin weight ratio of 5:1. Each type of chitosan was solubilised in distilled water at a concentration of 10 mg ml^{-1}. The chitosan solutions (0.5 ml) were further diluted with distilled water to a volume of 46 ml. Melatonin-loaded lecithin/chitosan nanoparticles (MLC113, MLC114, MLC213 and MLC214) that differed with respect to the type of chitosan used for preparation were obtained via the injection of 4 ml of the ethanolic lecithin/melatonin solution (syringe inner diameter of 0.75 mm) into 46 ml of a diluted chitosan solution (C113, C114, C213 or C214) with magnetic stirring (900 rpm). A final chitosan concentration of 100 μg ml^{-1} and a lecithin-to-chitosan weight ratio of 20:1 was obtained in the prepared nanoparticle suspensions. For comparison, melatonin-loaded lecithin nanoparticles (ML) were prepared via the injection of ethanolic melatonin/lecithin solutions into distilled water. Non-entrapped melatonin was separated from the melatonin-loaded nanoparticles using a dialysis technique, as described in Section 2.3. Melatonin-free nanoparticles were prepared following the same procedure described for melatonin-loaded nanoparticles, omitting melatonin (L, LC113, LC114, LC213 and LC214 nanoparticles).

2.3. Determination of melatonin loading

The encapsulation efficiency and drug content in the suspension of nanoparticles were measured by determining the amount of non-entrapped melatonin using the dialysis technique. The dialysis of nanoparticle suspensions was performed as reported previously (Hafner et al., 2009). Briefly, a 4 ml aliquot of a melatonin-loaded nanoparticle suspension was placed in a cellulose acetate dialysis bag and sealed (Spectra/Por® 4 Dialysis Tubing, MWCO 12–14 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, California, USA). The samples were dialysed against 100 ml of distilled water (receiver phase), with continuous magnetic stirring at 30 rpm and under sink conditions. At scheduled time intervals, 2 ml samples were withdrawn from the receiver phase and replaced with the same amount of distilled water. The dialysis was stopped when constant drug concentration values were detected in subsequent withdrawals from the receiver phase, taking into account the progressive dilution of the receiver phase. The withdrawn samples were assayed for melatonin content using a UV–vis spectrophotometer (λ = 278 nm) (Cary 50, Varian, Inc., USA) after dilution with 1 ml of distilled water. The dialysed nanoparticle suspension samples were used in all further studies.

2.4. Physical characterisation of the particle size and surface charge

The size and zeta potential of the nanoparticles were measured via photon correlation spectroscopy (PCS) using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). The nanoparticle samples were diluted with 0.45 μm filtered distilled water and a 10 mM NaCl solution prior to the measurement of the size and zeta potential, respectively. The pH of the diluted samples ranged from 5.8 to 6.0. The zeta potential measurements were performed at 25 °C. The samples were placed in the electrophoretic cell, where a potential of 150 mV was established.