Carbohydrate Polymers 156 (2017) 86–96

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Hyaluronan polymeric micelles for topical drug delivery

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ARTICLE INFO

Article history:
Received 8 August 2016
Received in revised form 30 August 2016
Accepted 4 September 2016
Available online 5 September 2016

Keywords:
Skin penetration
Polymeric micelle
Hyaluronan

ABSTRACT

Nanosized materials offer promising strategy for topical drug delivery due to their enhancing effect on drug percutaneous transport across the *stratum corneum* barrier. In this work, polymeric micelles made from hydrophobized hyaluronic acid (HA) were probed for skin delivery. Compared to non-polymeric micelle solutions containing similar drug amount, *in vitro* skin penetration analysis indicated 3 times larger deposition of drug in the epidermis and 6 times larger drug deposition in the dermis after 5 h of topical treatment in Franz diffusion cells. The drug deposition was further increased with prolonged time of topical treatment. Laser confocal microscopy revealed the accumulation of both, the HA forming the vehicle and the payload, in the epidermis and dermis. Although fluorescent labeling of the HA would suggest co-transport of the HA and the drug, loading FRET pair dyes in the micellar core clearly demonstrated gradual micelle disruption with increasing skin depth. Transcellular penetration was the predominant pathway for the loaded drug. The HA polymeric micelles also demonstrated increased bioactivity of loaded compound *in vitro* and *in vivo*. In addition, the loaded micelles were found to be stable in cream formulations and thus they have great potential for topical applications for cosmetic and pharmaceutical purposes.

1. Introduction

Nanosized materials such as liposomes, micelles, nanoparticles, nanoemulsions and polymeric suspensions are often reported to improve skin penetration of otherwise skin impermeable drugs into the *stratum corneum*, the topmost skin layer barrier (Karande & Mitragotri, 2009; Kong, Chen, Kweon, & Park, 2011; Lapteva, Mondon, Möller, Gurny, & Kalia, 2014; Lauterbach & Müller-Goymann, 2014; Yang, Bugno, & Hong, 2013). For this reason, colloidal nanosized systems have attracted great attention for topical administration of both cosmetically and pharmaceutically interesting compounds either for local or systemic delivery. At the same time, clinical applicability of the nanostructured systems as skin passive penetration enhancers is still under debate mainly due to the insufficient understanding of the interaction of nanosized drug delivery vehicles with the skin. This also includes limited knowledge about the fate and deposition of the vehicle material and drug in the skin layers after application (Wu, Price, & Guy, 2009). Compared to larger particles, nanosized systems are more likely to penetrate the *stratum corneum.* In this way, the nanoparticles have higher probability to access in the deeper skin layers including the circulatory system (Wu, Price, & Guy, 2009) and for this reason the health risk should be always assessed, mainly in case of long term skin exposure to nanosized systems (Bolzinger, Briancon, & Chevalier, 2011).

A number of attempts to make general rules regarding particle penetration from published results pointed out apparent inconsistences among the concluded observations (Bolzinger, Briancon & Chevalier, 2011; Contri, Fiel, Pohlmann, Guterres &, Beck, 2011; Prow et al., 2011). The reason for the data contradiction is probably the variety of nanomaterial constituents, used skin models and applied methodologies (Prow et al., 2011). The issue is quite complex and there is a number of potentially relevant parameters (Bolzinger, Briancon, & Chevalier, 2011). Besides the nanomate-

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http://dx.doi.org/10.1016/j.carbpol.2016.09.013
0144-8617/

rial size, other physico-chemical properties of the system including surface chemistry, shape, charge, amphiphilicity, porosity, aggregation and elasticity significantly affect interaction of nanomaterials with skin (Bolzinger, Briancon, & Chevalier, 2011; Yang, Bugno, & Hong, 2013).

To minimize the risk of non-biodegradable foreign particles accumulation in the body, a major area of polymer based drug delivery research is focused in the design of biodegradable polymer systems. Among these, hyaluronan (HA) represents an interesting material as topical drug delivery vehicle, especially in skin drug delivery, because HA is present in both the epidermis and dermis and constitutes a large fraction of skin extracellular matrix. In fact, the skin is believed to be a reservoir of about 1.7 g of HA, which is approximately one third of the total amount of HA expected to be present within the entire human body (Brown & Jones, 2005; Papakonstantinou, Roth, & Karakiulakis, 2012). HA is a natural polyanionic polysaccharide composed of repeating disaccharides that consist of *N*-acetyl-β-D-glucosamine and β-D-glucuronic acid linked by a 1 → 4 glycosidic bond. The disaccharides are linked by 1 → 3 bonds to form HA chain. HA hydrogels have been reported to be able to localize water soluble therapeutic agents in the superficial layers of the skin (Brown & Jones, 2005) and to improve protein drug delivery (Witting et al., 2015). Hyalurosomes, prepared from HA and soy phosphatidyl choline, improved curcumin deposition in the epidermis and dermis (Manca et al., 2015). Although there are still many questions regarding the mechanism of HA enhanced drug penetration, molecular weight and concentration of HA seem to play a crucial role in drug delivery. Regarding the delivery of hydrophilic drugs, shorter HA fragments (tens to hundreds of kDa) were more efficient in drug penetration (Brown & Jones, 2005; Cilurzo et al., 2014; Witting et al., 2015). Other factors, including active transport via HA receptors, specific structure of hydrated HA, and possible cotransport are also often discussed (Witting et al., 2015). HA in its native form contains hydrophobic patches formed in the secondary structure and so although being highly hydrophilic, it may non-covalently bind a limited amount of non-polar drugs (Scott, Cummings, Brass, & Chen, 1991). The binding capacity of HA for non-polar drugs may be enhanced by HA hydrophobization (Smejkalova et al., 2014), for example by esterification with fatty acids (Huerta-Angeles, Bobek, Prikopova, Smejkalova, & Velebny, 2014). The hydrophobized HA undergoes self-assembling in aqueous solutions while forming core-shell structures (polymeric micelles), where HA forms the hydrophilic shell, while the acyl chains aggregate in the hydrophobic core. This core may accommodate non-polar drugs through non covalent interactions.

Since both polymeric micelles and HA in its native form are often described as skin penetration enhancers (Witting et al., 2015; Yang, Bugno, & Hong, 2013), one would expect HA based micelles to improve skin penetration of hydrophobic drugs. To address this issue, two acylated derivatives of HA with short (hexyl HA, HAC6) and medium chain length (oleyl HA, HAC18:1) were prepared, transformed into polymeric micelles and at first loaded with Nile Red (NR) to simulate the hydrophobic active substance. The loaded micelles were topically (*in vitro*) applied to porcine skin and the fate of NR was followed and compared with control samples, qualitatively and quantitatively. In addition to the fate of loaded drug, we also tried to evaluate the penetration of the acylated HA, forming the drug delivery vehicle, by covalent labeling of acylated HA with Nile Blue and following its fluorescence signal in skin. Finally, the HA polymeric micelles were loaded with cosmetic active (coenzyme Q10), added in o/w cream formulations and tested for *in vivo* efficacy on human volunteers.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (HA) (M_w = 15,000 g/mol) was provided by Contipro a.s. (Dolní Dobrouč, Czech Republic). Isopropanol (IPA), triethylamine (TEA), *cis*-oleic acid (C18:1), sodium chloride (NaCl), tetrahydrofurane (THF) and chloroform were obtained from Lach-ner (Czech Republic). Hexanoic acid (C6), benzoyl chloride (BC), 4-dimethylaminopyridine (DMAP), 4-acetamido-2,2,6,6-tetramethylpiperidine1-oxyl (4-Ac-TEMPO), disodium hydrogen phosphate ($NaHPO_4.12\,H_2O$), sodium bromide (NaBr), sodium hydroxide, sodium hypochlorite, Nile Red (NR) and Nile blue A (NB) are commercially available products from Sigma-Aldrich. 3,3′-dioctadecyloxacarbocyanine, perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate (Dil) were obtained from Biotium Inc. (USA). Triglycerides (caprylic/capric triglyceride), glycerine, cream maker blend (glyceryl stearate, PEG-100-stearate), gel maker EMU (sodium acrylate, acryloyldimethyl taurate copolymer, isohexadecane, polysorbate 80), vitamin E acetate and benzyl alcohol for cream composition were purchased from MakingCosmetics (Snoqualmie, WA, USA). The cream composition consisted of 75.2% demineralized water, 12% triglycerides, 5% cream maker blend, 4% glycerine, 2% gel maker EMU, 1% vitamin E acetate and 0.8% benzyl alcohol.

2.2. Synthesis of oxidized hyaluronan (HA-ox)

HA (20.0 g, 50 mmol, M_w = 15,000 g/mol) was dissolved in water (1000 mL). Afterwards, sodium bromide (2.6 g, 25 mmol) and disodium hydrogen phosphate (38.8 g, 108 mmol) were added to the reaction mixture. The pH was adjusted to 9.0 with 0.1 M of sodium hydroxide. The solution was cooled to 5 °C for 2 h, bubbled with nitrogen and 4-Ac-TEMPO previously dissolved in 1 mL of water (106.7 mg, 0.5 mmol) was added. Finally, 6 mL of sodium hypochlorite (12.5 mmol) was added to the reaction. The oxidation was carried out for 15 min and stopped by adding 250 mL of isopropanol and diluted with water (1000 mL). The solution was ultrafiltered through a 5 kDa cut-off cassette (Merck-Millipore). The product was recovered after precipitation with isopropanol, decantation and dried in an oven at 60 °C for 24 h. The obtained degree of substitution (DS) of the aldehyde was 5.5 %, calculated by integration of the signal at 5.2 ppm with respect to the $-NH-COCH_3$ located at 2.1 ppm. Yield of the reaction was 86.5%. M_w of the product (SEC-MALLS): 15,800 g/mol and polydispersity: 1.6. FT-IR (KBr, cm^{-1}): 3419 (υ, C—OH), 2923, 1656, 1614 (—C=O), 1413 (-O—C=O), 1153, 1080 υ (C—OH), 1039, 607. ^{1}H NMR (500 MHz, NaOD): δ 2.1 (s, 3H, $-NH-COCH_3$), 3.4–4.0 (m, 10H, skeletal, CH), 4.4–4.6 (m, 2H, anomeric, CH), 5.2 (s, 1H,-$CH(OH)_2$).

2.3. Synthesis of oleyl hyaluronan grafted with Nile blue (HAC18:1-NB)

At first, oxidized HA (HA-ox) was grafted with Nile Blue, to produce HA-NB (Fig. S7). 500 mg of HA-ox (M_w: 15,800 g/mol and DS of 5.5%, 1.25 mmol) was dissolved in distilled water (25 mL). To that solution, Nile Blue (88 mg, 0.12 mmol), previously dissolved in 10 mL of DMSO was added. The reaction was allowed to react for 5 h at 25 °C in darkness. After that time, a reduction of the imine was carried out for 24 h at 25 °C by using $NaBH_3CN$ (78 mg, 1.25 mmol). After that time, the product was isolated by precipitation using excess of isopropanol (250 mL) and saturated NaCl solution, washed with anhydrous isopropanol (10 × 50 mL). The obtained precipitate was dissolved in demi-water and dialyzed (cut off 3.5 kDa) against 1% NaCl and 1% $NaHCO_3$ aqueous solution (2 × 5 L), distilled water (4 × 5 L). The product was isolated by freeze-drying.