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Self-assembled hyaluronic acid nanoparticles for controlled release of agrochemicals and diosgenin

Javier Pérez Quiñones[a,*], Oliver Brüggemann[a], Carlos Peniche Covas[b], Dmitri A. Ossipov[c]

[a] Institute of Polymer Chemistry, Johannes Kepler University Linz, Linz, Austria
[b] Center of Biomaterials, University of Havana, Havana, Cuba
[c] Department of Chemistry-Angstrom, Uppsala University, Uppsala, Sweden

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ABSTRACT

Commercial sodium hyaluronate (HA) and synthetic hydrazide-modified HA were functionalized with diosgenin and two agrochemicals (brassinosteroids DI31 and S7) with degree of substitution ranging from 5.6 to 13.1%. The HA-steroid conjugates were studied with FTIR, ^{1}H NMR and differential scanning calorimetry. Dynamic light scattering revealed self-assembly of the HA-steroid conjugates into stable negatively charged nanoparticles of around 159 nm–441 nm in water, which after drying appeared as 140 nm–370 nm spherically shaped nanoparticles according to transmission electron microscopy. These nanoparticles exhibited almost constant release rates of steroids for the first 8 h, demonstrating sustained steroids delivery for 72 h in acidic medium. The nanoparticles formed from HA-steroid conjugates were not cytotoxic to human microvascular endothelial cells (HMVEC), while the HA- brassinosteroid nanoparticles showed in vitro agrochemical activity that was superior to the activity observed for the parent brassinosteroids DI31 and S7 at 10^{-5} to 10^{-7} mg mL^{-1}.

1. Introduction

Hyaluronic acid (HA) is a linear high molecular weight natural biopolymer which consists of repeating → 4)-β-D-glucuronic acid- (1 → 3)- β-*N*-acetyl-D-glucosamine-(1 → disaccharide units. Molecular weight of HA ranges from hundreds to millions of Daltons (Chytil & Pekar, 2009). This only non-sulfated glycosaminoglycan has been mainly found in extracellular matrix of connective tissues (vitreous humour of the eye and the synovial fluid in the joints) and it was isolated from human umbilical cord residues (Lago et al., 2005). A comparative study on in vitro organogenesis in protocorm-like body (PLBs) of *Cymbidium dayanum* revealed that hyaluronic acid could act as a plant growth regulator with superior agrochemical activity compared to other saccharides including chitosan and *N*-acetyl glucosamine. Particularly, HA enhanced both PLBs and shoots formation at concentrations of 10^{-3} and 10^{-6} mg mL^{-1} within 30 days of culture (Syeda, Shimasaki, Huang, & Naruemol, 2011). Moreover, hyaluronic acid with molecular weight from 8 to 11.7 kDa has been reported as effective in rhizome growth and shoot formation of *Cymbidium kanran* at a concentration of 10^{-5} mg mL^{-1} (Kamal, Shimasaki, & Akter, 2014), while applications of HA at higher concentrations of 10^{-2} mg mL^{-1} showed a negative impact on organogenesis in PLBs of *Dendrobium kingianum* (Habiba, Shimasaki, Ahasan, & Alam, 2014). In addition, HA has been found to suppress pathogens in cucumber, pepper and tomato (Park, Paul, Kim, & Joseph, 2008) and to show antioxidant activity in PLBs of hybrid *Cymbidium* (da Silva, Uthairatanakij, Obsuwam, Shimasaki, & Tanaka, 2013). On other hand, studies on thermal degradation and stability of sodium hyaluronate of molecular weights between 0.4 and 2.3 MDa in solid state showed that the biopolymer depolymerization is reduced at neutral pH and low temperatures (negligible at 5 °C, ca. 10% at 40 °C after 3 months) (Caspersen et al., 2014). Therefore, solid formulations based on HA should remain stable and not depolymerized if stored properly at low temperatures over moderate times (1–12 months). However, hyaluronic acid is a biodegradable polymer, which is gradually degraded by enzymes hyaluronidases in mammals, invertebrates (insects, crustaceans), and some pathogenic fungi and bacteria (El-Safory, Fazari, & Cheng-Kang, 2010; Goodarzi, Varshochian, Kamalinia, Atyabi, & Dinarvand, 2013). Nevertheless, it has been shown that clinical efficacy of hyaluronans, and especially high molecular weight hyaluronan derivatives and cross-

* Corresponding author.
E-mail addresses: javenator@gmail.com, bulldogcu@fastmail.fm (J.P. Quiñones).

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linked HA, injected in the knee during osteoarthritis treatments is preserved for months despite the clearance of exogenous intra-articular hyaluronan in few days (Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012).

HA industrial production is based on the extraction from animal tissues, mostly from combs of cockerels, and microbial fermentation using bacterial strains of *Streptococcus zooepidemicus* (Boeriu, Springer, Kooy, van den Broek, & Eggink, 2013; Muzzarelli et al., 2012). Recent research was focused on reduction of HA production cost by optimizing the conditions of microbial production from recombinant microorganisms (Kaur & Jayaraman, 2016). Importantly, the use of cheap crude materials from the other industrial process such as renewable agricultural or marine resources for conversion into valuable HA bioproducts is expected to satisfy the needs of sustainable society. Particularly, mussel processing wastewaters, a glycogen-rich residual material from canning companies, and peptone from tuna viscera by-products were used as culture media for HA production by *Streptococcus zooepidemicus,* which resulted in more than 30% reduction of the production cost (Vázquez, Montemayor, Fraguas, & Murado, 2010). For another example, cashew apple juice was a promising medium for the microbial HA production (Pires, Macedo, Eguchi, & Santana, 2010).

HA has been prepared as films and hydrogels crosslinked with carbodiimide (Grundelova et al., 2015) or glutaraldehyde or divinyl sulfone (Collins & Birkinshaw, 2008). HA polyelectrolyte complexes with chitosan formed microspheres (Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004) and hydrophobic modification of HA resulted in generation of nanoparticles in aqueous environment (Chytil, Strand, Christensen, & Pekar, 2010), which was used in medical and cosmetic applications. Specific interactions of hyaluronans with CD44 cell-surface receptors, which are over-expressed in tumor cells, laid a ground for the development of controlled anticancer drug delivery systems with active targeting to some tumors. For example, HA dually functionalized with chemically orthogonal groups allowed formation of reactive HA hydrogels which were further modified with pyrene (Ossipov, Yang, Varghese, Kootala, & Hilborn, 2010). Enzymatic degradation of such hydrogel resulted in the formation of nanoparticles and their subsequent active uptake by cancer cells. The same nanoparticles obtained in a bottom-up approach were applied for loading and release of the anticancer drug doxorubicin (Yang, Kootala, Hilborn, & Ossipov, 2011). Dual modification of HA with trifluoroethyl acrylate and carbazate groups allowed to obtain an injectable hyaluronic acid hydrogel with the potential of ^{19}F magnetic resonance in animals (Yang et al., 2014).

Diosgenin ((25R)-spirost-5-en-3β-ol) is a steroidal sapogenin prepared by acidic, basic or enzymatic hydrolysis of several saponins, but mostly from dioscin, the most common steroidal saponin. It exhibits hypocholesterolemic, antioxidant, anti-inflammatory and estrogen activity (Patel, Gadewar, Tahilhany, & Patel, 2012; Selim and Jaouni, 2015). In addition, diosgenin and dioscin show cytotoxic, antitumor and anti-metastatic activities to different cancer cell lines (Chen, Shih, Huang, & Cheng, 2011; Selim & Jaouni, 2015; Srinivasan et al., 2009). Diosgenin is a starting compound in chemical synthesis of several steroids (i.e. progesterone, corticosteroids and contraceptives), because this suitable chemical has the required basic backbone and stereochemistry (Selim and Jaouni, 2015). For example, diosgenin is the synthetic precursor of two Cuban analogues of brassinosteroids (DI31 and S7) employed as agrochemicals over the last twenty years under the commercial trademark of Biobras-16. These two synthetic brassinosteroids stimulate plants growth and defense mechanism of the crops once applied, with increases in harvest of 5% to 30% (Serrano et al., 2015; Terry et al., 2012). However, the expected agrochemical benefits are not completely realized in plants because these compounds are quickly metabolized. Therefore, up to three foliar spray applications are usually needed, which increases economic cost of the brassinosteroids employment (Terry et al., 2012). On other hand, the hydrophobicity of DI31 and S7 also limits their bioavailability to plants and current commercial formulations include ethanol, *N*,*N*-dimethylformamide and other environmentally unfriendly chemical additives. We hypothesized that preparation of novel biodegradable solid formulations of diosgenin and brassinosteroids DI31 and S7 by conjugation to hyaluronic acid via a hydrolysable ester bond should improve bioavailability of the parent steroids and provide their sustained release over several days. Moreover, production of agrochemicals based on HA-steroid conjugates from inexpensive agrochemical resources was envisaged to contribute into building of sustainable society. In this work, we aimed at the development of HA-based delivery systems for the sustained release of agrochemicals. For this purpose, we prepared HA-steroid conjugates with three different steroids linked via two types of linkers, characterized the HA-steroid conjugates by Fourier transform infrared (FTIR) and proton nuclear magnetic resonance (^1H NMR) spectroscopy, as well as examined self-assembly of the conjugates by dynamic light scattering (DLS) and transmission electron microscopy (TEM). In vitro release of the steroids from the obtained nanoparticles was investigated under the conditions of hydrolysis of ester bond. Bioactivity of the nanoparticles towards radish (*Raphanus sativus*) was first investigated. To the best of our knowledge, this is the first report on the employment of HA matrix or particles for the delivery of agrochemicals. Additionally to agrochemical applications the prepared nanoparticles were also envisioned as nanomedicines in possible cancer treatment.

2. Experimental

2.1. Materials

Sodium hyaluronate with molecular weight of 7 kDa (average $M_n = 7180\ g\ mol^{-1}$, $M_w/M_n = 1.03$ as determined by gel permeation chromatography (GPC)) was purchased from Lifecore Biomedical. Hemisuccinates of diosgenin and two synthetic analogues of brassinosteroids with agrochemical activity (DI-31 and S7) were synthesized by base- catalyzed traditional esterification in pyridine with succinic anhydride (Abe, Hasunuma, & Kurokawa, 1976). The diosgenin and synthetic analogues of brassinosteroids (DI31 and S7) were supplied by the Center of Natural Products at University of Havana, Cuba. For modification of HA with hydrazide groups, linker **1** [disulfanediylbis(ethane-2,1-diyl) bis(2-(6-((hydrazinecarbonyl)oxy)-hexanoyl) hydrazinecarboxilate)] (see structure in Fig. 1) was synthesized according to our reported procedure (Ossipov, Piskounova, Varghese, & Hilborn, 2010). Solvents and chemicals employed were purchased from Sigma-Aldrich and used without further purification.

2.2. Synthesis of steroid intermediates for modification of HA

Steroid hemisuccinate *N*-hydroxysuccinimide esters (Fig. 1) required for functionalization of HA were synthesized in accordance to our previous protocol. To this end, ca. 60–65 mg (0.1 mmol) of steroid hemisuccinates were dissolved in 6 mL of dried CH_2Cl_2, 30 mg (0.15 mmol) of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 20 mg (0.17 mmol) of *N*-hydroxysuccinimide (NHS) were added, and the mixture was stirred for 24 h at room temperature. The precipitated *N*-dicyclohexylurea was removed by filtration trough 0.2 μm Nalgene syringe filter. An aliquot corresponding to one third of the filtered volume was taken and the solvent (CH_2Cl_2) was evaporated with nitrogen flow.