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Eco-friendly microwave-assisted protocol to prepare hyaluronan-fatty acid conjugates and to induce their self-assembly process

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ABSTRACT

An environmentally sustainable and energy-efficient synthetic process has been developed to prepare hyaluronan-based nano-sized material. It consists in a microwave-promoted acylation of the hydroxyl function of the polysaccharide with natural fatty acids, performed under solvent-free conditions. The efficient interaction of the solid reagents with the MW radiation accounts for the obtained high yielded products. The self-assembly process of the obtained compounds very fast occurred in an aqueous medium under MW-radiation, thus allowing the development of a green protocol for the nano-particles preparation.

1. Introduction

HA, member of the glycosaminoglycan family, consists of a linear polysaccharide with repeating beta-1,4-linked D-glucuronic acid (GlcA) and beta-1,3-linked N-acetyl-D-glucosamine (GlcNAc) disaccharide units. Due to its inherent biocompatibility and biodegradability, as well as its susceptibility to chemical modification, several HA-based biomaterials with promising biomedical potential have been developed (Lee, Mok, Lee, Oh, & Park, 2007; Manju & Sreenivasan, 2011; Mironov et al., 2005; Peer & Margalit, 2004). In particular, by chemical conjugation of hydrophobic moieties to the hydrophilic HA backbone, several nano-aggregates were prepared and studied as nano-sized drug delivery system for cancer treatment (Cho et al., 2011, 2012; Choi et al., 2009, 2010; Ganesh, Iyer, Morrisey, & Amiji, 2013; Huang et al., 2014; Schanté, Zuber, Herlin, & Vandamme, 2011).

In this regard, we have recently developed amphiphilic HA-derivatives by chemical conjugation of natural fatty acids to the backbone of the polysaccharide, and the obtained materials were able to self-assemble, forming spherical nano-particles with a quite small size (Calce et al., 2015). The smallest aggregates have been considered for a potential employment as stable and effective drug delivery system for biomedical applications. The employed synthetic procedure uses solvent-free conditions and microwaves of domestic oven for activation, in other words it consists in a MW-induced solid-state reaction that provides the great advantage of a clean, fast and energy-efficient synthetic method. Compared to the solution-phase MW synthesis, the solid-state MW-activation has experienced a narrow application into the field of organic chemistry, this is due to the less comprehensive MW-solid interactions (Kappe, 2013; Kitchen et al., 2014). Moreover, there are serious difficulties associated with monitoring temperature conditions inside the solid. On the other hand, it has been hypothesized an increased mobility of polar solid substrates under MW radiations that increases the heating rate. Therefore, milder reaction conditions can be used for chemical process to produce high yielded final products (de la Hoz, Diaz-Ortiz, & Moreno, 2005).

Herein, we report on a microwave-assisted synthetic protocol to prepare HA-fatty acid conjugates characterized by defined degrees of substitution. The reaction parameters were deeply investigated and a tuned synthetic protocol was developed to prepare HA-oleate and HA-linoleate. This procedure not only shortens the reaction time, but also improves its yield and reproducibility. Moreover, also the self-assembly process of the polysaccharides conjugates

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was driven by the MW irradiation. The obtained materials were characterized for their properties, like the degree of substitution, the particle size, zeta potential and also for their morphology.

2. Experimental

2.1. Synthesis and characterization of HA-fatty acids conjugated

2.1.1. Synthesis

Fatty acid anhydrides were synthesized as follows. The appropriate fatty acid (10 mmol) was dissolved in dichloromethane (2 mL), the solution was cooled in an ice-water bath and stirred vigorously under argon atmosphere. The dicyclohexylcarbodiimide (5 mmol), previously dissolved in the minimum volume of dichloromethane, was added and the stirring was continued at ice bath temperature for 2 h. The white solid N,N′-dicyclohexylurea was removed by filtration and the solvent was evaporated in vacuum to give the final anhydride.

By using an agate mortar, hyaluronan and the appropriate fatty acid anhydride, at the same weight ratio, were manually milled in presence of catalytic amount of K_2CO_3 to obtain several hyaluronan derivatives.

The samples, placed in a 0.5–2 mL microwave vial, were irradiated for 2 min in a microwave oven (Initiator, Biotage Sweden AB, Uppsala, Sweden) under the following conditions:

- 80 °C, P max = 200 W (compounds **1a** and **2a**);
- 120 °C, P max = 220 W (compounds **1b** and **2b**);
- 160 °C, P max = 250 W (compounds **1c** and **2c**),
- 200 °C, P max = 300 W (compounds **1d** and **2d**);
- 100 W, T max = 53 °C (compounds **1e** and **2e**);
- 200 W, T max = 81 °C (compounds **1f** and **2f**).

After cooling at room temperature, the obtained solid was dissolved in water, placed in a 250 mL separatory funnel and extracted with ethyl acetate in order to remove the unreacted fatty acids. Subsequently, the aqueous layer was neutralized by adding 0.5 N HCl solution in water and then dialyzed (membrane cut off 6000–8000 Da) for 1 day in Milli-Q water.

The final product was collected after lyophilization process.

2.1.2. NMR characterization

1D [^{1}H] NMR spectra were recorded for HA-oleate and HA-linoleate in the temperature range 298–303 K either on a Varian Unity Inova 400 MHz NMR spectrometer equipped with *z*-axis pulsed-field gradients and a triple resonance probe or a Varian 600 MHz spectrometer with a cold probe. To prepare NMR samples, compounds were dissolved in 600 μL of D_2O (99.9% D, Sigma–Aldrich, Milan, Italy). 1D [^{1}H] spectra were acquired with a relaxation delay of 2 s and 512–2048 scans, without water suppression. Spectra were processed with Varian software (VNMRJ). The water signal was set at 4.75 ppm for proton chemical shifts referencing.

The degree of substitution (DS) was estimated from analysis of 1D [^{1}H] experiments by calculating the ratio $(I_a/3)/(I_b/2)$ where I_a indicates the integral of the peak corresponding to the CH_3 protons of the newly introduced fatty acid chains (close to 0.86 ppm) and I_b the area (=integral) covering the peaks of sugar anomeric protons in the spectral region between 4.37 and 4.65 ppm. Each integral was normalized for the corresponding number of protons in one HA disaccharide unit.

For HA-linoleate (**2d**) and HA-oleate (**1d**) 2D [^{1}H, ^{1}H] TOCSY (Griesinger, Otting, Wuthrich, & Ernst, 1988) experiments (2048*256 total data points and 64 scans pet t1 increment, 70 ms mixing time) were acquired as well. Proton chemical shifts values for these compounds have been previously reported (Calce et al., 2015).

2.1.3. FT-IR characterization

All modified hyaluronan samples were analyzed by FT-IR spectroscopy. The FT-IR spectra were recorded on a Jasco FT/IR 4100 spectrometer. Samples were ground into a fine powder using an agate mortar before being compressed into KBr discs. The characteristic peaks of IR transmission spectra were recorded at a resolution of 4 cm^{-1} over a wavenumber region of 400–4000 cm^{-1}. The bands relevant for the structural organization are: HA-oleate (**1d**): FT-IR (cm^{-1}): 3428 ν(O—H), 2923 ν(C—H), 1737 ν(C=O fatty acid ester), 1648 $\nu_{as}(COO^-)$, 1413 $\nu_{as}(COO^-)$, 1078 ν and 1040 $\nu(COC)_{glycosidic\ bond\ ring}$. HA-linoleate (**2d**): FT-IR (cm^{-1}): 3419 ν(O—H), 2926 ν(C—H), 1734 ν(C=O fatty acid ester), 1648 $\nu_{as}(COO^-)$, 1415 $\nu_{as}(COO^-)$, 1078 ν and 1040 $\nu(COC)_{glycosidic\ bond\ ring}$.

2.2. Preparation and characterization of HA-fatty acids nano-particles

2.2.1. Synthesis of HA-fatty acids nano-particles

HA-oleate and HA-linoleate nano-particles were prepared as follow: 1 mg of the respective conjugate was dissolved in 1 mL of H_2O 0.9% wt NaCl in a 0.5–2 mL microwave vial. Then, the solution was irradiated by microwaves at a constant power of 40 W for 10 s and stirred at 900 rpm.

All solutions were filtered through a membrane filter (pore size 0.40 μm, Millipore).

2.2.2. Size distribution and zeta potential of nano-particles

The particle size distribution and zeta potential of the HA-fatty acid nano-particles were measured at 25 °C by dynamic light scattering (DLS) technique with a Malvern Zetasizer (Nano ZS, Malvern Instruments, Westborough, MA) with NIBS optics.

The scattered light was measured at an angle of 173° and was collected on an autocorrelator. The hydrodynamic diameters (d) of micelles were calculated by using the Stokes–Einstein equation.

All data were averaged over three measurements.

The stability of HA-fatty acid conjugates was evaluated by size change over the course of 7 days at room temperature.

2.2.3. Transmission electron microscopy

Transmission electron microscopy observation was performed on a microscope Tecnai G2 Spirit TWIN operating at an acceleration voltage of 120 kV. The specimen was prepared as follows. One drop of dilute latex was delivered on a copper EM grid covered with a thin holey carbon film and dried at room temperature.

3. Results and discussion

In a previous study we developed a MW-assisted esterification reaction to prepare hyaluronan derivatives. It consisted in a solvent-free process for generating conjugates of the polysaccharide, via OH esterification with natural fatty acids (Calce et al., 2015). We also tried to modulate the degree of hyaluronan hydroxyl functionalization by varying several reaction conditions, like the MW time exposure and the reacting amount of fatty acid anhydride and polysaccharide.

Unfortunately, no control on the synthetic process was registered by acting on these parameters.

However, moving from a domestic MW oven to a MW source apparatus, specifically designed for laboratory use, we decided to investigate the effects of the reaction temperature on the process efficiency. In particular, we studied the synthetic process to