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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 $\nu$(O—H), 2923 $\nu$(C—H), 1737 $\nu$(C=O fatty acid ester), 1648 $\nu_{as}(COO^-)$, 1413 $\nu_{as}(COO^-)$, 1078 $\nu$ and 1040 $\nu(COC)_{\text{glycosidic bond ring}}$. HA-linoleate (**2d**): FT-IR (cm$^{-1}$): 3419 $\nu$(O—H), 2926 $\nu$(C—H), 1734 $\nu$(C=O fatty acid ester), 1648 $\nu_{as}(COO^-)$, 1415 $\nu_{as}(COO^-)$, 1078 $\nu$ and 1040 $\nu(COC)_{\text{glycosidic bond ring}}$.

### 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

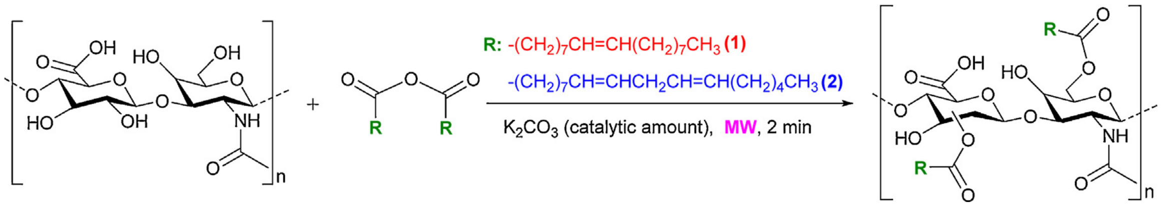
Transmission electron microscopy observation was performed on a microscope Tecnai G2 Spirit TWIN operating at an acceleration voltage of 120 kV. The specimen was prepared as follows. One drop of dilute latex was delivered on a copper EM grid covered with a thin holey carbon film and dried at room temperature.

## 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



**Scheme 1.** Schematic view of the preparation of HA-conjugates.

prepare HA-oleate and HA-linoleate, since their aggregates revealed a particularly interesting narrow size (Calce et al., 2015).

We designed several experiments characterized by a controlled temperature and unconstrained microwave irradiation power, and the temperature values were selected taking in consideration the oil bath temperature, generally used for conventional heating (oil bath $T = 160\,^{\circ}C$ for 15 min). Moreover, being our reaction performed in absence of solvent, we had to explore a range of temperature, since we could not refer to the solvent settings generally transferred from the traditional heating to a MW-activated process (Kappe, 2004).

Oleic and linoleic acids activated as anhydrides were mechanically milled with the hyaluronan in the presence of catalytic amount of $K_2CO_3$ (Scheme 1). The obtained mixture was placed into a MW reactor and a selected temperature value was applied for 2 min (Table 1). In order to be as reliable and reproducible as possible, the same experimental conditions (reactants amount, vessel, etc.) were always applied. After cooling, each sample was treated as already reported and fully characterized by FT-IR and NMR spectroscopy.

Typical spectra of HA-oleate and HA-linoleate (a spectrum of the native HA is also included for comparison), are shown in Fig. 1 and clearly revealed, in the region featuring the carboxylic groups (1740–1600 cm$^{-1}$), the occurred esterification of the employed fatty acid with the appearance of a new band in the C=O ester stretching region (1735–1740 cm$^{-1}$). By evaluating the intensity of this band, the samples resulted to be characterized by an increased degree of substitution, if compared to FT-IR spectra of the same conjugates prepared with conventional oil bath heating and also with domestic MW oven irradiation (Calce et al., 2015). In particular, these data were confirmed by NMR analysis performed on HA-oleate and HA-linoleate samples.

As we have already reported (Calce et al., 2015), after insertion of an acyl chain, NMR spectra of the HA-adducts become principally affected by line broadening rather than chemical shifts changes. In detail a few chemical shifts variations can be obviously seen and involve mainly signals from HA, whereas excessive enlargement of lines provokes low signal intensity for the hydrophobic acyl chains from oleate and linoleate moieties (Fig. 2). In fact, due to aggregation, only a few of the expected signals from oleate and linoleate can be clearly identified in the 1D [$^1$H] spectra (Figs. 2 and 3). A degree of substitution for most of the synthesized molecules could be evaluated by NMR spectroscopy (Calce et al., 2015) by integrating the peak corresponding to the $CH_3$ methyl group of the fatty acid chains, that in spite of signal loss due to aggregation, is always clear in the NMR spectra and the peak from anomeric HA protons (Fig. 3). In a few compounds (Table 1), the anomeric proton signals in the NMR spectra resulted too close to the water peak to allow a reliable evaluation of the integrals, thus the DS could not be precisely estimated.

**Table 1**
Reaction conditions and DS values of the obtained HA-conjugates.

| Entry | Reaction conditions | | Degree of substitution (%) | |
|---|---|---|---|---|
| | | | HA-oleate (*1*) | HA-linoleate (*2*) |
| **a** | 2 min | 80 °C | 3.40 | 5.3 |
| **b** | | 120 °C | 4.00 | 3.40 |
| **c** | | 160 °C | 3.00 | 3.90 |
| **d** | | 200 °C | 5.3 | 5.9 |
| **e** | | 100 W | n.d. | 4.69 |
| **f** | | 200 W | n.d. | 3.76 |

To better understand the obtained results, it is worth remembering that MW activation uses the ability of some compounds (liquids or solids) to transform electromagnetic energy into heat. In major details, the energy transmission is produced by dielectric losses, which is in contrast to conduction and convection processes observed for the conventional heating. Hence MW heating is volumetric and fast, being the whole reaction mixture simultaneously heated. On the basis of these considerations, we expected that our solid state mixture is more efficiently activated by MW-radiation compared to the conventional heating, which slower occurs from the surface to the inner.

Moreover, MW-activation avoids the degradation of the material in contact with the vessel wall. All these arguments supported the increased yield observed for all the prepared hyaluronan conjugates, in terms of the increased efficiency of the polysaccharide functionalization. In this regard, it is worth noting that the domestic

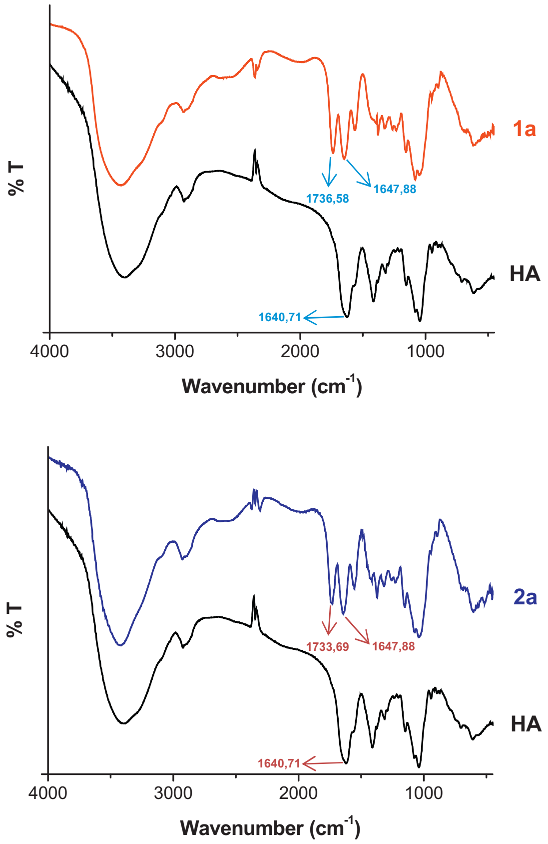


**Fig. 1.** Transmittance FT-IR spectra of HA conjugates.

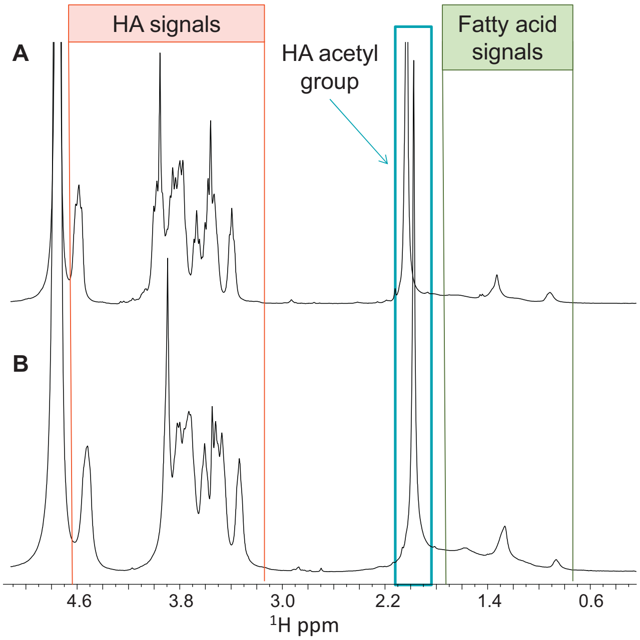


**Fig. 2.** 1D [$^{1}$H] NMR spectrum of HA-linoleate (2d) (A) and HA-oleate (1d) (B).

MW oven is characterized by a low repeatability of the experimental conditions, due to the essentially random electric field pattern applied, and it is very difficult to accurately take under control the power delivered into the reaction vessel. It can explain the better results reached by employing MW apparatus (Biotage Initiator), specifically designed for chemical reactions.

Moreover, for reactions performed in polar solvent, the MW radiations are strongly absorbed by the solvent, so that the thermal energy is still transmitted to reagents by conventional mechanism. In case of solid state reactions, since a solvent that could transfer energy/heat lacks from the reaction mixture, the radiation can only be absorbed by the reactants.

Therefore, our process is effectively MW-assisted, in such mechanism different from what happened with the oil bath, where the heating is transferred to reagents from the vessel wall (de la Hoz et al., 2005).

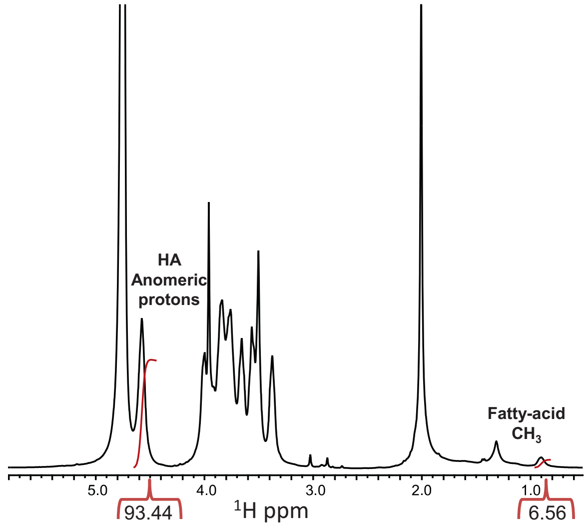


**Fig. 3.** 1D [$^{1}$H] NMR spectrum of HA-linoleate (2e), measured integrals are indicated on the corresponding peaks.

All the performed experiments at different temperatures showed a very similar final yield, estimated in terms of DS (Table 1). Since they were characterized by high initial power, we decided to investigate the effect of this parameter, to tune the experimental conditions of the process under investigation. In general, MW-induced solid state reactions are far less comprehensive than those in solution, due to the complexity of heating process and also to the significant difficulties associated with monitoring the temperature inside the sample (Kappe, 2013; Tang et al., 2014). Starting from these considerations, we performed several experiment by changing the MW irradiation power, while living unconstrained the temperature (Fig. 4). We found that a quite low power was necessary for obtaining polysaccharide conjugates with a DS value already observed for the reaction performed at high temperatures (Table 1).

These results confirmed that the hyaluronan esterification reaction is very efficient, but does not increase the degree of substitution by varying the reaction parameters.

The temperature values registered by the MW-radiation system are quite low, if compared to those necessary by the conventional heating (oil bath $T = 160\,^{\circ}$C for 15 min), it suggested that heating induced by MW is very efficient (Fig. 4) (Rodriguez et al., 2015).

In other words, the interaction between the material and the MW radiation very efficiently induce the reactants transformation and for these same reasons it can involve a selective heating of same reagents compared to others. In particular, our reaction mixture consists of two polar/ionic components, like the hyaluronan and the potassium carbonate that very efficiently absorbs the MW radiations, and the less polar fatty acid anhydride which is expected to be less sensitive, on the basis of the MW activation principles. Therefore, we can hypothesize a hot spot solvent-free

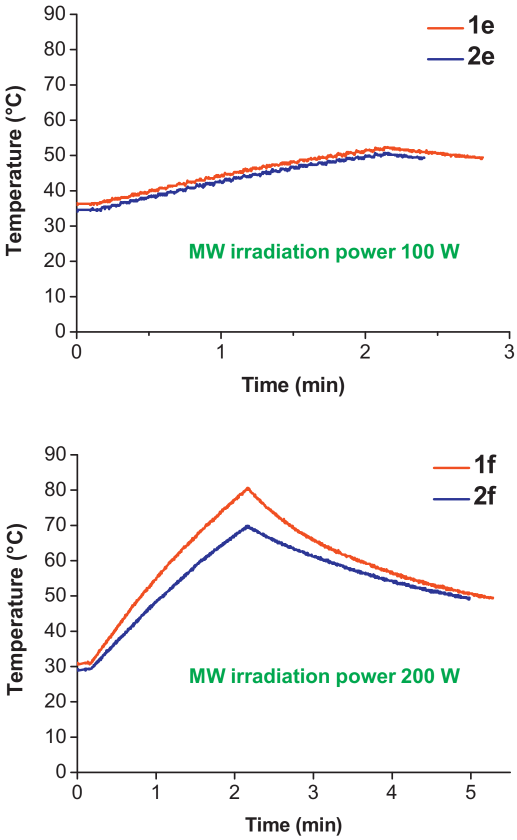


**Fig. 4.** Profile of the time vs the temperature at a fixed power value.

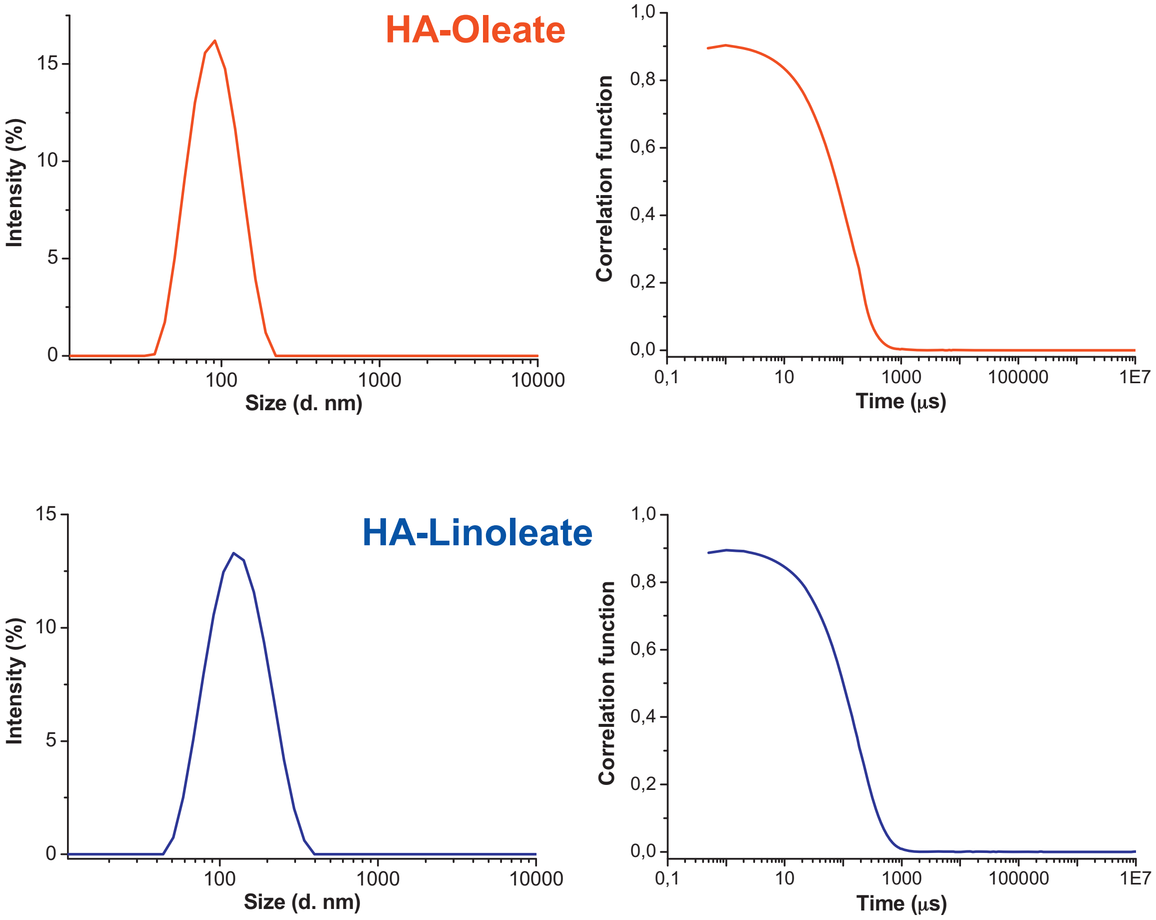


**Fig. 5.** Size distribution vs intensity and intensity correlation of HA-oleate nano-particles (upper panel) and of HA-linoleate nano-particles (lower panel).

process, which reduces the decomposition of compounds, like the fatty acid anhydrides, thermally unstable.

The nano-particles of HA-oleate and HA-linoleate were also prepared by employing a MW irradiation, the samples were dissolved in an aqueous solution of NaCl 0.9% and irradiated with a power of 40 W for 10 s. The obtained aggregates revealed a mean diameter of 80.57 nm for HA-oleate and of 114.1 nm for HA-linoleate and a narrow size distribution (Fig. 5), as confirmed by the measured polydispersity values (Table 2).

Among the different methods developed for preparing nanoparticles of natural polysaccharide, the aqueous self-assembly process is one of the most successful for amphiphilic polymer. It allows the spontaneous association between hydrophobic moieties, hence a core–shell structural organization leads to the formation of micellar aggregates (Yang, Han, Zheng, Dong, & Liu, 2015). Generally, the ultrasonic bath is used to speed up this process, by breaking molecular interactions to allow the formation of new interactions, that can minimize the interfacial free energy of the system (Yang et al., 2015).

In the same way, we have hypothesized that the orientation induced on polar molecules by the microwave radiations could favor the micellar self-aggregation, so that the previous method used to prepare the nano-particles of HA-oleate and HA-linoleate, based on the emulsion evaporation of organic/water system solvent, was replaced by a MW-assisted aqueous route. It allowed us to replace the chloroform with a green solvent like the water, which also greatly adsorbs the MW radiation and transfers the thermal energy to other compounds (Yang et al., 2015). The obtained nanoparticles resulted stable under physiological conditions (7 days), thus proving that the developed method was plenty successful. At the best of our knowledge, this is the first example of protocol which employs MW radiation to promote the spontaneous self-association of amphiphilic polymers. All other methods employed for a controlled MW-assisted nanoparticle synthesis are, however, at an initial stage, in terms of understanding of the process mechanism and optimization of its parameters (Yang et al., 2015; Duan, Wang, & Li, 2015).

**Table 2**
Characteristic parameters of HA-fatty acid nano-particles.

| | HA-oleate | HA-linoleate |
|---|---|---|
| Mean diameter (nm) | 80.57 ± 0.27 | 114.1 ± 0.91 |
| Polydispersity | 0.154 | 0.188 |
| $\zeta$ (mV) | −33.2 | −25.1 |

## 4. Conclusions

We tuned a MW-assisted reaction in order to prepare hyaluronan conjugates by acylation of the polysaccharide alcoholic functions with oleic and linoleic acids, activated as anhydride. The material-radiation interaction is believed responsible of the efficient thermal effect. Indeed, this effect is not masked or limited by the solvent, since the reaction is performed under solvent-free conditions. Moreover, harsh reaction parameters could be avoided under MW irradiation, in fact we demonstrated that with a power of 100 W for 2 min (maximum temperature reached 50 °C) a good degree of hyaluronan functionalization already occurred. When compared to modified hyaluronan obtained under conventional heating, our results showed that microwave radiations improved the chemical process, both in terms of yield and also purity of the final product. In conclusion, taking in consideration the energy aspects, an already biocompatible chemical process, when performed under MW-activation, further moved toward "green chemistry". In this regard, the MW method employed to induce the HA-oleate and HA-linoleate self-assembly offered the great advantage of eliminating the use of organic solvent, which makes the whole process even more eco-compatible in terms of chemical consumption.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2016.02.001.

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