Supporting Information

**Biocompatible process to prepare hyaluronan-based material able to self-assemble in stable nano-particles**

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1. Experimental section

1.1. Materials

The Hyaluronic acid sodium salt from *Streptococcus equi* mol wt 15-30 kDa, the fatty acids and all solvents were purchased from Sigma–Aldrich.

1.2 Synthesis and characterization of HA-fatty acids conjugated

1.2.1 Synthesis of HA-fatty acid conjugated

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 (10 mg) and the appropriate fatty acid anhydride (10/30 mg) at different weight ratio (HA: oleic anhydride, 1:3, 1a, and 1:1, 1b; HA: linoleic anhydride, 1:3, 2a, and 1:1, 2b; HA: palmitic anhydride, 1:1, 3a, and 2:1, 3b) were manually milled in presence of K$_2$CO$_3$ (0.1 equiv.) to obtain several hyaluronan derivatives.

The reaction was carried out by using a domestic microwave oven, 900 W (2450 MHz), DeLonghi, Treviso, Italy. A Teflon cavity was used as reaction vessel, accurately placed always in the same position of the rotary dish. The reaction mixtures were irradiated with a potency of 900 W for two cycles of 3 min each, in order to avoid polysaccharide degradation that, however, was never appreciably observed. Importantly, a significant change of the degree of substitution (DS) was not observed by extending the MW exposure time (5 min). The synthesis of each selected sample was repeated in order to tune the reaction conditions, in Table S1 the technical parameters and the DS calculated for the performed reactions are
reported. The HA-palmitate DS were not evaluated, in this case only FT-IR spectra were examined (see next paragraph). 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) for 1 day in Milli-Q water.

The final product was collected after lyophilization process.

Yield: around 40%

Table S1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (min)</th>
<th>Potency (W)</th>
<th>DS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Oleate (1a)</td>
<td>3+3</td>
<td>900</td>
<td>2.5/1.9/2.9/2.6</td>
</tr>
<tr>
<td>HA-Oleate (1a’)</td>
<td>3+5</td>
<td>900</td>
<td>1.8</td>
</tr>
<tr>
<td>HA-Linoleate (2a)</td>
<td>3+3</td>
<td>900</td>
<td>1.9/1.5/2.5/1.8</td>
</tr>
<tr>
<td>HA-Linoleate (2a’)</td>
<td>3+5</td>
<td>900</td>
<td>1.7</td>
</tr>
<tr>
<td>HA-Palmitate (3a)</td>
<td>3+3</td>
<td>900</td>
<td>n.d.</td>
</tr>
<tr>
<td>HA-Palmitate (3a’)</td>
<td>3+5</td>
<td>900</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

1.2.2 NMR characterization

NMR spectra were acquired for HA-oleate, HA-linoleate and HA-palmitate at 298 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 provided with a cold probe. NMR samples consisted of hyaluronan-derivatives dissolved in 99.9 % D₂O (Armar Chemicals, Switzerland) with volumes equal to 600 µL. 1D [¹H] spectra were generally recorded with a relaxation delay of 2 sec and 1-4 k scans. 2D [¹H, ¹H] TOCSY (Total Correlation Spectroscopy) experiments (2048*256 total data points and 128 scans
pet 1t increment) were acquired with a mixing time of 70 ms. No water suppression was implemented. Spectra were processed with Varian software (vnmrj_1.1D). The program NEASY\textsuperscript{[S2]} that is included in the Cara (http://www.nmr.ch/) software package, was used to analyze 2D spectra. Proton chemical shifts were referenced to the water signal at 4.75 ppm. For HA-palmitate excessive line broadening avoided carrying out detailed analysis of NMR spectra. The degree of substitution (DS) was evaluated with the equation \( DS = \frac{I_a}{3} / \frac{I_b}{2} \) where \( I_a \) represents the integral of the peak corresponding to the CH\(_3\) protons of the inserted fatty acid chains (at 0.86 ppm) and \( I_b \) the area (=integral) covering the peaks of sugar anomeric protons (approximate spectral region between 4.37 and 4.65 ppm). Each integral was normalized for the corresponding number of protons in one HA disaccharide unit.

- NMR \(^1\text{H}\) assignments. HA-oleate (1a). \(^1\text{H}\)-NMR (ppm, D\(_2\)O) hyaluronan protons: series of broad signals in the range 3.34-3.87, sugar anomeric protons 4.50-4.54, N-acetyl group 2.00; fatty acid chain: 0.86 (CH\(_3\)), 1.26-1.30 (CH\(_2\)), 1.58 (CH\(_2\)CH\(_2\)COOR), 1.99 (CH\(_2\)CH=CHCH\(_2\)), 2.25-2.36 (m, CH\(_2\)COOR), 5.29 (CH=CH). HA-linoleate (2a). \(^1\text{H}\)-NMR (ppm, D\(_2\)O) hyaluronan protons: series of broad signals in the range 3.34-3.87, sugar anomeric protons 4.50-4.54, N-acetyl group 2.00; fatty acid chain: 0.86 (-CH\(_3\)), 1.28-1.32 (-CH\(_2\)-), 1.58 (-CH\(_2\)CH\(_2\)COOR), 2.04 (CH\(_2\)CH=…=CHCH\(_2\)), 2.74 (-CH=CHCH\(_2\)CH=CH-), 2.37 (CH\(_2\)COOR), 5.29 (-CH=CH-).

1.2.3 FT-IR characterization

All modified hyaluronan samples were analyzed by FT-IR spectroscopy. The FT-IR spectra were recorded on a Jasco 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 (1a): FT-IR (cm\(^{-1}\)): 3410 \(\nu\)(O–H), 2924 and 2853 \(\nu\)(C–H), 1743 \(\nu\)(C=O fatty acid ester), 1643 \(\nu\)\(_{\text{as}}\)(COO\(^{-}\)), 2924 and 2853 \(\nu\)(C–H), 1743 \(\nu\)(C=O fatty acid ester), 1643 \(\nu\)\(_{\text{as}}\)(COO\(^{-}\)).
1.2.4 Determination of Critical Aggregation Concentration (CAC)

The critical aggregation concentration (CAC) of the HA derivatives was determined by a fluorescence method using pyrene as probe. A 3.0 x $10^{-2}$ M pyrene solution in acetone was diluted with water (12.0 x $10^{-7}$ M), then the acetone was removed by a rotary evaporator at 60 °C for 1h, until a final concentration of 5 x $10^{-7}$ M was reached. This solution was used to dissolve the HA-fatty acid derivatives with a final concentration in the range of 1.8-0.002 mg/mL; each sample was sonicated for 4-5 minutes and then allowed to stand for 3 h.

The fluorescence emission spectra (340-500 nm) were recorded using a Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 337 nm. The ratio of the emission intensities of the first (373 nm) and the third (384 nm) peak was plotted against the concentration of the HA conjugate and the CAC was determined as inflection point of the obtained curve.

1.3 Preparation and characterization of HA-fatty acids nano-particles
1.3.1 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 2 mL of a mixture CHCl₃/H₂O 0.9% wt NaCl (50/50); the two phases were emulsified by stirring over-night at room temperature. Under continuous stirring the organic solvent evaporated and the nano-particles formed into the aqueous layer.

HA-palmitate nano-particles were prepared by suspending 1 mg of the compound in 1 mL of 10 mM HEPES (pH 7.4) aqueous solution and sonicating the obtained mixture for 20 min at room temperature.

All solutions were filtered through a membrane filter (pore size 0.20 μm, Millipore).

1.3.2 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.

1.3.3 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 cast on a copper EM grid covered with a thin holey carbon film and dried at room temperature.

References

2. NMR spectra

-Supplemental Figure S1. (A) 1D [$^1$H] proton spectra of HA (black) and HA-oleate 1a (red). (B) 1D [$^1$H] NMR spectra of HA-oleate 1a (green) and oleic-acid (red). Aggregation phenomena produce extensive line broadening that mainly involve lipid moieties (i.e. signals in between 0.8 and 2.5 ppm).
-Supplemental Figure S2. (A) Comparison of 1D [^1H] proton spectra of HA (black) and HA-linoleate 2a (Red). (B) 1D [^1H] NMR spectra of HA-linoleate 2a (green) and linoleic-acid (black). In the spectrum of HA-linoleate signals from the fatty acid tail can be barely seen likely due to aggregation processes.
Supplemental Figure S3. 2D $[^1H, ^1H]$ TOCSY spectrum of HA-oleate 1a. Different spectral regions containing NMR signals from either ialuronic acid and fatty acid chains are indicated. The * are pointing to small impurities present in the sample.
3. FT-IR spectra

-Supplemental Figure S4. Transmittance FT-IR spectra of HA-oleate 1b

-Supplemental Figure S5. Transmittance FT-IR spectra of HA-linoleate 2b
Supplemental Figure S6. Transmittance FT-IR spectra of HA-palmitate 3b
4. Critical aggregation concentration (CAC)

-Supplemental Figure S7. Intensity ratio $I_{373}/I_{384}$ from pyrene emission spectra as a function of HA-fatty acid conjugates concentration
5. DLS measurements

Supplemental Figure S8. The size distribution of HA-oleate (1a, panel A), HA-linoleate (2a, panel B) and HA-palmitate (3a, panel C) nano-particles measured by DLS.
-Supplemental Figure S9. The size distribution of HA-oleate 1b nano-particles
Supplemental Figure S10. The size distribution of HA-linoleate 2b nano-particles
Supplemental Figure S11. The size distribution of HA-palmitate 3b nano-particles
6. Stability of HA-fatty acid conjugates

-Supplemental Figure S12. The stability of HA-fatty acid conjugates evaluated by size change within one week
7. TEM images

-Supplemental Figure S13. TEM image of HA-Oleate 1a nano-particles

-Supplemental Figure S14. TEM image of HA-Linoleate 2a nano-particles
Supplemental Figure S15. TEM image of HA-Palmitate 3a nano-particles.