Supporting Information

Hyaluronic acid-based formulation with simultaneous local drug delivery and antioxidant ability for active viscosupplementation

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## Table with a complete list of acronyms and their full form

| Full name | Acronym |
|-----------|---------|
| Hyaluronic Acid | HA |
| Tocopheryl acetate (Vitamin E) | VE |
| Hydroxypropyl-β-cyclodextrin | CD |
| Diclofenac Sodium | DF |
| Hyaluronic Acid - Tocopheryl acetate (Vitamin E) | HV |
| Hyaluronic Acid - Hydroxypropyl-β-cyclodextrin | HC |
| Hyaluronic Acid - Tocopheryl acetate (Vitamin E) - Hydroxypropyl-β-cyclodextrin | HCV |
| 2,2-diphenyl-1-picrylhydrazyl | DPPH |
| Optical Density | OD |
| Extracellular matrix | ECM |
| Synovial fluid | SF |
| Osteoarthritis | OA |
| Non-steroidal anti-inflammatory drugs | NSAIDs |
| Diclofenac | DF |
| Differential Scanning Calorimetry | DSC |
| Interleukin-10 | IL-10 |
| Molecular weight | Mw |
| Phosphate-buffered saline | PBS |
| Room temperature | RT |
| Scavenging ability | SA |
| Solubilized fraction percentage | SF% |
| Dulbecco’s Modified Eagle’s Medium | DMEM |
| Fetal bovine serum | FBS |
| Alamar blue assay | AB |
| 4',6-diamidino-2-phenylindole | DAPI |
| Lipopolysaccharides | LPS |
| Non-freezing water | NFW |
| Freezing water | FW |
| Autoclaved | AC |
Sample calculation with each of the equations presented in the paper

Rheological synergism

The effect of the interaction among the components on the viscoelastic properties of HCV was evaluated by calculating the synergistic contribution ($\Delta G'_{\text{synergistic}}$) to the elastic modulus of the formulation, as expressed in the Equation 1. The calculation of $\Delta G'_{\text{synergistic}}$ for HCV before being sterilized in autoclave was reported as example.

$$\Delta G'_{\text{synergistic}} = G'_{\text{HCV}} - (G'_{\text{HV}} + G'_{\text{CD}}) = 130 \text{ Pa} - (53 \text{ Pa} + 0.013 \text{ Pa}) = 77 \text{ Pa}$$

(1)

Scavenging Ability

The antioxidant properties of HCV were evaluated the 2,2-diphenyl-1-picrylhydrazy (DPPH) assay, through which it has been possible to determine the scavenging ability (SA) of the material, expressed as reported in Equation 2. The assay consisted of measuring the UV absorbance at 517 nm of a DPPH solution in ethanol ($A_{\text{control}}$) and of a solution at the same DPPH put in contact with our material ($A_{\text{sample}}$). The assay has been performed at two different concentrations of HCV, and the calculation for the system at 300 $\mu$g/mL have been reported.

$$SA (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 = \frac{0.4445 - 0.382}{0.4445} \times 100 = 0.140607424$$

(2)

Drug solubility

Diclofenac (DF) was loaded in the formulation HCV by preparing the system using a DF solution in PBS (20 mg/mL). The efficiency of the drug loading was evaluated by centrifuging he HCV formulation, measuring the UV-Visible absorption of the supernatant at wavelength 256 nm, and subtracting the amount of the drug in the supernatant from that of the Diclofenac solution used to prepare the formulation. The concentration of DF in the formulation resulted 16 mg/mL, and the drug loading efficiency, expressed as solubilized fraction (DF), resulted being 80% respect to the total amount of drug used to prepare the system.

$$SF\% = \frac{\text{solubilized DF}}{\text{Total DF}} \times 100 = \frac{16 \text{ mg mL}^{-1}}{20 \text{ mg mL}^{-1}} \times 100 = 80\%$$

(3)

Drug release

The release of Diclofenac sodium (DF) from HCV was studied by inserting 1 g of the formulation containing DF at 1% w/w in a dialysis bag (cut off 500 to 1000 Da) that has been immersed in PBS (18 ml) at the temperature of 37 $^\circ$C. At different times, 1 mL of external solution has been withdrawn and the DF concentration was evaluated through UV-Vis absorption (absorption peak at 276 nm). In
this way the amount of released drug at each time \( Q_t \) was evaluated. The released fraction of DF was calculated by dividing \( Q_t \) for the amount of released drug at equilibrium \( Q_\infty \).

The relationship between the released DF fraction \( Q_t/Q_\infty \) and the time \( t \) has been studied using the MATLAB function lsqcurvefit. lsqcurvefit is a nonlinear least-squares solver, that, given a data set (xdata,ydata) and a written function (fun), calculates the parameters vector \( x \) with the method of the least squares:

\[
x = \text{lsqcurvefit}(\text{fun},x0,xdata,ydata)
\]  

(4)

where \( x0 \) is the first attempt values vector.

In our work the function used was the Korsmeyer–Peppas kinetic model (Equation 5), and the kinetic constant \( k_k \) and the diffusional exponent \( n \) were the two parameters that lsqcurvefit provided as output.

\[
Q_t/Q_\infty = k_k t^n
\]  

(5)

**Alamar Blue Assay**

Data are expressed as the percentage difference between treated and control to evaluate the percentage of reduction (Reduction %) is calculated with the following formula (Equation 6):

\[
\text{Reduction} \% = \left( \frac{(O_2 \times A_1) - (O_1 \times A_2)}{(O_2 \times P_1) - (O_1 \times P_2)} \right) \times 100
\]  

(6)

where \( O1 \) and \( O2 \) are the molar extinction coefficient \( (E) \) of oxidized AB at 570 nm and 600 nm; \( A1 \) is the absorbance of test wells at 570 nm; \( A2 \) is the absorbance of test wells at 600 nm; \( P1 \) is the absorbance of control well at 570 nm; and \( P2 \) is the absorbance of control well at 600 nm.

Sample calculation for HC has reported below:

| 570 nm | \( A1 \) |
|--------|--------|
| CTRL   | 0.540  |
| HC     | 0.508  |
|        | 0.490  |
|        | 0.497  |

| 600 nm | \( A2 \) |
|--------|--------|
| CTRL   | 0.074  |
| HC     | 0.093  |
|        | 0.070  |
|        | 0.072  |

\( O1 \) (570nm)= 80586
\( O2 \) (600nm)= 117216
\[ \text{Reduction (\%)} = \frac{(117216 \times 0.508) - (80586 \times 0.093)}{(117216 \times 0.560) - (80586 \times 0.541)} \times 100 = 235.7836 \quad (7) \]

| % reduction | CTRL    | HC      |
|-------------|---------|---------|
|             | 259.6932| 235.7826|
|             | 234.086 | 208.9296|
|             | 237.4021| 216.2583|
| AVERAGE     | 243.7271| 220.3235|

The percentage reduction for each sample was normalized to the percentage reduction for the mean of the untreated controls to obtain the percentage of cell viability:

| % viability | CTR      | HC      |
|-------------|----------|---------|
|             | 106.5508 | 96.7404 |
|             | 96.0443  | 85.72277|
|             | 97.40489 | 88.72972|
| AVERAGE     | 100      | 90.39763|
| SD          | 5.713813 | 5.695041|

\[ \text{Cell Viability (\%)} = \frac{\text{% reduction sample}}{\text{% reduction average of sample control}} \times 100 = \frac{235.7826}{243.7271} \times 100 = 96.7404 \quad (8) \]
**Statistical Analysis**

**Percentage Viability: Ordinary one-way ANOVA**

| Number of families | 1 |
|-------------------|---|
| Number of comparisons per family | 4 |
| Alpha | 0.05 |

Dunnett's multiple comparisons test

| Summary | Test details | Mean 1 | Mean 2 | Mean Diff, SE of diff | n1 | n2 | q | DF |
|---------|--------------|-------|-------|----------------------|----|----|---|----|
| CTR vs. HA | 98,00 | 96,04 | 1,961 | 4,185 | 3 | 3 | 0,4687 | 10 |
| CTR vs. HC | 98,00 | 90,40 | 7,607 | 4,185 | 3 | 3 | 1,818 | 10 |
| CTR vs. HV | 98,00 | 96,07 | 1,933 | 4,185 | 3 | 3 | 0,4619 | 10 |
| CTR vs. HCV | 98,00 | 111,3 | -13,27 | 4,185 | 3 | 3 | 3,171 | 10 |

**Anti-inflammatory IL-10 expression: Ordinary one-way ANOVA**

| Number of families | 1 |
|-------------------|---|
| Number of comparisons per family | 3 |
| Alpha | 0.05 |

Dunnett's multiple comparisons test

| Summary | Test details | Mean 1 | Mean 2 | Mean Diff, SE of diff | n1 | n2 | q | DF |
|---------|--------------|-------|-------|----------------------|----|----|---|----|
| CTR vs. HA | -39,64 | -83,19 to 3,909 | No | ns |
| CTR vs. HCV | -82,91 | -126,5 to -39,36 | Yes | ** |
| CTR vs. HCV+DF | -295,2 | -338,8 to -251,7 | Yes | **** |

Test details

| Mean 1 | Mean 2 | Mean Diff, SE of diff | n1 | n2 | q | DF |
|--------|-------|----------------------|----|----|---|----|
| CTR vs. HA | 15,82 | 55,46 | -39,64 | 15,12 | 3 | 3 | 2,621 | 8 |
| CTR vs. HCV | 15,82 | 98,73 | -82,91 | 15,12 | 3 | 3 | 5,482 | 8 |
| CTR vs. HCV+DF | 15,82 | 311,1 | -295,2 | 15,12 | 3 | 3 | 19,52 | 8 |