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 ($\Delta G'_{synergistic}$) to the elastic modulus of the formulation, as expressed in the **Equation 1**. The calculation of $\Delta G'_{synergistic}$ for HCV before being sterilized in autoclave was reported as example.

$$\Delta \boldsymbol{G'_{synergistic}} = \boldsymbol{G'_{HCV}} - (\boldsymbol{G'_{HV}} + \boldsymbol{G'_{CD}}) = 130\ Pa - (53\ \text{Pa} + 0.013\ \text{Pa}) = \boldsymbol{77\ Pa} \quad (1)$$

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 ($A_{control}$) and of a solution at the same DPPH put in contact with our material (A_{sample}). The assay has been performed at two different concentrations of HCV, and the calculation for the system at 300 μg/mL have been reported.

$$SA\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 = \frac{0.4445 - 0.382}{0.4445} \times 100 = 0.140607424 \quad (2)$$

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.

$$SF\% = \frac{solubilized\ DF}{Total\ DF} \times 100 = \frac{16\ mg\ mL^{-1}}{20\ mg\ mL^{-1}} \times 100 = 80\% \quad (3)$$

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 ˚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_∞.

The relationship between the released DF fraction Q_t/ Q_∞ 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 = lsqcurvefit(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.

$$Q_t/Q_\infty = k_k t^n \quad (5)$$

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**):

$$Reduction\ (\%) = \frac{(O_2 \times A_1) - (O_1 \times A_2)}{(O_2 \times P_1) - (O_1 \times P_2)} \times 100 \quad (6)$$

where $O1$ and 02 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
P1	CTRL	HC
0.560	0.540	0.508
	0.490	0.462
	0.497	0.474
600 nm		A2
P2	CTRL	HC
0.541	0.074	0.093
	0.070	0.099
	0.072	0.096
O1 (570nm)=	80586	
O2 (600nm)=	117216	

$$Reduction\ (\%) = \frac{(117216 \times 0.508) - (80586 \times 0.093)}{(117216 \times 0.560) - (80586 \times 0.541)} \times 100 = 235.7836 \quad (7)$$

% riduction	CTRL	HC
	259.6932	235.7826
	234.086	208.9296
	237.4021	216.2583
AVERAGEg	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:

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

$$Cell\ Viability\ (\%) = \frac{\%\ \text{reduction sample}}{\%\text{reduction average of sample control}} \times 100 = \frac{235.7826}{243.7271} \times 100 = 96.7404 \quad (8)$$

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	Mean Diff,	95% CI of diff,	Significant?	Summary
CTR vs. HA	1,961	-10,13 to 14,06	No	ns
CTR vs. HC	7,607	-4,489 to 19,70	No	ns
CTR vs. HV	1,933	-10,16 to 14,03	No	ns
CTR vs. HCV	-13,27	-25,37 to -1,175	Yes	*

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	Mean Diff,	95% CI of diff,	Significant?	Summary
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