

Supplementary information

Multilayer Nanocarrier for the Codelivery of Interferons: A Promising Strategy for Biocompatible and Long-Acting Antiviral Treatment

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1. Results

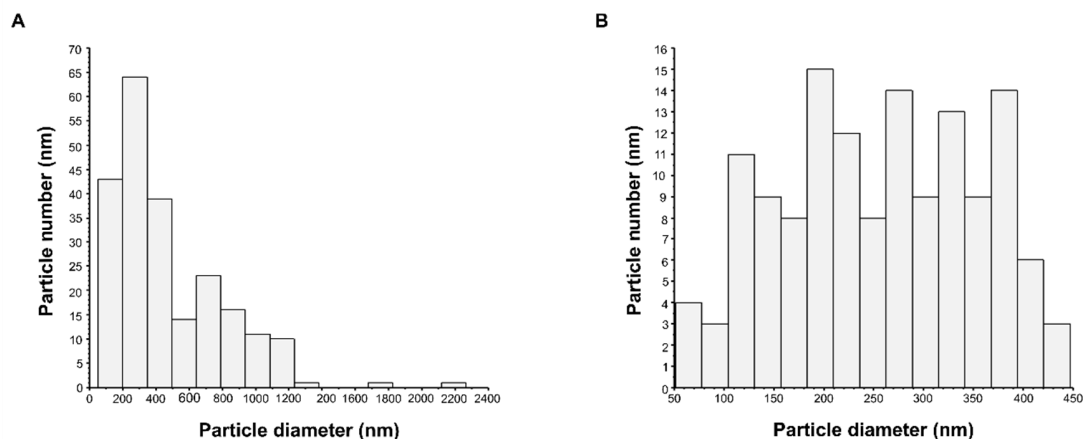


Figure S1. Characterization of empty and rhIFN α -2b and rhIFN- γ encapsulating CS NPs morphology and size. **A)** Histogram of the size distribution of the CS NPs with rhIFN α -2b and rhIFN- γ represented with the obtained data. **B)** Histogram of the size distribution of the CS NPs with rhIFN α -2b and rhIFN- γ by applying a filter at 450 nm to the data.

The histograms showed that the different formulations were heterogeneous regarding size distribution (Figure S1a). In addition to the above result, the assumption of whether or not the NPs measurement data followed a normal distribution was considered. The size values were taken with the filter at 450 nm (Figure S1b).

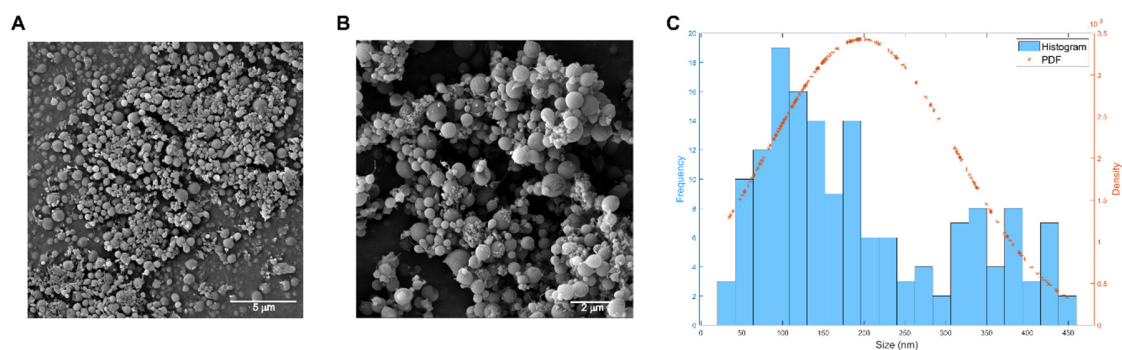


Figure S2. Characterization of empty and rhIFN α -2b encapsulating QS NPs morphology and size. **A)** SEM micrograph of empty QS NPs. **B)** SEM micrograph of QS NPs encapsulating rhIFN α -2b. **C)** Histogram of the CS NPs with rhIFN α -2b and rhIFN- γ based on the determination of the probability density function.

Table S1. Size distribution of QS NPs by electron microscopy and Dynamic Light Scattering (DLS).

Nanoformulations	No. of measured parts	Average particle size	Histogram filter 450 nm Fiji© Software	Shapiro Wilk statistical test	DLS		
					Zeta potential	Average diameter	
Empty QS NPs	234	301.2 ± 224.3 nm	188.2 ± 101.7 nm	W=0.911 p=0.000	+ 40.2 ± 3.73 mV	128.4 nm	100%
QS NPs + rhIFN α -2b	244	391.8 ± 340.5 nm	191.6 ± 91.1 nm	W=0.944 p=0.021	+ 40.7 ± 4.12 mV	207.9 nm	100%

Average of the measurements applying the 450 nm filter, Shapiro Wilk statistical test and the p value for each formulation.

Table S2. Encapsulation efficiency results for CS NPs encapsulating rhIFN α -2b and rhIFN- γ .

Total rhIFNs (rhIFN α -2b and rhIFN- γ)	326.3	Area [mV.Min]
Non-encapsulated rhIFNs	76	Area [mV.Min]
Encapsulated rhIFNs = total rhIFNs - non-encapsulated rhIFNs	250.3	Area [mV.Min]
EE = encapsulated rhIFNs / total rhIFNs	0.767085504	
EE percentage	76.7%	
Percentage of proteins at surface level	23.3%	

Table S3. Encapsulation efficiency results for QS NPs encapsulating rhIFN α -2b.

Total rhIFN α -2b	296.5	Area [mV.Min]
Non-encapsulated rhIFNs	136	Area [mV.Min]
Encapsulated rhIFNs = total rhIFNs - non-encapsulated rhIFNs	160.5	Area [mV.Min]
EE = encapsulated rhIFNs / total rhIFNs	0.541315346	
EE percentage	54.1%	
Percentage of proteins at surface level	45.9%	

*This encapsulation efficiency refers to the core of the CS NPs, composed of QS and rhIFN α -2b.

The amount of protein loaded in a nanoparticulate system can be determined by calculating the encapsulation efficiency percentage of protein retained in the NPs relative to the total protein used for nano encapsulation. The determination was performed using an indirect method, SEC-HPLC, quantifying the free recombinant proteins (rhIFN α -2b and rhIFN- γ) in the supernatant of the nanoparticle batches using *TSK gel G2000SW* matrix. The encapsulation efficiency analysis determined 76.7% for CS NPs encapsulating rhIFN α -2b and rhIFN- γ , and 54.1% for QS NPs encapsulating rhIFN α -2b.

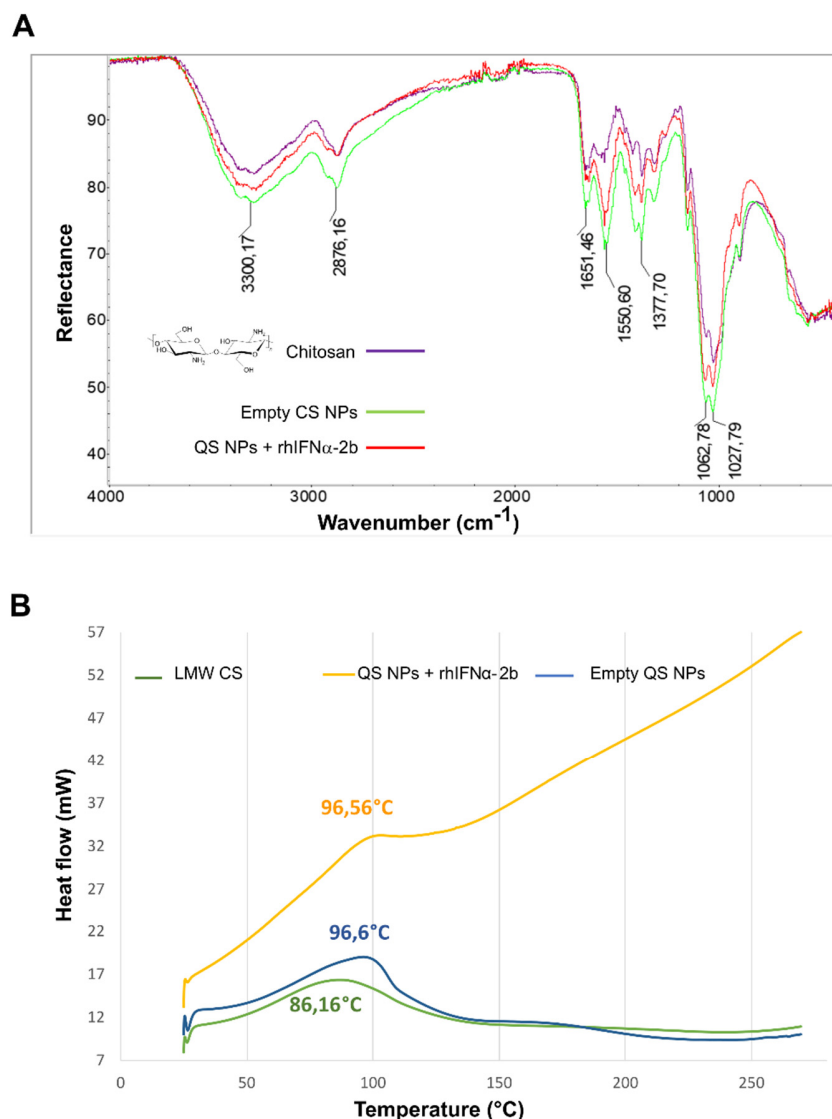


Figure S3. Physicochemical characterization of QS nanoformulations. **A)** ATR-FTIR of empty QS and QS NPs encapsulating rhIFN α -2b. **B)** Thermal analysis of the QS NPs. The graph describes the temperatures reached in Differential Scanning Calorimetry for empty NPs and encapsulating rhIFN α -2b.

For the formulation QS empty NPs and QS encapsulating rhIFN α -2b, corresponding to the core of the CS NPs, the ATR-FTIR spectra show that in the nanoparticles with chitosan the polymer signals have only small variations in displacement, even after the encapsulation process (Figure S3A). This implies that there is no structural variation in the formulation.

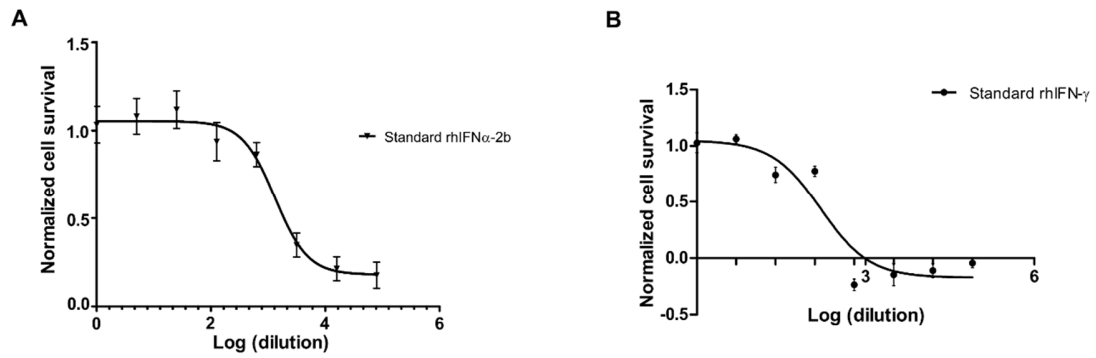


Figure S4. Evaluation of the antiviral activity of the nanoformulations. Log (Dilution) graph, to which HEP-2 cells were exposed to counteract the cytotoxic effect of Mengo virus vs. Cell survival, normalized with respect to 100% viability (cell control) and maximum lethality (virus control). **A)** Antiviral activity of standard rhIFNα-2b. **B)** Antiviral activity of standard rhIFN-γ.

Table S4. Determination of antiviral activity under accelerated conditions for rhIFN α -2b from CS NPs encapsulating rhIFN α -2b and rhIFN- γ .

Antiviral activity of CS NPs + rhIFN α -2b & rhIFN- γ	Log EC ₅₀	rhIFN α -2b titer (IU/mL)	rhIFN α -2b specific activity (IU/mg)
Standard rhIFN α -2b	3.515	1.2×10^4	
rhIFN α -2b activity of CS NPs + rhIFN α -2b & rhIFN- γ . 4°C	2.133	7.2×10^3	2.2×10^8
rhIFN α -2b activity of CS NPs + rhIFN α -2b & rhIFN- γ . 16°C	1.918	6.5×10^3	2.0×10^8
rhIFN α -2b activity of CS NPs + rhIFN α -2b & rhIFN- γ . 25°C	2.413	8.2×10^3	2.5×10^8
rhIFN α -2b activity of CS NPs + rhIFN α -2b & rhIFN- γ . 30°C	2.388	8.1×10^3	2.5×10^8
rhIFN α -2b activity of CS NPs + rhIFN α -2b & rhIFN- γ . 37°C	1.724	5.9×10^3	1.7×10^8

Table S5. Determination of antiviral activity under accelerated conditions for rhIFN- γ from CS NPs encapsulating rhIFN α -2b and rhIFN- γ .

Antiviral activity of CS NPs + rhIFN α -2b & rhIFN- γ	Log EC ₅₀	rhIFN α -2b titer (IU/mL)	rhIFN- γ Specific activity (IU/mg)
Standard rhIFN- γ	2.202	1.1×10^7	6.6×10^{10}
rhIFN- γ activity of CS NPs + rhIFN α -2b & rhIFN- γ . 4°C	2.133	1.0×10^7	6.4×10^{10}
rhIFN- γ activity of CS NPs + rhIFN α -2b & rhIFN- γ . 16°C	1.918	9.5×10^6	5.7×10^{10}
rhIFN- γ activity of CS NPs + rhIFN α -2b & rhIFN- γ . 25°C	2.413	1.2×10^7	7.3×10^{10}
rhIFN- γ activity of CS NPs + rhIFN α -2b & rhIFN- γ . 30°C	2.388	1.2×10^7	7.2×10^{10}
rhIFN- γ activity of CS NPs + rhIFN α -2b & rhIFN- γ . 37°C	1.724	8.0×10^6	5.2×10^{10}

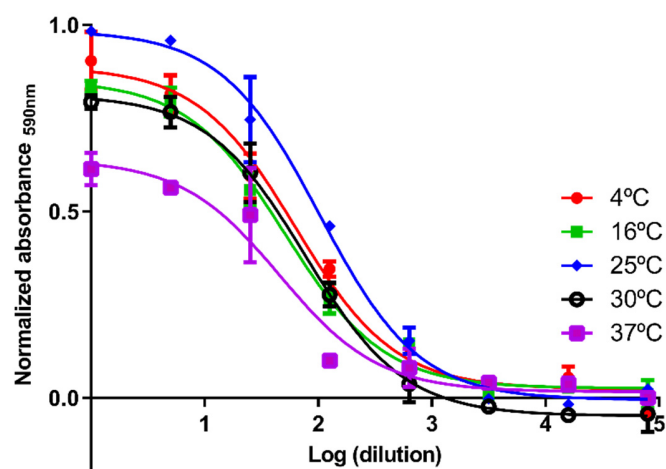


Figure S5. Stability under accelerated conditions of QS NPs + rhIFN α -2b. Log (dilution) plot of the stability results of QS NPs encapsulating rhIFN α -2b. Error bars represent mean \pm SD (three replicates).

Table S6. Determination of antiviral activity under accelerated conditions for rhIFN α -2b from QS NPs encapsulating rhIFN α -2b.

Antiviral activity of QS NPs + rhIFN α -2b	Log EC50	rhIFN α -2b titre (IU)/mL	rhIFN α -2b specific activity (IU)/mg
Standard rhIFN α -2b	3.515	1.2×10^4	
rhIFN α -2b activity of QS NPs + rhIFN α -2b, 4°C	1.802	6.2×10^3	1.8×10^8
rhIFN α -2b activity of QS NPs + rhIFN α -2b, 16°C	1.710	5.8×10^3	1.8×10^8
rhIFN α -2b activity of QS NPs + rhIFN α -2b, 25°C	2.015	6.9×10^3	2×10^8
rhIFN α -2b activity of QS NPs + rhIFN α -2b, 30°C	1.885	6.4×10^3	1.9×10^8
rhIFN α -2b activity of QS NPs + rhIFN α -2b, 37°C	1.663	5.7×10^3	1.7×10^8

Table S7. Mucosal irritability test in rabbits. Animal weight behaviour: Data are expressed as mean \pm SD. Treatment groups were compared through repeated measures ANOVA, as there were more than two evaluation times, and Bonferroni error correction was considered. In addition, a student's t-test for dependent samples was performed. The statistical significance set was $\alpha = 0.05$.

Weight (g) / Treatment		Group I	Group II	Group III	Group IV	Group V	ANOVA F (p)
Total		5	5	5	5	5	
Week 1 / Mean \pm SD		1.97 \pm 0.34	2.02 \pm 0.45	1.84 \pm 0.31	1.94 \pm 0.45	2.39 \pm 0.51	1.234 (0.328)
Week 2 / Mean \pm SD		2.11 \pm 0.39	2.10 \pm 0.34	2.08 \pm 0.71	0.91 \pm 0.32	2.59 \pm 0.78	1.079 (0.393)
Week 3 / Mean \pm SD		2.13 \pm 0.40	2.18 \pm 0.40	2.23 \pm 0.83	2.01 \pm 0.31	2.48 \pm 0.46	0.556 (0.697)
Week 4 / Mean \pm SD		2.39 \pm 0.48	2.43 \pm 0.66	2.14 \pm 0.48	2.16 \pm 0.42	2.50 \pm 0.64	0.434 (0.782)
p (t-test)	Week 1 vs Week 4	0.022	0.067	0.053	0.307	0.516	

The rabbits were evaluated for the weight variable for 4 weeks, and the relationship between the body weight variable and the treatment groups per week was established. The control groups (Group I and Group II) had similar behaviour in the four weeks. Group II Placebo (saline) as Group III (empty NPs) constituted controls for Group IV CS NPs (rhIFN α -2b-rhIFN- γ) and Group V (interferons in solution). Groups III and IV showed a trend toward weight recovery throughout the study. Group V started with higher weight values than the other groups and remained with similar figures until the end of the trial. The mean weight at week 1 vs week 4 was compared between the groups, and no significant differences were found ($p > 0.0125$) (Data not shown). The proposed treatment scheme did not affect the weight of the animals, but rather, there was a trend towards weight gain without statistical significance.

Table S8. Mucosal irritability test in rabbits. Organ weight values for each treatment group. Data are expressed as mean \pm SD.

Organs/ groups	Group I Control (g)	Group II Placebo (saline solution) (g)	Group III Empty CS NPs (g)	Group IV CS NPs + rhIFN α - 2b & rhIFN- γ (g)	Group V rhIFN α -2b & rhIFN- γ (g)
n	5	5	5	5	5
Liver	69.2 \pm 10.03	71.8 \pm 13.24	67.0 \pm 13.21	68.0 \pm 13.40	75.2 \pm 13.16
Kidneys	12.2 \pm 1.79	12.2 \pm 2.28	11.6 \pm 2.51	11.8 \pm 2.49	13.0 \pm 2.35
Lung	5.0 \pm 0.71	4.8 \pm 0.84	4.4 \pm 0.89	4.6 \pm 0.89	5.0 \pm 1.0
Heart	5.0 \pm 0.71	5.0 \pm 0.71	4.6 \pm 0.89	5.0 \pm 1.22	5.6 \pm 0.89

This test analysed the respiratory, digestive, urinary, circulatory, lymphatic, and skeletal muscle systems. Some internal organs were weighed for anatomic-morphological characterization (liver, kidney, heart, and lung). Organ weights were grouped according to treatment groups. Liver (F=0.35, p=0.84); kidney (F=0.29, p=0.88), lung (F= 0.44 p=0.77) and heart (F=0.87, p=0.51) weights showed no significant differences between treatment groups. No specific macroscopic lesions were observed in organs and systems.

Table S9. Safety study of the nanoformulations in higher organisms (sheep). Animal weight behaviour among the groups. Data are expressed as mean \pm SD. Treatment groups were compared through repeated measures ANOVA with Bonferroni correction. In addition, a dependent t-test was performed. The statistical significance set was $\alpha = 0.05$.

Weight (g) / Treatment		Group I	Group II	Group III	Group IV	ANOVA F (p)
Total		4	4	4	4	
Start		52.2 \pm 8.7	45.8 \pm 8.3	52.2 \pm 5.9	48.0 \pm 3.9	0.858 (0.489)
End		52.6 \pm 9.7	46.9 \pm 8.7	53.4 \pm 5.5	48.7 \pm 4.4	0.703 (0.568)
p (t-test)	Start vs End	0.657	0.047	0.037	0.102	

The weight behaviour during the study when comparing the beginning and the end of the treatment (F=0.858, p= 0.489; F=0.703 p= 0.568) did not show significant differences in the two times evaluated. For the average weights between the groups at each time evaluated, it was shown that there were no significant differences. Still, when comparing the average weight at the beginning vs. the end in each of the treatment groups, significant differences were found for Group 3 (CS NPs + rhIFN α -2b + rhIFN- γ) (p=0.037 < 0.05). However, the animals weight increased at the end of the treatment, which speaks in favour of the formulation's safety.