Supplementary Materials: Micellar formulations of cyclosporine for ocular delivery: investigating micelles' penetration mechanisms for anterior and posterior segment targeting

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1. Analytical methods

1.1. Cyclosporine and linolenic acid quantification method

Cyclosporine and linolenic acid quantification was performed using an HPLC-UV system (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA), with a reverse-phase column (Aeris™ WIDEPORE XB-C8 column, 150 x 4,60 mm, 3,6 µm, Phenomenex, Torrance, CA, USA) thermostated at 65°C. The mobile phase was composed of CH_3CN:water with TFA 0.1% in ratio 55:45 (v/v). The flow was maintained at 1.6 ml/min and the injection volume was equal to 100 µl. The retention time of cyclosporine and linolenic acid, both quantified by UV absorbance at 230 nm, was respectively of 4.9 and 3.1 minutes. Details on calibration curves, RSD%, RE% and LOQ values of HPLC methods for the quantification of cyclosporine and linolenic acid are reported in table S1. The chromatogram of a standard solution composed of alpha-linolenic acid and cyclosporine A is showed in Figure S1.

Table S1. Range of calibration curves, RSD% (relative standard deviation %) and RE% (relative error %) of cyclosporine and linolenic acid.

COMPOUND	CONCENTRATIONS INTERVAL (µg/ml)	RSD%	RE%	LOQ		
				LOQ (µg/ml)	RSD%	RE%
Cyclosporine	1 – 50	< 1.8	<1	1	1.2	3.2
Linolenic acid	5-100	<5.6	<5.6	5	2.3	21

To determine the solubility of cyclosporine and linolenic acid in the micellar formulation, the samples were analysed by HPLC after dilution in CH_3CN:water with TFA 0.1% in ratio 55:45. For the quantification of cyclosporine in the tissues, the extraction samples were diluted with water to obtain more solved chromatographic peaks. Indeed, rounded and poorly solved peaks were collected after direct injection of the extraction mixture due to the very high percentage of organic solvent (CH_3CN:CH_3COOH 1% in 87:13 ratio). Thus, prior to the analysis, samples were diluted by adding 60 µl of water to 200 µl of extraction sample. The same dilution was done for the preparation of the standards for the calibration curve.

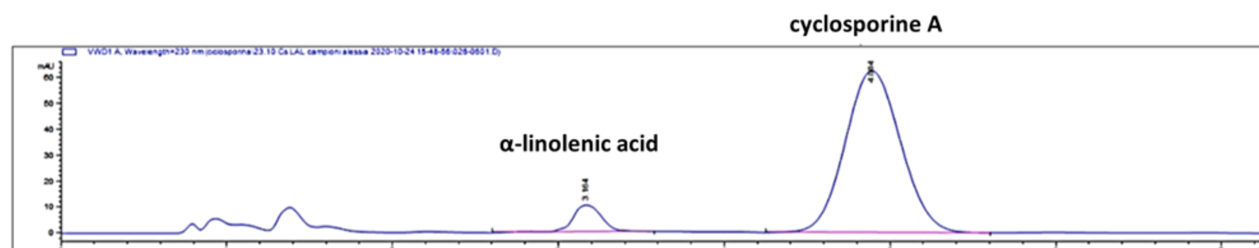


Figure S1. HPLC chromatogram of a standard solution of alpha-linolenic acid and cyclosporine A at concentration 50 µg/ml.

1.2. TPGS quantification method

TPGS quantification was done using an HPLC-UV system (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA), with a reverse-phase C18 column (Waters, Symmetry300 C18, 5 µm, 4.6 × 250 mm) and a C18 guard column (SecurityGuard Widepore C18, Phenomenex, Torrance, CA, USA), thermostated at 40 °C. The mobile phase, composed of acetate buffer pH 4.8:methanol in 3:97 (v:v) ratio, was pumped at 2 mL/min. The retention time of TPGS, quantified by UV absorbance at 215 nm, was 4 minutes while the injection volume was 100 µL. Calibration curves were built in the concentration interval 1-25 µg/ml. For the construction of the calibration curve, a stock solution was prepared by dissolving TPGS in water at final concentration of 100 µg/ml. The standards were obtained by diluting the stock solution in CH_3CN:CH_3COOH 1% in 87:13 ratio. Samples were injected without previous dilution.

1.3. Sodium fluorescein quantification method

The samples fluorescence (λ_{exc}=490 nm, λ_{em}=535 nm) was measured via microplate reader (SPARK10 M, TECAN, Mannendorf, CH). The calibration curve was built in the range 5-500 ng/ml by dissolving fluorescein sodium in HEPES buffer. The RSD% value was lower than 4.13 while the RE% value was lower than 6.42. The LOQ was 5 ng/ml with RSD% of 0.28 and RE% of 2.21.

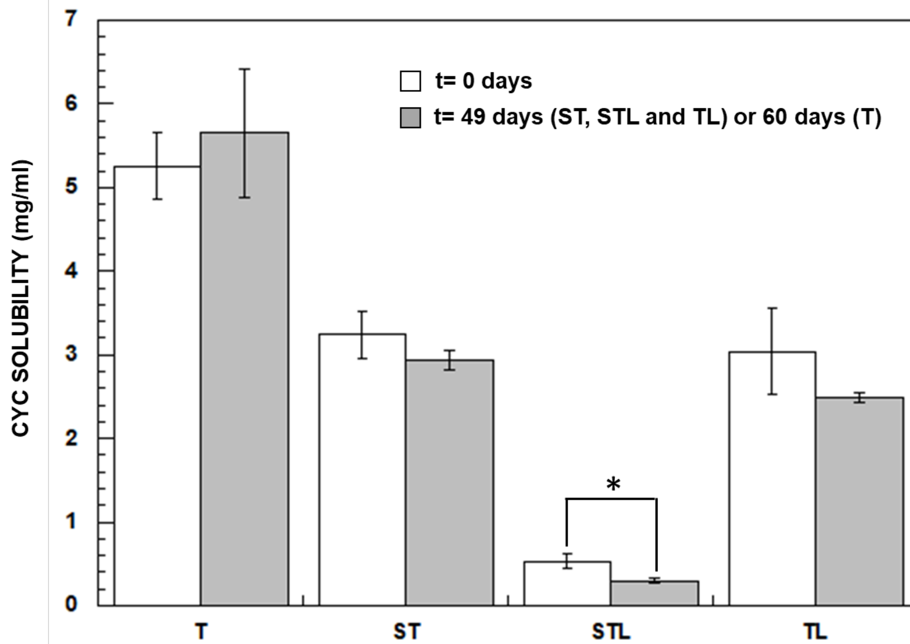
2. Micelles characterization

2.1. Micelles size

Table S2. Size (mean ± SD), intensity and PDI of blank and cyclosporine-loaded micelles at 0 days from preparation. Micelles were diluted 1:10 with high purity water before the analysis.

	BLANK			**CYC-LOADED**		
MICELLAR SOLUTION	**SIZE (nm)**	**INTENSITY (%)**	**PDI**	**SIZE (nm)**	**INTENSITY (%)**	**PDI**
S	14.09 ± 4.80	99.00	0.15	13.20 ± 2.37 444.9 ± 130.3	22.90 77.1	1.00
ST	15.10 ± 4.79	96.20	0.20	15.65 ± 5.90	94.6	0.21
T	13.86 ± 4.30 [1]	100.00	0.13	12.96 ± 4.03 [1]	100	0.09
STL	14.08 ± 3.21 341.9 ± 120.5	49.20 50.8	0.76	16.01 ± 5.88	92.5	0.23
TL	13.91 ± 3.60	72.80	0.42	14.47 ± 4.91	96.00	0.20

59 ## 2.2. Micelles stability study



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61 Figure S2. Concentration of cyclosporine in formulations T, ST, STL and TL after 0 and 49 days from preparation.
62 Micelles T, ST and TL were stable, while formulation STL showed a significant reduction of cyclosporine
63 concentration at 49 days.Data are reported as mean ± SD, $n \geq 2$. ($p^* < 0.05$)

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3. Two-photon microscopy

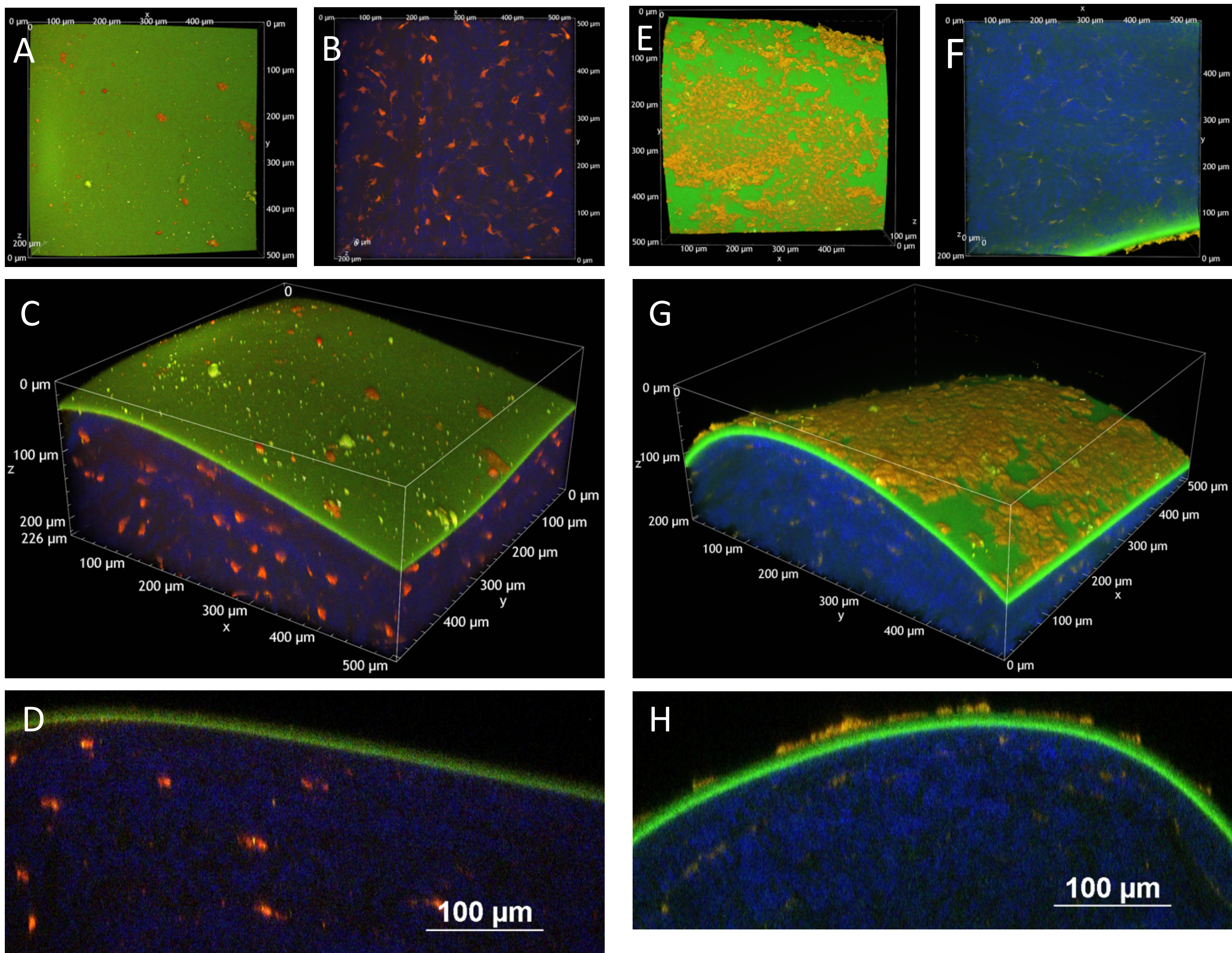

Figure S3. Volume renderings of corneal tissue (endothelium side) reconstructed from Z-stack, acquired with an excitation wavelength of 860 nm (SHG from collagen in blue, nile red fluorescence detected in both red and green channels). Panel A-D: sample treated with TPGS micelles loaded with NR (Z-step: 1 µm, total depth: 226 µm). Panel A: XY view, Panel B: -XY view, Panel C: 3D overview, Panel D: XZ slice (512 µm x 226 µm). Panel E-H: reference treated with NR aqueous solution (Z-step: 1.5 µm, total depth: 203 µm). Panel E: XY view, Panel F: -XY view, Panel G: 3D overview, Panel H: XZ slice (512 µm x 203 µm). All the images in the two Z-scans were acquired with the same detector gains and laser power. The thin layer characterized by a fluorescence signal which falls mainly in the green channel is the Descemet membrane, that is usually characterized by an autofluorescence signal in the green region when irradiated at 860 nm. Attached to this layer, some residual cells of the endothelium are present and have been clearly stained by nile red. The lack of integrity/absence of the endothelium can be attributed to the experimental procedure and particularly to Franz cells dismantling, which can highly affect the fragile structure of this layer.

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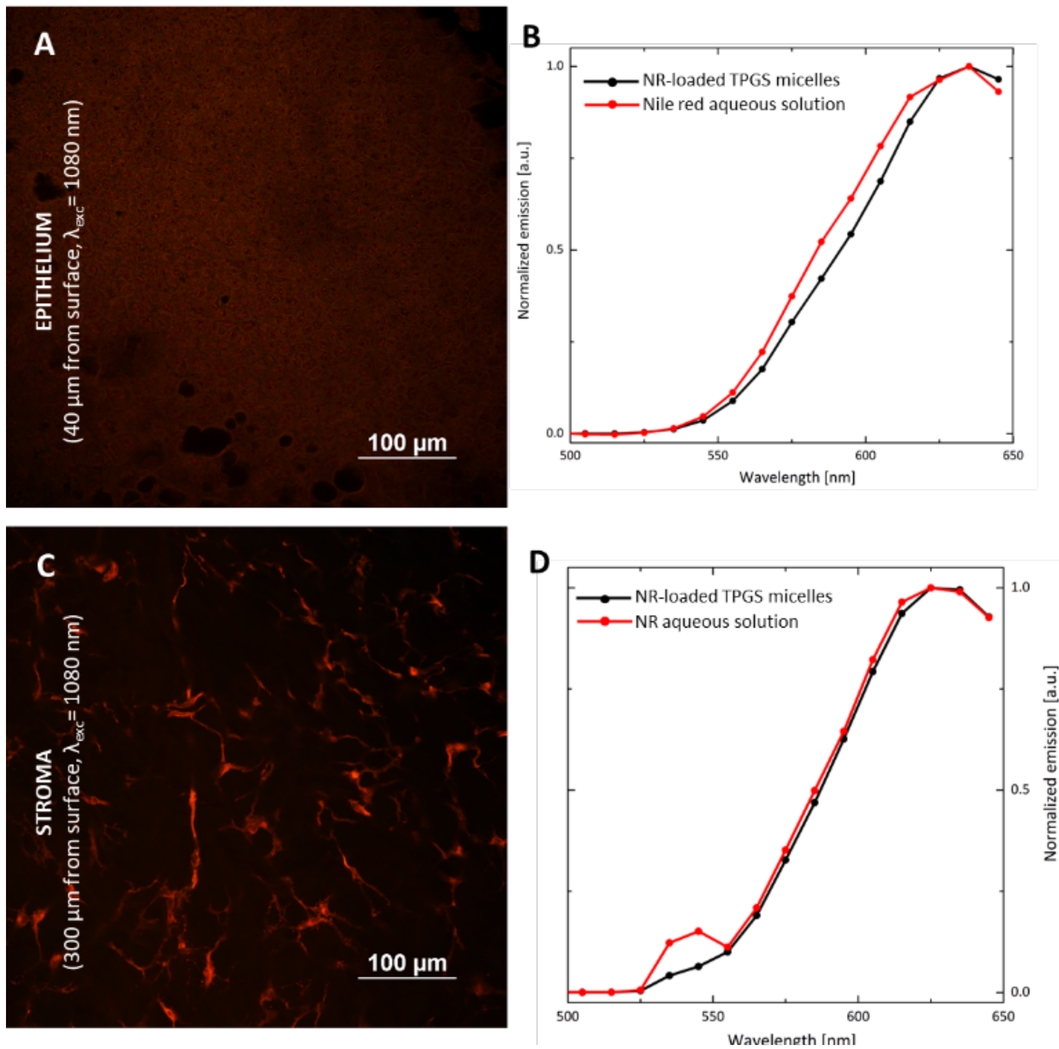

116 Figure S4. Panel A and C represents respectively the corneal epithelium and stroma treated with the NR aqueous
117 saturated solution when irradiated at 1080 nm (channels red and green channel overlay). Panel B and D shows the
118 comparison between the emission spectra recorded in the tissue in case of the NR-loaded TPGS micelles (black line) and
119 the reference aqueous saturated solution (red line), in the epithelium (B) and in the stroma (D) respectively. All the
120 images have been acquired with the same detector gain for the red and green channels and same laser power. The
121 emission profile recorded with (black line) and without TPGS (red line) micelles are almost superimposable for both
122 epithelium and stroma indicating that the environment experienced by NR is similar in terms of polarity. In panel B, the
123 emission spectrum of the NR aqueous solution (red line) is probably characterized by a higher contribution from the
124 tissue autofluorescence (due to the lower nile red concentration) that could explain the small blue-shift observed.

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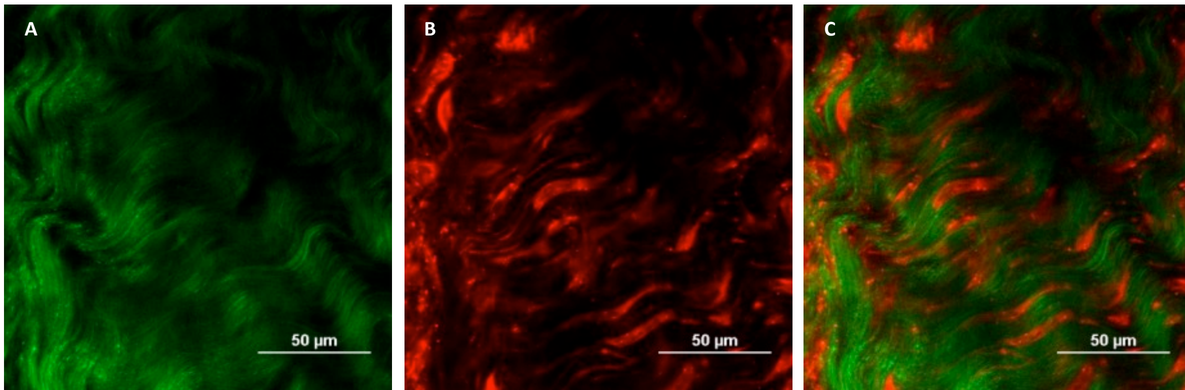

131 Figure S5. Porcine sclera treated with NR-loaded micelles upon excitation at 1080 nm. Panel A corresponds to the SHG
132 signal from collagen fibers (signal collected in the spectral region 510-540 nm using the spectral detector). Panel B
133 corresponds to NR fluorescence (signal collected in the spectral region 580-650 nm using the spectral detector) . Panel
134 C shows the image overlay: green and red images are complementary, indicating that fluorescence stems from pores
135 inside the collagen structure and further confirming that the dye can exploit the interfibrillar matrix for its penetration.
136
137
138 **References**

[1] S. Pescina, L.G. Lucca, P. Govoni, C. Padula, E.D. Favero, L. Cantù, P. Santi, S. Nicoli, Ex Vivo Conjunctival Retention and Transconjunctival Transport of Poorly Soluble Drugs Using Polymeric Micelles, Pharmaceutics, 11 (2019) 476.