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Polymethine dyes-loaded solid lipid nanoparticles (SLN) as promising photosensitizers for biomedical applications

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(Article begins on next page)

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Polymethine dyes-loaded Solid Lipid Nanoparticles (SLN) as promising photosensitizers for biomedical applications.

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Abstract:	<p>Polymethine dyes (PMD) have proved to be excellent candidates in the biomedical field for potential applications in both diagnostic and therapeutic. However, PMD application in biomedicine is hindered by their poor solubility and stability in physiological conditions. Therefore, the incorporation of these dyes inside nanosystems could be extremely important to prevent the formation of dye aggregates in aqueous environment and protect the photophysical characteristics. In the present work two PMD based on the benzoinolenine ring (bromine benzo-cyanine-C4 and bromine benzo-squaraine-C4) were incorporated into Solid Lipid Nanoparticles (SLN) to solubilize and stabilize them in aqueous solutions. Obtained SLN showed a high incorporation efficiency for both PMD ($\approx 90\%$) and not only preserved their spectroscopic properties in physiological conditions but also enhanced their excellent photophysical and photochemical properties in the NIR region. Viability assays showed good biocompatibility of both empty and loaded nanocarriers while the cellular uptake and intracellular localization showed the effective internalization in MCF-7 cells, with a partial mitochondrial localization for CY-SLN. Moreover, in vitro phototoxicity assay showed that cyanine loaded-SLN (CY-SLN) is more photoactive than the free dye.</p>
Suggested Reviewers:	<p>Nora Ventosa ICMAB: Institut de Ciència de Materials de Barcelona ventosa@icmab.es For her expertise in the field of nanocarriers.</p> <p>Anatoliy Tatarets SSI Institute for Single Crystals NAS of Ukraine: Naukovo-tehnologichnij kompleks Institut monokristaliv Nacional'na akademija nauk Ukraini tatarets@isc.kh.ua For his expertise in the synthesis of polymethine dyes for biological applications</p> <p>Atanas Kurutos Bulgarian Academy of Sciences: B'lgarska akademija na naukite ohtak@chem.uni-sofia.bg For his expertise in the synthesis of polymethine dyes for biological applications</p>



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Editor
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

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Dear Editor,

We are hereby submitting a manuscript entitled: **“Polymethine dyes-loaded Solid Lipid Nanoparticles (SLN) as promising photosensitizers for biomedical applications”** by Chinigò *et al.* for your consideration for publication in Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.

Cancer is still one of the greatest challenges facing our world and the biological significance of this work perfectly matches these challenges, i.e. the urgent need for more effective diagnostics tools and therapies. Polymethine dyes (PMD) have proved to be excellent candidates for potential biomedical applications in both diagnostic and therapeutic fields. First of all, thanks to their excellent spectroscopic properties exhibited in the near-infrared region, these dyes proved promising diagnostic agents in the field of *in vivo* optical imaging. In addition, recent studies have revealed that some PMD are potential photosensitizers for the photodynamic treatment of some types of tumor. However, due to their highly hydrophobic structure, PMD's application in biomedicine is hindered by their poor solubility and stability in physiological conditions. Hence the need to develop new approaches that allow PMD to be solubilized or screened for their hydrophobicity, thus overcoming the challenges of bioavailability.

Here we investigated the possibility to use Solid Lipid Nanoparticles (SLN) as nanocarriers to improve PMD's solubility and bioavailability in physiological conditions. In particular, we entrapped into SLN a **bromo-functionalized benzoindolenine ring-based cyanine** recently published by our group as a potential photosensitizer for PDT and its **newly synthesized squaraine** derivative which shows enhanced fluorescence performances but which is completely insoluble in aqueous solutions. To the best knowledge of the authors, this paper is the **first example in literature of squaraine's incorporation into SLN**. In summary, we demonstrated that **SLN are a valuable delivery strategy**

for PMD in biomedical applications. In fact, SLN not only allows to solubilize otherwise insoluble and unusable PMD in physiological conditions like our squaraine derivative but even **enhance their spectroscopic performances.** Overall our work demonstrated that PMD incorporation into SLN may increase the value of these dyes as candidates for future *in vivo* imaging and / or PDT applications.

Taking into account the topics covered by Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy and in particular the novel applications in photochemistry and photobiology, we believe that our work will be of interest to the readers of the Journal, especially those interested in the potential biomedical applications of dyes. Based on the novelty and impact of our findings, we hope that you will consider our manuscript for publication in Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.

Here are reported the most significant papers published by our groups about PMD's spectroscopic properties and/or PDT applicability:

Ciubini B., Visentin S., Serpe L., Canaparo R., Fin A., and Barbero N., "Design and synthesis of symmetrical pentamethine cyanine dyes as NIR photosensitizers for PDT," *Dye. Pigment.*, **160**, 806–813 (2019)

doi: 10.1016/j.dyepig.2018.09.009

Serpe L. *et al.*, "Squaraines bearing halogenated moieties as anticancer photosensitizers: Synthesis, characterization and biological evaluation," *Eur. J. Med. Chem.*, **113**, 187–197 (2016)

doi: 10.1016/j.ejmech.2016.02.035

Butnarasu C., Barbero N., Barolo C., and Visentin S., "Interaction of squaraine dyes with proteins: Looking for more efficient fluorescent turn-on probes" *Dye. Pigment.*, **184**, 108873 (2021)

doi.org/10.1016/j.dyepig.2020.108873

Barbero N., Butnarasu C., Visentin S., and Barolo C., "Squaraine Dyes: Interaction with Bovine Serum Albumin to Investigate Supramolecular Adducts with Aggregation-Induced Emission (AIE) Properties" *Chem. - An Asian J.*, **14**, 896–903 (2019)

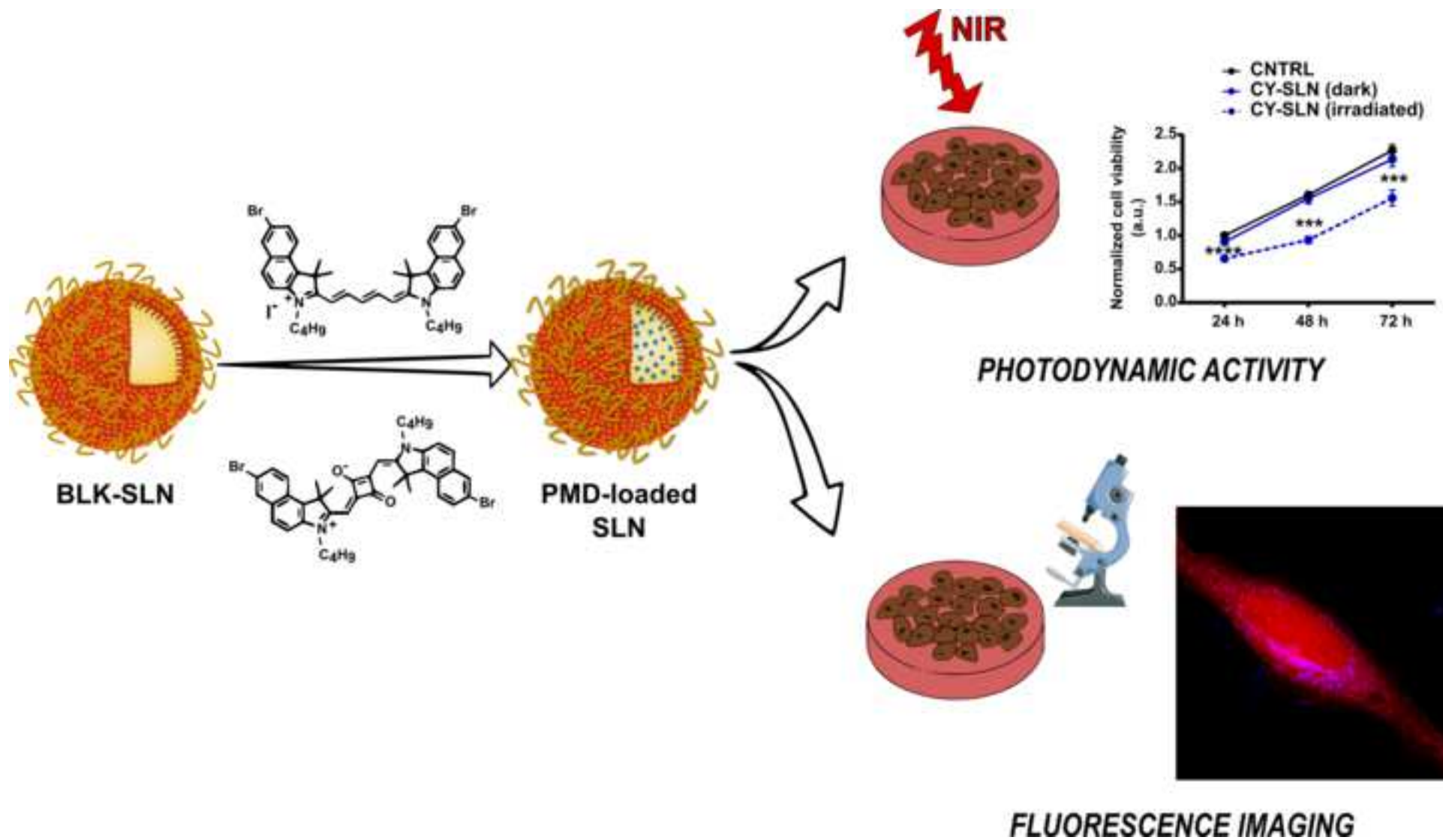
doi: 10.1002/asia.201900055

Alberto G. *et al.*, "Solid silica nanoparticles as carriers of fluorescent squaraine dyes in aqueous media: Toward a molecular engineering approach," *Colloids Surfaces A. Physicochem. Eng. Asp.*, **568**, 123–130 (2019)

doi: 10.1016/j.colsurfa.2019.01.052

Yours sincerely,

Giorgia Chinigò and co-authors



Highlights

- Polymethine dyes are promising diagnostic and therapeutic agents
- Polymethine dyes show poor solubility and stability in physiological conditions
- Solid Lipid Nanoparticles allow dyes' solubilization in physiological conditions
- Dye-loaded nanoparticles show low cytotoxicity and an efficient cellular uptake
- Nanoparticles increase Squaraines' quantum yield and Cyanines' photoactivity

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1 Polymethine dyes-loaded Solid Lipid Nanoparticles (SLN) as promising 2 photosensitizers for biomedical applications.

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16

17 **

18

19 1 ABSTRACT

20 Polymethine dyes (PMD) have proved to be excellent candidates in the biomedical field for potential
21 applications in both diagnostic and therapeutic. However, PMD application in biomedicine is hindered by their
22 poor solubility and stability in physiological conditions. Therefore, the incorporation of these dyes inside

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**Abbreviations

PMD: polymethine dye; NIR: near-infrared; PDT: photodynamic treatment; SLN: solid lipid nanoparticles; CY: cyanine; SQ: squaraine; PS: photosensitizer; DLS: dynamic light scattering; BW: before wash; A3W: after three washes; PDI: polydispersity index; EPR: enhanced permeability and retention; PEG: polyethylene glycol; AF4: asymmetric flow field-flow-fractionation

23 nanosystems could be extremely important to prevent the formation of dye aggregates in aqueous environment
24 and protect the photophysical characteristics. In the present work two PMD based on the benzoindolenine ring
25 (bromine benzo-cyanine-C4 and bromine benzo-squaraine-C4) were incorporated into Solid Lipid Nanoparticles
26 (SLN) to solubilize and stabilize them in aqueous solutions. Obtained SLN showed a high incorporation efficiency
27 for both PMD ($\approx 90\%$) and not only preserved their spectroscopic properties in physiological conditions but also
28 enhanced their excellent photophysical and photochemical properties in the NIR region. Viability assays showed
29 good biocompatibility of both empty and loaded nanocarriers while the cellular uptake and intracellular
30 localization showed the effective internalization in MCF-7 cells, with a partial mitochondrial localization for CY-
31 SLN. Moreover, *in vitro* phototoxicity assay showed that cyanine loaded-SLN (CY-SLN) is more photoactive than
32 the free dye.

33

34 KEYWORDS: polymethine dyes, solubility, nanocarrier, SLN, optical imaging, photodynamic activity

35

36 2 INTRODUCTION

37

38 In the last two decades, strong interest has been attracted to bioimaging and therapeutics of near-infrared
39 (NIR) probes. These dyes, thanks to their emission in the NIR region of the electromagnetic spectrum (650-900
40 nm), which is characterized by minimal scattering of the excitation light and low self-fluorescence of biological
41 molecules, allow deeper tissue penetration with minimal background interference [1]. Among NIR fluorescent
42 probes, NIR polymethine dyes (PMD), such as pentamethine and heptamethine cyanines (CY) and squaraines
43 (SQ), have been extensively studied for many biomedical applications [2], including *in vivo* optical imaging [3],
44 thanks to their peculiar spectroscopic properties, the easiness in designing, the simplicity of synthesis and the
45 close relationship between structure and properties [5,6]. Some NIR PMD have been shown to enhance *in vivo*
46 characterization of tumors, by significantly improving tumor visualization and allowing detection and
47 identification of small pre-neoplastic lesions and metastasis [4,7]. Moreover, it has recently been shown that a
48 unique group of NIR fluorescent heptamethine cyanine preferentially accumulate in cancer cells without the

49 need for chemical conjugation in many different types of cancer cells including cultured, circulating and
50 disseminated tumor cells [8, 9, 10], as well as in preclinical models, including mice and dogs [8–12]. More
51 interestingly, many studies have recently highlighted an intrinsic anticancer activity of some PMD, which may be
52 used as efficient agents for photothermal and photodynamic therapy (PDT) [2, 13], alternative strategies for the
53 treatment of both oncological and non-oncological diseases [13]. Indeed both cyanine [16–18] and squaraine
54 [19–24] derivatives have shown excellent light-induced toxicity on different types of tumors. Moreover, from
55 data reported in literature, it seems that the presence of a heavy atom in the heterocyclic ring of these dyes
56 leads to greater photodynamic activity, due to the enhancement of the inter-system conversion process, which
57 underlies the singlet oxygen production [18,24,25].

58 However, the main challenges in the biomedical application of NIR PMD are associated with their structural
59 characteristics, which result in poor solubility and low chemical stability, especially in aqueous solutions. In order
60 to overcome this limit, a valid alternative to the synthetic approach is represented by their incorporation into
61 different nanoparticle systems, which may solubilize or shield hydrophobicity of this class of compounds and,
62 therefore, overcome bioavailability challenges [2,26,27]. Indeed, PMD can be successfully loaded into organic
63 nanoparticles, such as micelles [28,29], liposomes [28] and lipid nanoparticles [31–33]. Among these organic
64 nanosystems, in the last decades, solid lipid nanoparticles (SLN) have proved themselves excellent candidates
65 for the targeting and delivery of various diagnostic [34,35] and therapeutic [34] agents, including photosensitizers
66 (PS) [37,38]. Besides the biocompatibility of the excipients used for their formulation and the possibility of being
67 synthesized through relatively simple and inexpensive processes, they also possess great kinetic stability [34].
68 Very few examples of incorporation of PMD within SLN are currently reported in literature, regarding
69 indocyanine green (ICG) and some cyanine derivatives based on the indolenine ring (DiO, DiI, DiD, DiR and IR-
70 780) [33,35,39].

71 Here, we investigated the possibility of using SLN to increase solubility and stability of some NIR PMD in
72 aqueous solutions in view of possible future applications in the biomedical field. In particular, we entrapped into
73 SLN two symmetrical polymethine dyes (CY and SQ) based on an identical heterocyclic moiety, i.e. a bromo-
74 functionalized benzoindolenine ring. The two dyes differ in the polymethine bridge resulting in a positively

75 charged dye (CY) and a zwitterionic one (SQ) which, in turn, provoke quite different solubility properties in an
76 aqueous solution at physiological pH. In particular, SQ dye, designed following the successful series of Br-
77 indolenine squaraines showing excellent PDT activity [22], suffers from a very low solubility in aqueous media,
78 preventing its use for biological applications. In the present paper, we showed that SLN not only allow to
79 solubilize PMD in aqueous solution, as aimed, but even enhance their spectroscopic performances, making PMD-
80 SLN potential and appealing candidates for *in vivo* imaging and/or PDT applications.

81

82 3 MATERIALS AND METHODS

83

84 3.1 Materials

85 All the chemicals for SQ and CY synthesis were purchased from Merck and were used without any further
86 purification. Epikuron® 200 (soybean lecithin with a phosphatidylcholine content $\geq 92\%$ - Cargill) was purchased
87 from AVG (Italy), trilaurin and benzylalcohol from Fluka, 2-phenylethanol and PEG-40 stearate (Myrj 52) from
88 Sigma Aldrich. NaCl was supplied by ACEF (Italy). Only freshly distilled water and ultra-pure water (Milli-Q,
89 Millipore, USA) were used. Ethanol 96%, Tetrahydrofuran and Acetonitrile (HPLC grade) were purchased from
90 Scharlab (Italy).

91 NovaChem Surfactant 100 (special mix of non-ionic and ionic detergents for Asymmetric Flow Field-Flow
92 Fractionation (AF4) applications) was purchased from Postnova Analytics GmbH (Germany).

93 3.2 Synthesis

94 (CY) was synthesized as previously reported by some of us [38]. To obtain (SQ), compound **2** (0.45 g, 0.95 mmol)
95 and squaric acid (0.05 g, 0.48 mmol) were introduced in a 10-20 mL microwave vial with a toluene/n-butanol
96 mixture (1:1, 14 mL) and heated at 160 °C for 30 min. After solvent evaporation, Flash column chromatography
97 with 100% CH₂Cl₂ allowed to remove all the impurities. Then elution of 100% acetone afforded the squaraine as
98 blue-green crystals (28%).

99 MS (ESI) [M-H]⁻: 765.08

100 ¹H NMR (200 MHz, DMSO-d₆) δ: 8.07 – 7.93 (m, 4H), 7.73 (d, J = 8.8 Hz, 2H), 7.59 (dd, J = 9.1, 2.0 Hz, 2H), 7.26
101 (d, J = 8.8 Hz, 2H), 6.15 (s, 2H), 4.10 (s, 4H), 1.97 (s, 14H), 1.89 – 1.69 (m, 5H), 1.46 (dd, J = 15.3, 7.6 Hz, 5H),
102 0.95 (t, J = 7.2 Hz, 7H).

103 ¹³C NMR (50MHz, DMSO-d₆) δ: δ 182.02, 171.28, 139.71, 134.54, 132.13, 131.39, 130.48, 128.61, 126.88,
104 123.93, 117.82, 111.03, 86.58, 50.88, 43.60, 29.24, 26.61, 20.16, 13.70.

105 All microwave reactions were performed in single-mode Biotage Initiator 2.5. TLC were performed on silica gel
106 60 F254 plates. ESI-MS spectra were recorded using LCQ Thermo Advantage Max spectrometer, with
107 electrospray interface and ion trap as mass analyzer. The flow injection effluent was delivered into the ion source
108 using N₂ as sheath and auxiliary gas. ¹H NMR (200 MHz) and ¹³C NMR (50 MHz) spectra were recorded on a Bruker
109 Avance 200 NMR.

110

111 3.3 Solid Lipid Nanoparticles (SLN) preparation

112

113 Blank (BLK-SLN) and dye-loaded SLN (CY-SLN and SQ-SLN) were prepared in collaboration with Nanovector
114 s.r.l. (Turin, Italy). SLN were prepared by oil-in-water (O/W) warm microemulsion method reported elsewhere
115 [41,42], slightly modified. Briefly, the lipid matrix (trilaurin) was heated above its melting point (52°C) together
116 with phosphatidylcholine and short-chain alcohols, then this lipid phase was mixed under magnetic stirring with
117 an aqueous phase at the same temperature containing the surfactant PEG-40 stearate in order to stabilize O/W
118 interface and promote spontaneous microemulsion formation. When clear microemulsion was formed, it was
119 immediately dispersed into water at the same temperature (52°C) (ratio 1:10 v/v) under mechanical stirring
120 (1900 rpm) and left to achieve room temperature (1900 rpm). Dye-loaded SLN were prepared in the same way
121 by adding the PMD (cyanine or squaraine) to the oil phase of the microemulsion. Obtained SLN dispersions were
122 then washed (3X) by tangential flow filtration (TFF) (Vivaflow 50, RC membrane, 100 KDa MWCO, Sartorius
123 Stedim Italy S.r.l.), for purification, all residual solvents were finally reduced at regulatory acceptable

124 concentrations [41]. Each formulation (BLK-SLN, CY-SLN and SQ-SLN) was prepared in triplicate to verify the
125 reproducibility of the synthesis procedure and increase the reliability of data.

126

127 3.4 Physicochemical characterization

128

129 3.4.1 Determination of particle size and surface charge

130

131 The average dimension of SLN was evaluated by batch mode Dynamic Light Scattering (DLS), while Zeta
132 potential was measured by Laser Doppler Velocimetry (LDV). For size measurement, SLN dispersions were diluted
133 1:100 in ultrapure water, whereas for zeta potential measurements samples were diluted 1:50 in NaCl 1mM. All
134 measurements were carried out on Zetasizer-Nano ZS (Malvern Instruments, UK), in triplicate, at 25°C.

135

136 3.4.2 Asymmetric Flow Field-Flow fractionation (AF4) characterization

137 To deeper characterize SLN's size distribution, they were analyzed by AF2000 Asymmetric Flow Field-Flow
138 Fractionation (AF4) instrument (Postnova Analytics GmbH, Germany), which was coupled online to an SPD-20A
139 UV-vis spectrophotometer (Postnova), and a Zetasizer Nano ZS (Malvern Instruments). AF4 was performed in a
140 PMMA channel with a spacer of 350 μm width at the inlet, lined with a regenerated cellulose membrane (cut off
141 10 KDa). Filtered (0.1 μm Durapore membrane) 0.05% NaCl solution in ultrapure water added with 0.05%
142 Novachem Surfactant 100 was used as the carrier. Samples were diluted 1:2 in the carrier and then manually
143 injected into the system (20 μl). Channel tip flow and focus flow was set to 1 mL/min. The flow rate to the
144 detector was kept at 0.5 mL/min, and the cross flow was set to decrease from 1 mL/min to 0.1 mL/min in 20
145 minutes and then to remain constant at 0.1 mL/min for 20 minutes. The UV detector was set at 280 nm with a
146 sensitivity of 0.001, and the acquisition time for each autocorrelation function in the Zetasizer Nano detector
147 was 3 seconds. The intensity of scattered light (kcount/s) and ζ -average mean diameter (nm) were elaborated
148 using Zetasizer Nano Software. The dimensional range for each analyzed sample was obtained from the
149 integration of the DLS distribution curve.

150

151 3.5 Entrapment efficiency and chemical composition

152 Dyes entrapment efficiency was calculated as the ratio between dye concentration in SLN dispersion
153 before washing (BW) by TFF and after 3 washing cycles (A3W) according to the following equation:

$$154 EE (\%) = \frac{[dye]_{A3W}}{[dye]_{BW}} \times 100$$

155 Washing steps were performed by addition and removal of a fixed amount of water, equal to the volume of
156 dispersion.

157 Dye concentration was determined after SLN disruption by dilution in tetrahydrofuran (1:100). Absorbance was
158 measured by spectrophotometric titration in 99% tetrahydrofuran at 692 nm and 672 nm for cyanine and
159 squaraine samples respectively (UV-visible spectrophotometer - HITACHI UH5300). Dyes concentration inside
160 SLN was calculated by comparing the absorbance of the unknown sample with a calibration curve prepared with
161 standards of known concentrations.

162 Phosphatidylcholine (PC) content was also determined by HPLC-UV analysis using a method previously described
163 [42], to confirm the final composition of SLN (i.e. after washing steps) and their chemical stability overtime.

164 3.6 Optical properties of dyes and dye-loaded SLN

165 Absorbance spectra were recorded on HITACHI UH5300 spectrophotometer (quartz cuvettes, 1 cm
166 pathway length) in ethanol with increasing water content (from 0% to 90%) for dyes' spectroscopic
167 characterization and in 100% ultrapure water for dye loaded SLN. Cyanine's molar extinction coefficient (ϵ) in
168 absolute ethanol was obtained from Ciubini *et al.* [38], whereas squaraine's molar extinction coefficient (ϵ) in
169 absolute ethanol was determined by spectrophotometric titration. The analysis was performed in duplicate.
170 Results were considered acceptable once the difference between the determined $\log \epsilon$ was less or equal to 0.02
171 in relevancy to their average.

172 Fluorescence measurements were recorded on a HORIBA FluoroLog2 (Jobin-Yvon) fluorimeter. Diluted
173 solutions with absorbance around or lower than 0.1 units were used to avoid the presence of aggregates.
174 Because of the low Stokes shift (20 nm for CY and 6 nm for SQ), typical of this class of compounds, emission

175 spectra were obtained by exciting dyes at the wavelength corresponding to the hypsochromic shoulder showed
176 in absorption spectra ($\lambda_{\text{ex}}= 620 \text{ nm}$ and 640 nm for cyanine and squaraine respectively). Dyes' fluorescence
177 spectra were recorded in ethanol with increasing water content (from 0% to 90%) and dye-loaded SLN's emission
178 spectra in 100% ultrapure water. Fluorescence lifetimes (τ_f) were measured in DMSO using a nanoLED source
179 (emission at 636 nm , Horiba Jobin-Yvon) and a single photon counting detector (TBX04 Horiba Jobin-Yvon).
180 Fluorescence quantum yields (ϕ) were determined in DMSO using the same instrument with the integrating
181 sphere Quanta- ϕ (Horiba) and De Mello method. Values reported in results correspond to the average of three
182 independent measurements.

183 3.7 Cell culture and cell viability assay

184 Human microvascular endothelial cell line (HMEC-1, American Type Culture Collection ATCC) were
185 cultured in EndoGRO™ MV-VEGF complete medium (Merck Millipore), complemented with 0.5% gentamicin
186 antibiotic and 5 mM L-glutamine; human breast adenocarcinoma cell line (MCF-7, ATCC) were cultured in
187 Dulbecco's Modified Eagle's Medium-high glucose (DMEM from Euroclone), complemented with 10% Fetal
188 Bovine Serum (FBS from Euroclone), 0.5% gentamicin antibiotic and 5 mM L-glutamine. All cell cultures were
189 maintained in an incubator at $37 \text{ }^\circ\text{C}$ and 5% CO_2 atmosphere, using Falcon™ plates as supports.

190 For cell viability, cells ($0.5 \cdot 10^4$ cells/well) were seeded in 96-well plates (Sarstedt, Germany). After 18 h
191 incubation at 37°C , different dyes concentrations of dye-loaded SLN dispersions (from 10 nM to $1 \text{ }\mu\text{M}$), were
192 added for 24 and 48 h to the culture medium. In order to compare the cytotoxicity of the dye in the free form or
193 after encapsulation, cytotoxicity of cyanine in the free form was tested (diluted from a stock 1mM in DMSO). SQ
194 alone was not tested due to its insolubility in these conditions. For each condition, eight replicates were
195 performed. Cytotoxicity was assessed 24 and 48 h after SLN treatment using CellTiter 96 AQueous Non-
196 Radioactive cell proliferation assay (Promega, USA) following manufacturer instruction. Briefly, MTS was added
197 (10%) to the culture medium and kept in an incubator for 2 h. Cells without SLN and incubated with complete
198 medium or basal medium without serum and grow factor (EndoGRO starve for HMEC samples and DMEM 0%
199 FBS for MCF-7 samples) were used as positive and negative controls, respectively. Absorbance was then recorded
200 at 490 nm (soluble formazan absorbance) using a microplate reader (FilterMax F5, Multi-Mode Microplate

201 Reader, Molecular Devices). Absorbance values obtained were analyzed with Excel software (Office, Microsoft)
202 to determine the mean absorbance for each condition after subtraction of the average background (absorbance
203 value of cell medium alone treated with MTS was considered as background). Absorbance values obtained are
204 directly proportional to the number of viable cells.

205 3.8 Photodynamic treatment and phototoxicity assay

206 To evaluate the photodynamic activity, MCF-7 cells were plated in 96 well plates ($0.5 \cdot 10^4$ cell/well in 200 μ L
207 of DMEM 10% FBS) and after six hours were treated with CY-SLN, SQ-SLN and CY at different concentrations
208 (from 10 to 200 nM). Cells were incubated O/N at 37°C and 5% CO₂ and then were irradiated with a light beam
209 intensity of 8 mW/cm² for 15 minutes. For cell irradiation, a compact LED array-based illumination system with
210 a homogeneous illumination area was used. The system was specifically designed and produced by Cicci Research
211 s.r.l (Italy) for *in vitro* PDT tests on cells grown in standard multiwell plates (96-wells). The proposed illumination
212 system includes a RED-LED array (light source with excitation wavelength: 640 nm, and irradiance: 8 mW/cm²)
213 composed of 96 LEDs in a 12 × 8 arrangement. In addition, both LED-array and 96-multiwell plates were placed
214 into a case to isolate the system during irradiation. 24 h, 48 h and 72 h after LED treatment MTS assay were
215 performed in order to evaluate cell viability. For each time monitored (24 h, 48 h and 72 h) two 96-well plates
216 were prepared: one plate was treated with the light beam and the other one was used as control (not irradiated).

217 3.9 Cellular uptake and intracellular localization

218 Cells ($50 \cdot 10^4$ cells/dish) were seeded in 10 cm diameter Petri dishes and after three days culture media
219 were removed and replace with fresh culture media. Cells were incubated for 2 h, 6 h, O/N and 24 h with fresh
220 culture media containing the same concentration (100 nM) of cyanine in the free form (CY) or encapsulated into
221 SLN (CY-SLN) and of squaraine encapsulated into SLN (SQ-SLN). Following incubation at 37°C, the medium was
222 removed, cells were trypsinized and the pellet was washed twice with PBS. Proteins were subsequently extracted
223 using 50 μ L of RIPA buffer (Pierce® RIPA Buffer, Thermo scientific) and left 1 h on ice. Then cell lysates were
224 sonicated and left on ice for 1 h. After quantification by the BCA Protein Assay proteins were diluted in ethanol

225 to a final concentration of 1 $\mu\text{g}/\mu\text{L}$. Then cell lysates were diluted 1:10 in ethanol to a final volume of 1 mL and
226 fluorescence emission of the sample was recorded on a spectrofluorimeter (FluoroLog2, Jobin-Yvon - HORIBA).

227 To assess the intracellular localization of dye-loaded SLN we used Calcein and MitoTracker Red
228 (Molecular probes[®], Invitrogen), in order to label and track the whole cellular volume and mitochondria in live
229 cells, respectively. Briefly, $10 \cdot 10^4$ cells were left to attach for 24 h on glass coverslips in a 6-well plate (Sarstedt,
230 Germany) and then incubated O/N with growth medium containing 100 nM of the dye incorporated into SLN
231 (CY-SLN or SQ-SLN) or of the dye in the free form (CY). After incubation, cells were washed and then incubated
232 with Calcein (250 nM) or MitoTracker Red (250 nM) for 30 min. After incubation, wells were washed twice with
233 Hanks' Balanced Salt Solution (HBSS) in order to wash off the excess probe and fixed in 4% paraformaldehyde
234 (PAF) at 37°C for 2 min. Coverslips were then mounted onto a glass slide by DABCO MIX (purchased from Sigma-
235 Aldrich) and observed using a Leica TCS SP8 confocal system (Leica Microsystems, Germany) equipped with a
236 HCX PL APO 63X/1.4 NA oil-immersion objective. Cyanine and squaraine dyes were excited with a HeNe laser at
237 633 nm, whereas Calcein and MitoTracker Red were excited with DPSS laser at 561 nm in order to simultaneously
238 detect the probes. Images were acquired on the three coordinates of the space (XYZ planes) with a resolution of
239 $0.081 \mu\text{m} \times 0.081 \mu\text{m} \times 0.299 \mu\text{m}$ and were processed and analyzed with ImageJ software (Rasband, W.S., U.S.
240 National Institutes of Health, Bethesda, MA). 3D images with Calcein allowed assessing whether dyes are within
241 the cell or not and Mitotracker Red signals allowed to understand whether dyes co-localize with cell
242 mitochondria (Pearson's correlation coefficient was measured by using ImageJ JACoP plugin).

243 3.10 Statistical analysis

244 Data shown are the average values of three independent pulled experiments \pm SEM (standard error mean).
245 Statistical analyses were performed using Graph-Pad Prism 6.0 software (La Jolla, CA, USA). Statistical significance
246 between populations was determined by analysis of variance (1way ANOVA-Kruskal Wallis test) followed by *post-*
247 *hoc* Dunn's multiple comparisons test. Differences with p-values <0.05 were considered statistically significant
248 and *: p-value < 0.05 , ***: p-value < 0.0005 , ****: p-value < 0001 .

249

250 4 RESULTS AND DISCUSSION

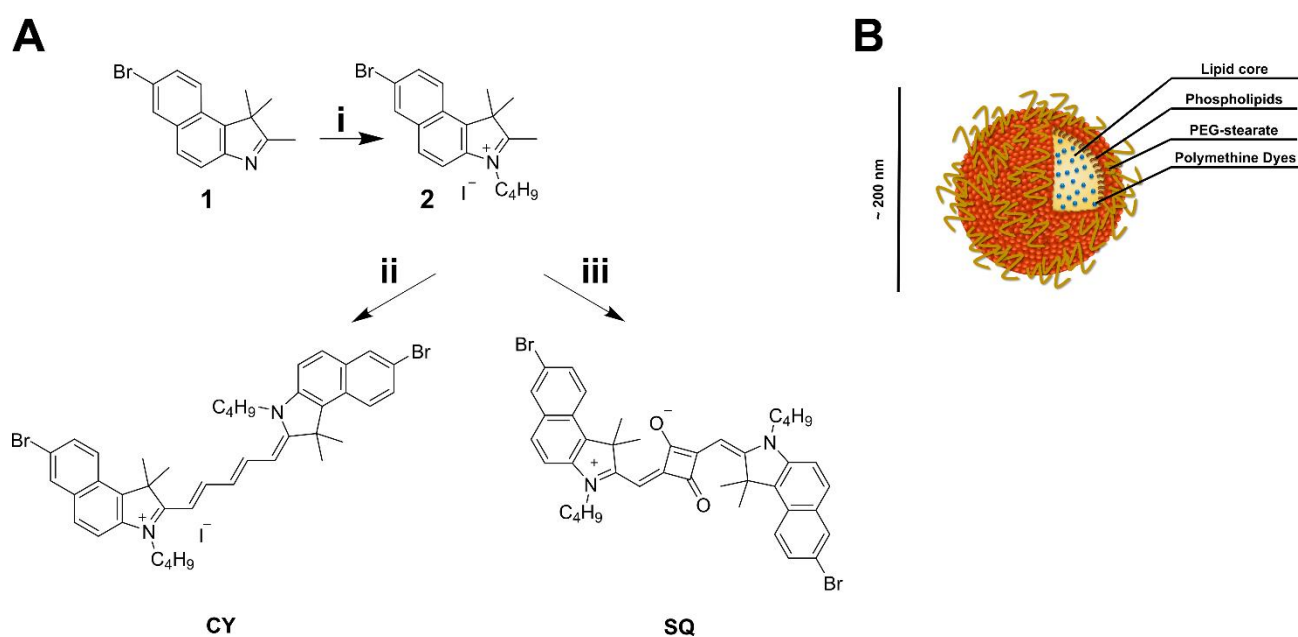
251

252 4.1 Synthesis of polymethine dyes

253

254 The synthesis of symmetrical brominated pentamethine cyanine and squaraine dyes involved the
255 condensation of the quaternary heterocyclic salts (**2**), bearing an activated methyl group, with a
256 malonodialdehyde derivative and squaric acid, respectively. Compound **1** was obtained from 7-bromo-1,1,2-
257 trimethyl-1Hbenzo[e]indole exploiting the Fischer indole synthesis by reacting (6-bromonaphthalen-2-yl)
258 hydrazine with 3-methylbutan-2-one in glacial acetic acid, as previously described [38]. The quaternization of the
259 benzoindolenine ring to get compound **2**, performed under microwave irradiation, led to an increased acidity of
260 the methyl group which enabled the following condensation reaction (Fig. 1 A) to obtain CY and SQ. The synthesis
261 of the symmetrical cyanine dye is already reported in our previous work [38], while the symmetrical squaraine
262 dye was synthesized in a one-step reaction under microwave heating following our well-established method for
263 indolenine-based squaraines [43] by reacting two equivalents of quaternary heterocyclic salts with squaric acid
264 (Fig. 1 A).

265



266

267 **Figure 1. PMD and SLN.**

268 A) PMD synthesis: (i) acetonitrile, iodobutane, MW, 45 min, 155 °C; (ii) sodium acetate, acetic anhydride, N-((1E,2E)-3-
269 (phenylamino)allylidene)benzenaminium chloride, MW, 10 min, 130 °C; (iii) squaric acid, toluene/n-butanol, MW, 30 min,
270 160 °C. B) Schematic representation of dye-loaded SLN synthesized.

271

272

273 4.2 Solid Lipid Nanoparticles (SLN) preparation and physicochemical characterization

274

275 Solid Lipid Nanoparticles (SLN) and dye-loaded SLN were prepared as reported in the experimental section.
276 Results of SLN physicochemical characterization through batch mode dynamic light scattering (DLS) for each
277 formulation after the purification procedure (A3W) are reported in Table 1. BLK-SLN exhibited a mean diameter
278 of 171 nm and a polydispersity index (PDI) lower than 0.20, which indicates the homogeneity of size distribution.
279 On the other hand, the characterization of dye-loaded SLN (CY-SLN and SQ-SLN) revealed higher sizes due to the
280 presence of the PMD within the nanoparticles and slightly higher PDI that remained anyway lower than 0.30. A
281 schematic representation of the dye-loaded SLN synthesized is represented in Figure 1 B. It is important to
282 highlight that synthesized particles have a mean diameter around 200 nm, which is highly interesting in cancer
283 applications. Indeed, previous studies on liposomes of different mean size [44] set at 200 nm the threshold size
284 for more effective extravasation of nanoparticles into the tumor tissues via the leaky vessels by the enhanced
285 permeability and retention (EPR) effect [45]. Moreover, the presence of the steric stabilizer PEG-40 stearate into
286 the formulation will probably improve the bioavailability of the nanosystem in body fluids and its
287 pharmacokinetic profile after administration for further potential *in vivo* studies [46]. Indeed, PEG chains
288 sterically stabilize nanoparticles and increase their plasma half-life, reducing binding to serum proteins and other
289 opsonic factors [47]. Moreover, both blank and dye-loaded SLN showed good stability under storage at different
290 temperatures (4, 25 and 40 °C): as shown in Figure S1 A, size and PDI of all SLN did not change over 30 days from
291 their synthesis at all three investigated temperatures.

292 Regarding the ζ -potential, BLK-SLN showed a value of -11.6 mV, comparable to other SLN prepared with
293 triglycerides [48], suggesting good stability of the dispersion. Comparing ζ -potentials measured on the loaded

294 SLN (Table 1), we observed a significant difference between the two formulations: SQ-SLN showed a negative ζ -
 295 potential as well as BLK-SLN, whereas CY-SLN showed an inversion of ζ -potential, which becomes positive.
 296 Considering the structures of the two PMD (Fig. 1 B) and the positive charge associated with the cyanine dye,
 297 the inversion of the potential observed in the case of CY-SLN can be due to a partial localization of the dye on
 298 the particle surface. This effect has been also described for other cyanine dyes incorporated in lipid nanocarriers
 299 [49].

300

301 **Table 1**

302 *Physicochemical properties of BLK-SLN, SQ-SLN and CY-SLN. PDI: polydispersity index; ZP: zeta potential, EE: entrapment*
 303 *efficiency; PC: phosphatidylcholine (mean \pm SD, n = 3).*

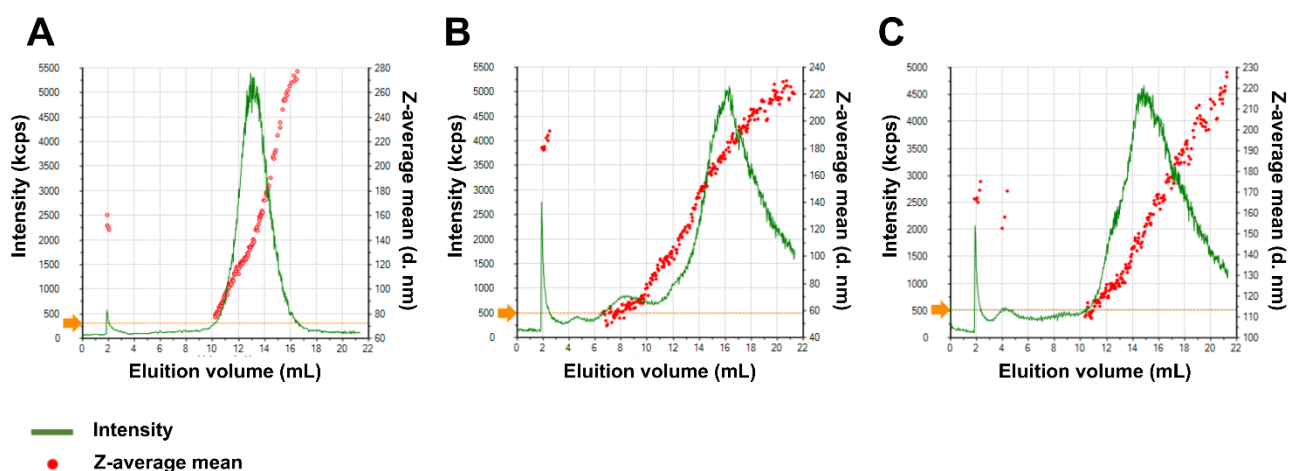
SLN	Size (nm)	PDI	ZP (mV)	EE (%)	PC (mg/mL)
BLK-SLN	170.9 \pm 22.84	0.152 \pm 0.052	-11.6 \pm 4.9	-	11.05 \pm 1.50
CY-SLN	194.7 \pm 27.43	0.258 \pm 0.036	+7.8 \pm 2.2	89.2 \pm 0.4	11.60 \pm 1.00
SQ-SLN	203.4 \pm 18.43	0.250 \pm 0.052	-6.6 \pm 2.3	88.9 \pm 7.4	9.96 \pm 1.78

304

305 Finally, in order to deeper physicochemical characterization and to verify the effective size distribution of
 306 the formulation obtained through batch mode DLS, SLN were also analyzed employing Asymmetric Flow Field-
 307 Flow-Fractionation (AF4), an elution-based particle separation technique that allows to separate, detect and
 308 measure any sub-populations eventually present in the colloidal sample. This technique is based on the
 309 application of a force field (cross flow) perpendicular to the particle transport direction, which allows smaller
 310 particles to be transported faster and eluted earlier [50]. AF4 analysis confirmed that all 3 types of SLN prepared
 311 were characterized by one main population: the fractograms, reported in Figure 2, show an upward trend of
 312 hydrodynamic size over the elution peaks. The diameters calculated by analysis of light scattered signals
 313 corresponding to peak elution were respectively 143 nm for BLK-SLN (Fig. 2 A), 182 nm for CY-SLN (Fig. 2 B) and
 314 157nm for SQ-SLN (Fig. 2 C). The elution volume (mL), corresponding to the elution time (min), and peak width
 315 are displayed in Table 2.

316 The difference with respect to the diameter obtained from the batch mode DLS measurement may be due
 317 to the different ionic composition of the buffer used in AF4, compared to ultrapure water used for the batch

318 mode. Indeed, the different ionic and surfactant composition of the medium alters the nature of the ionic sphere
 319 that surrounds the surface of nanoparticles and, consequently, their hydrodynamic radius, which includes not
 320 only the diameter of the nanoparticle itself but also the ions included within the slipping plane, which move
 321 together to it in Brownian motion. Moreover, a small shoulder was detected before the elution peak of CY-SLN
 322 (6.5 mL <elution volume <10.6 mL). Nevertheless, because of the low intensity of the peak close to the noise
 323 threshold, this signal is not attributable to the presence of a sub-population of SLN within the sample, but it
 324 probably indicates the formation of small micellar systems (64 nm) induced by the surfactants present in the
 325 elution medium used (Novachem 0.05% + NaCl 0.05%) and probably related to the different surface
 326 characteristics showed by positive ζ -potential of CY-SLN compared with the other formulations [51].



327

328 **Figure 2. SLN AF4 characterization.**

329 AF4-DLS fractograms of BLK-SLN (A), CY-SLN (B) and SQ-SLN (C) (red dots: z-average mean diameter in nm; green curve:
 330 intensity of scattered light in kcount/s; yellow line: noise threshold).

331

332

333 **Table 2**

334 *Particle size obtained from DLS analysis of AF4 fractograms, elution volume (mL) and peak width.*

335

SLN type	Z-average mean diameter (nm)	Elution volume (mL) (initial-final)	Peak width (nm)
BLK-SLN	143	10.5-16	32.0
CY-SLN	182	12.5-21	27.2
SQ-SLN	157	11-20	25.2

336

337 The entrapment efficiency of SLN was very high for both dyes, with values close to 90% (Table 1), showing
338 the efficacy of this type of lipid carrier for the incorporation of hydrophobic molecules. Moreover, the PC
339 concentrations displayed in Table 1 confirmed the final composition of SLN and negligible losses during the
340 purification process, as PC content after 3 washes was about 90% compared to that of non-washed formulations.

341

342 4.3 Dyes and dye-loaded SLN optical characterization

343

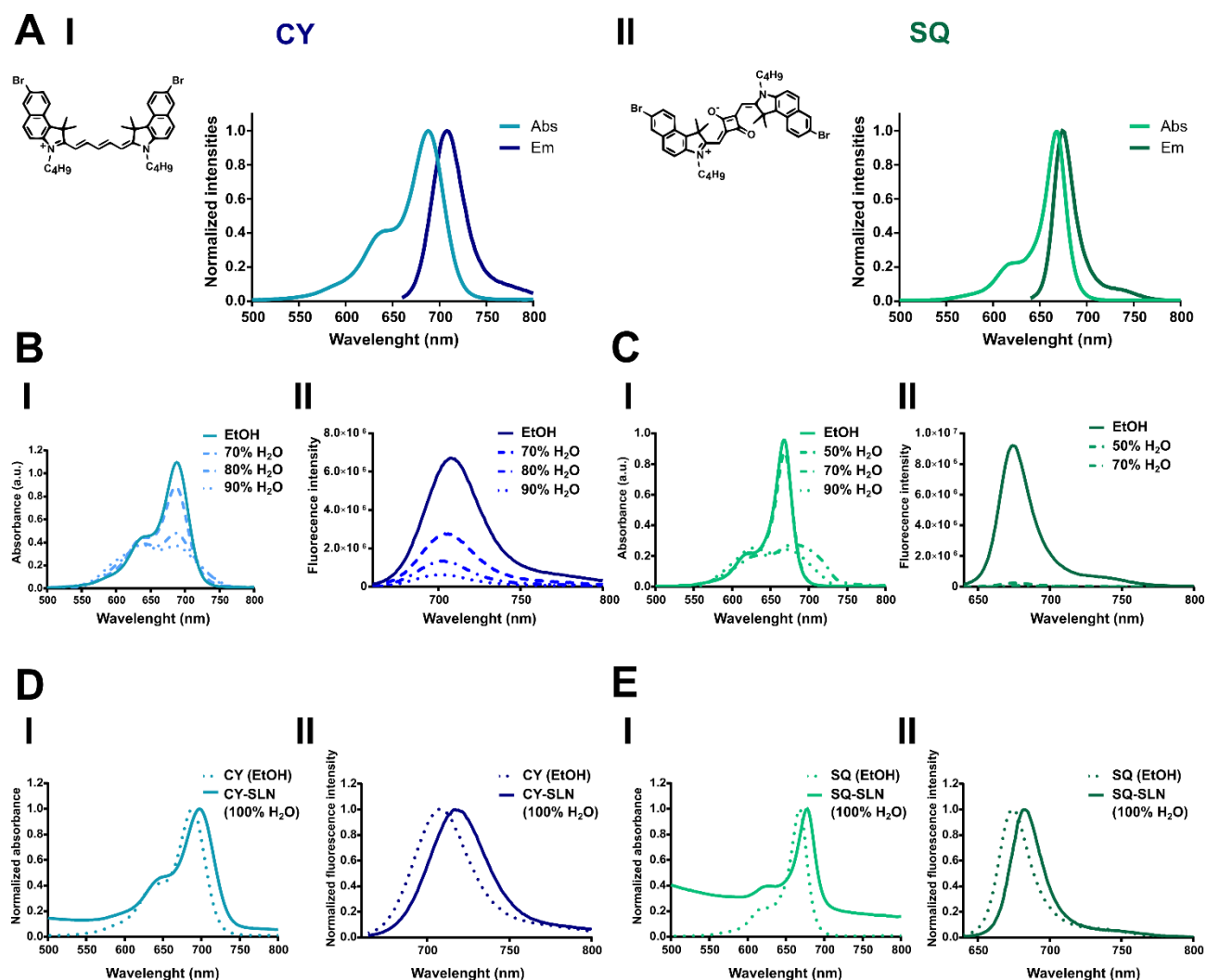
344 CY and SQ dyes show a narrow absorption band in the Far-Red, perfectly matching the phototherapeutic
345 window (Fig. 3 A). CY (Fig. 3 A-I) and SQ (Fig. 3 A-II) show similar optical properties in absolute ethanol with an
346 absorbance maximum at 688 (CY) and 668 (SQ) nm, preceded by a hypsochromic shoulder typical for PMD [24]
347 (at about 620 and 640 nm respectively) and emission maxima at 708 (CY) and 674 nm (SQ). CY and SQ exhibit
348 high molar extinction coefficients ($2.08 \cdot 10^5$ and $2.52 \cdot 10^5$ L·mol⁻¹ cm⁻¹, respectively, in ethanol) and good
349 quantum yields in organic solvent (36% and 31% respectively in DMSO). The higher molar extinction coefficient
350 showed by SQ as compared to CY is due to the greater rigidity of squaraine derivatives' polymethinic bridge,
351 which reduces photoisomerization phenomena leading to a more stable structure and an optimization of its
352 optical properties with respect to the corresponding cyanine derivative. The squaraine derivative's
353 absorption/emission spectrum shows a typical hypsochromic shift compared with the corresponding cyanine
354 derivative, because of the presence in its structure of the squaric acid core that balances the positive charge
355 present on the heterocyclic nitrogen.

356 However, both dyes are poorly soluble in aqueous solutions and this compromises their applicability in
357 the biomedical field, as well as that of other organic dyes. Indeed, as shown in Figures 3 B and 3 C, absorption
358 spectra of CY (Fig. 3 B-I) and SQ (Fig. 3 C-I) in ethanol show a significant change in shape and intensity upon
359 increasing the water content from 0 to 90%, leading to the bleaching of their fluorescent properties (Fig. 3 B-II
360 for CY and Fig. 3 C-II for SQ). The modifications of the absorption spectra upon water addition suggest the rapid

361 aggregate formation of the two dyes in aqueous conditions. This phenomenon is particularly evident in the case
362 of SQ dye, which exhibits a lower solubility than CY in aqueous solution due to its zwitterionic structure.

363 Interestingly, the incorporation of the PMD into SLN allows to preserve their standard spectroscopic
364 properties even in aqueous solution (Fig. 3 D and 3 E). Indeed, dye-loaded SLN reproduce the same spectroscopic
365 profile exhibited by dyes in the free form in organic solvent with a small bathochromic shift (Table 3). The
366 absorption below 600 nm, shown by dye-loaded SLN, may be due to the Rayleigh scattering from nanoparticles
367 and it is more pronounced in the case of SQ-SLN because of the lower dyes/lipids (w/w) ratio as compared to CY-
368 SLN's one. SQ-SLN quantum yield in H₂O is even higher than the one exhibited by free-dye in DMSO (52% versus
369 31%) (Table 3). We also evaluated and compared the fluorescence lifetime (τ_f) of the free-dyes and the dye-
370 loaded SLN. Free-dyes' fluorescence lifetimes (τ_f) show mono-exponential decay and are in the nanoseconds
371 range, accordingly with the previous results [38]. As regard dye-loaded SLN, the mono-exponential trend of the
372 τ_f indicates a homogeneous dispersion of the dyes within the lipidic nanosystem in both formulations. Moreover,
373 τ_f recorded for dye-loaded SLN in aqueous solution are similar to those obtained for free-dyes in organic solvent.
374 Interestingly, squaraine into SLN shows again enhanced optical performances with respect to its free form with
375 a fluorescence lifetime even doubled (2.571 versus 1.382). This means that the SQ derivative into SLN gives rise
376 to prolonged fluorescence emission, probably due to an increased stabilization of the fluorophore by the lipidic
377 microenvironment. Finally, we also investigated the stability of the optical properties of the dyes into SLN. Both
378 CY-SLN and SQ-SLN showed good stability until 30 days after formulation (at 4°C and 25°C) and only a small
379 decrease in fluorescence intensity was observed keeping the sample at 40°C (condition of accelerated stability)
380 for 30 days (Fig. S1 B).

381 These results clearly show that the incorporation of PMD into SLN allows a successful preservation of
382 their optical characteristics, making them suitable candidates for optical imaging.



384

385 Figure 3. Dyes and dye-loaded SLN optical characterization.

386 **A)** Normalized absorption (abs) and emission (em) spectra of CY (I) and SQ (II) in absolute ethanol. Insets show dyes'
 387 structures. **B)** Changes in the absorption spectra (I) and in the emission spectra (II) of CY (5×10^{-6} and 5×10^{-7} M respectively
 388 in absolute ethanol) upon increasing content of water from 0 to 90%. λ_{ex} for emission spectra = 620 nm. **C)** Changes in the
 389 absorption spectra (I) and in the emission spectra (II) of SQ (3×10^{-6} and 3×10^{-7} M respectively in absolute ethanol) upon
 390 increasing content of water from 0 to 90%. λ_{ex} for emission spectra = 640 nm. **D)** Normalized absorption (I) and emission
 391 (II) spectra of CY encapsulated into SLN (CY-SLN) in 100% water. Dot lines represent normalized absorption (I) and emission
 392 (II) spectra of CY in the free form in absolute ethanol. **E)** Normalized absorption (I) and emission (II) spectra of SQ
 393 encapsulated into SLN (SQ-SLN) in 100% water. Dot lines represent normalized absorption (I) and emission (II) spectra of SQ
 394 in the free form in absolute ethanol.

395

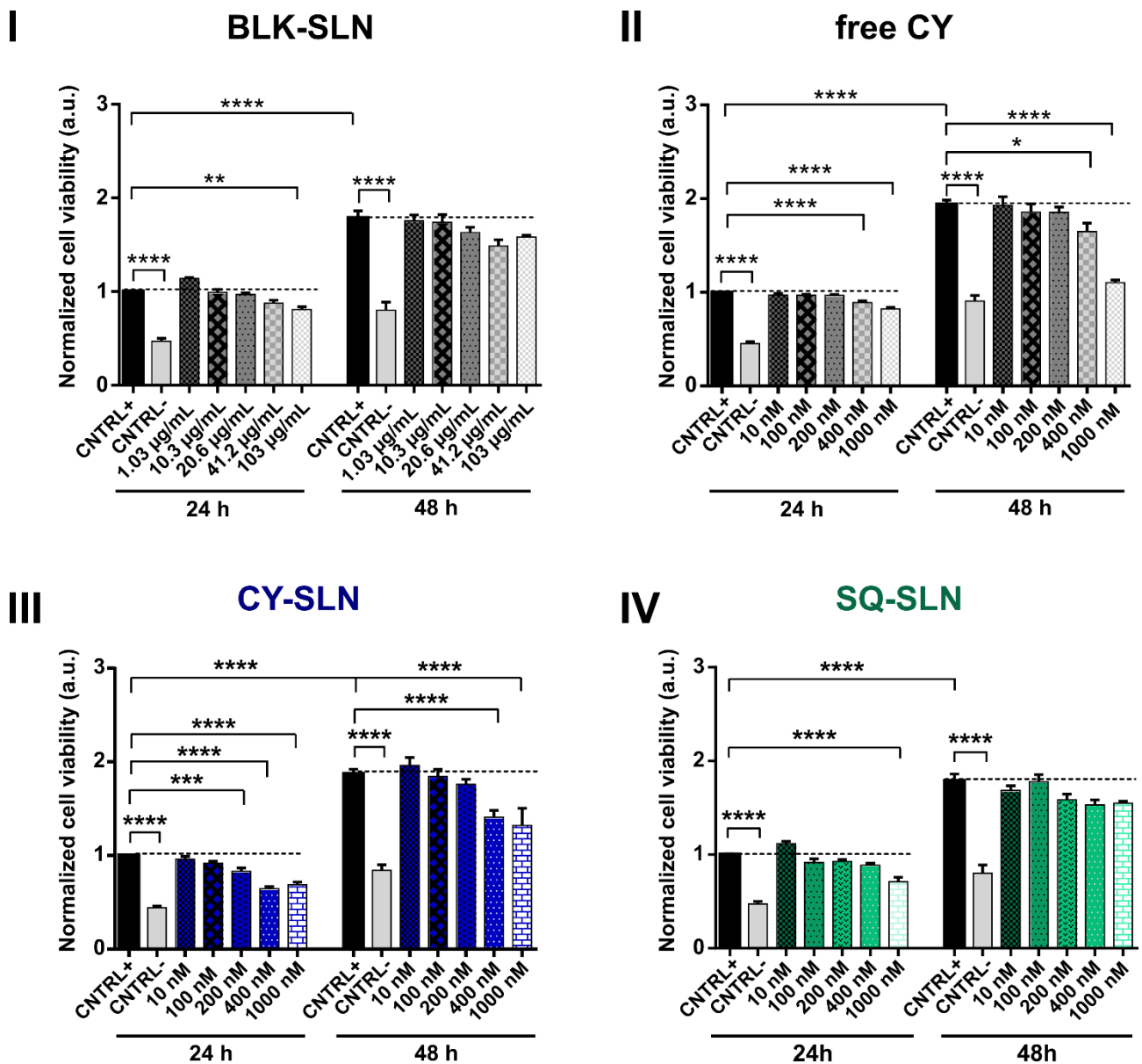
396 *Table 3*
 397 *Optical properties of free-dyes (CY and SQ) in organic solvent and dye-loaded SLN (CY-SLN and SQ-SLN) in aqueous solution.*
 398 *$\lambda_{max}(abs)$, $\lambda_{max}(em)$: dyes absorption and emission maxima, respectively, ϵ : molar extinction coefficient at the absorption*
 399 *maximum, ϕ_{fl} : fluorescence quantum yield, τ_f : fluorescence lifetime.*

	absorbance		emission				
	λ_{max} (nm)	$\epsilon \lambda_{max}$ (L mol ⁻¹ cm ⁻¹)	λ_{max} (nm)	ϕ_{fl} (%)		τ_f (ns)	
	EtOH/H ₂ O	EtOH	EtOH/ H ₂ O	DMSO	H ₂ O	DMSO	H ₂ O
CY	688	$2.08 \cdot 10^5$	708	36	-	1.570 ± 0.004	-
CY-SLN	698	-	717	-	36	-	1.372 ± 0.004
SQ	668	$2.52 \cdot 10^5$	674	31	-	1.382 ± 0.003	-
SQ-SLN	678	-	682	-	52	-	2.571 ± 0.004

403 4.4 *In vitro* cell viability of CY-SLN and SQ-SLN

405 We investigated the inherent cytotoxicity of BLK-SLN, CY-SLN and SQ-SLN using the MTS viability assay on
 406 MCF-7 cells. BLK-SLN showed good biocompatibility (Fig. 4 A-I), starting to affect cell viability 24 h after treatment
 407 only at the highest lipid concentration tested (about 100 μ g/mL, corresponding to a dye concentration of 1.5 μ M
 408 and 1 μ M for CY and SQ respectively). Regarding dye-loaded SLN, cell viability was tested by varying dyes'
 409 concentration into SLN in the nanomolar range, in order to identify the maximum concentration at which
 410 treatment can be administered without inducing cytotoxicity. CY cytotoxicity in its free form was also tested and
 411 Figure 4 A shows that CY (Fig. 4 A-II) started to affect cell viability from a concentration of 400 nM 24 h after
 412 treatment, whereas surprisingly, once incorporated into SLN its cytotoxicity increased starting from a
 413 concentration of 200 nM 24 h after treatment although with partial recovery at 48h (Fig. 4 A-III). On the contrary,
 414 SQ-SLN (Fig. 4 A-IV) are less cytotoxic and do not affect cell viability until a dye concentration of 1 μ M. To check
 415 the cell-type specificity of the observed CY-SLN cytotoxic effect, we measured CY-SLN cytotoxicity also on
 416 endothelial cells (HMECs) and, surprisingly, CY-SLN do not show the same cytotoxic profile shown on MCF-7 (Fig.
 417 S2). Indeed, HMECs treated with 200 nM of CY-SLN still show a slight increase in cell viability as compared to the
 418 control even 48 h after treatment (Fig. S2). Similar results were also found testing BLK-SLN cytotoxicity on HMECs
 419 at lipid concentrations corresponding to those used for CY-SLN treatment (Fig. S2). On the other hand, CY
 420 cytotoxicity in the free form did not show any differences between the two cell types, at least in the

421 concentration range investigated (till 200 nM) (Fig. S2). These results indicate that SLN's and CY-SLN's cytotoxicity
 422 is cell type-dependent and it may also suggest that tumor cells may be more sensitive to the dye-loaded
 423 nanosystem than normal cells. For subsequent characterizations of our nanosystems on tumor cell model (MCF-
 424 7) we used SLN with dye concentrations of 100 nM, in order to avoid results affected by any cytotoxic effects
 425 (Fig. 5 A-I).



426

427 **Figure 4. In vitro cytotoxicity of CY-SLN and SQ-SLN on MCF-7.**

428 Cell viability assays on MCF-7 24 h and 48 h after treatment with different concentrations of BLK-SLN (I), free CY (II), CY-SLN
 429 (III) and SQ-SLN (IV). For BLK-SLN concentrations refer to phosphatidylcholine content ([PC] from 1.03 to 103 µg/mL),
 430 whereas for dye-loaded SLN concentrations refer to dyes incorporated into SLN (from 10 to 1000 nM). Data are normalized
 431 on CNTRL+ (MCF-7 untreated) at 24 h and are represented as mean ± SEM. Data refer to a pull of at least 3 independent

432 experiments (eight replicates for each experiment). Statistical significance versus CNTRL+: *** P< 0.001, **** P< 0.0001
433 (Kruskal-Wallis test with *post-hoc* Dunn's test).

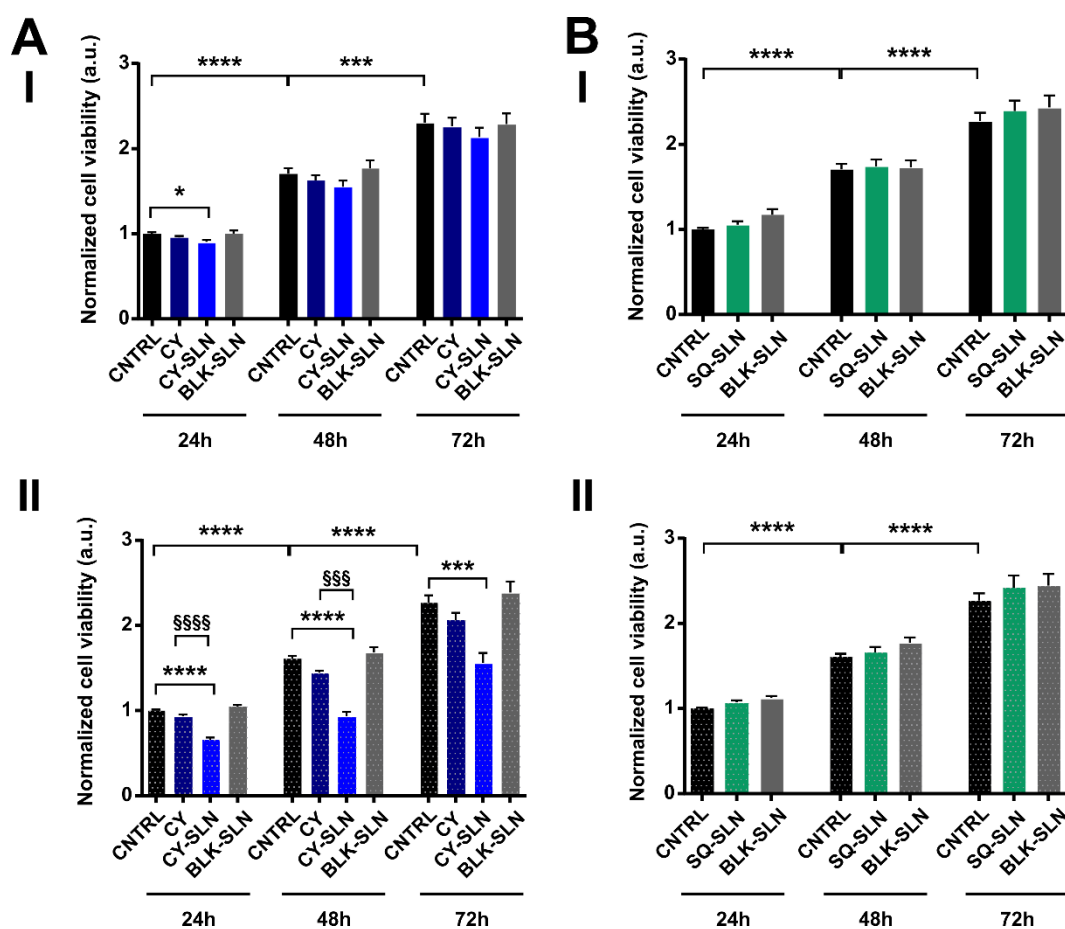
434

435 4.5 *In vitro* photoactivity of CY-SLN and SQ-SLN

436

437 *In vitro* phototoxicity results, illustrated in Figure 5 A-II, do not show any phototoxic effects of CY in the
438 free form (100 nM) on MCF-7, but interestingly, highlight a photoactivity of CY after encapsulation into SLN (100
439 nM) (Fig. 5 A-II, CY-SLN versus CNTRL 24 h and 48 h after light beam treatment: p-value < 0.0001 and after 72 h:
440 p-value < 0.001). This result may be due to the lower possibility of aggregation of CY encapsulated into SLN,
441 which leads to an increase in the lifetime of the triplet state leading to a more efficient photoactivity.
442 Alternatively, the photoactivity of CY-SLN could be due to a higher local concentration of the dye due to the
443 nanoencapsulation. Notably, BLK-SLN did not show any phototoxicity, meaning that the decrease in cell viability
444 after light beam treatment is exclusively due to the activity of the dye (Fig. 5 A-II). Our results are in agreement
445 with other studies about potential PS loaded into SLN, that have shown how the incorporation of these highly
446 hydrophobic molecules in lipid nanoparticles may increase their photostability and also their singlet oxygen
447 production capacity [37, 38]. On the other hand, it has to be noticed that the same CY previously tested in its
448 free form resulted in a significant phototoxic activity at 10 nM on HT-1080 [38]. Surely the higher energy fluency
449 rates applied in the previous work (18.0 J/cm² versus 7.2 J/cm²) can at least partially explain the difference in the
450 observed results. However, this difference is probably also attributable to a cell line-dependent sensibility to
451 PMD treatment that emerged even in the dark: indeed, HT-1080 cells showed significant cytotoxicity starting
452 from 100 nM [38], whereas MCF-7 viability was not affected by free CY till 400 nM (Fig. 5 A-II). Regarding the
453 squaraine derivative, it was not possible to investigate its phototoxicity in the free form because of its insolubility
454 in aqueous solutions. However, SQ-SLN do not show any phototoxic effects at the concentration investigated
455 (100 nM) (Fig. 5 B-I), suggesting that this nanosystem could be used as potential diagnostic tools for *in vivo*
456 fluorescence imaging, but not as a therapeutic tool in photodynamic treatment. On the contrary, preliminary
457 data on CY-SLN suggest that they may be tested in photodynamic treatment of some types of cancer, thanks to

458 its negligible cytotoxicity in the dark and its moderate activity after light beam treatment, enhanced by its
 459 incorporation in lipid nanocarriers (Fig. 5 A-I and II).



460

461 **Figure 5. Dyes and dye-loaded SLN *in vitro* phototoxicity**

462 **A)** Cell viability assays on MCF-7 O/N treated with 100 nM CY (in free form or encapsulated into SLN) kept in the dark (I) or
 463 24 h, 48 h and 72 h after LED irradiation (640 nm, 7.2 J/cm²) (II). **B)** Cell viability assays on MCF-7 O/N treated with 100 nM
 464 SQ (encapsulated into SLN) kept in the dark (I) and 24 h, 48 h and 72 h after LED irradiation (640 nm, 7.2 J/cm²) (II). Data are
 465 normalized on CNTRL at 24 h and represented as mean ± SEM. Data refer to 4 independent experiments (eight replicates
 466 for each experiment). Statistical significance versus CNTRL (MCF-7 untreated with dyes): * P< 0.05, *** P< 0.001, **** P<
 467 0.0001; statistical significance between free dyes and dye-loaded SLN: §§§ P< 0.001, §§§§ P< 0.0001 (Kruskal-Wallis test
 468 with *post-hoc* Dunn's test).

469

470 **4.6 Cell uptake and intracellular localization**

471

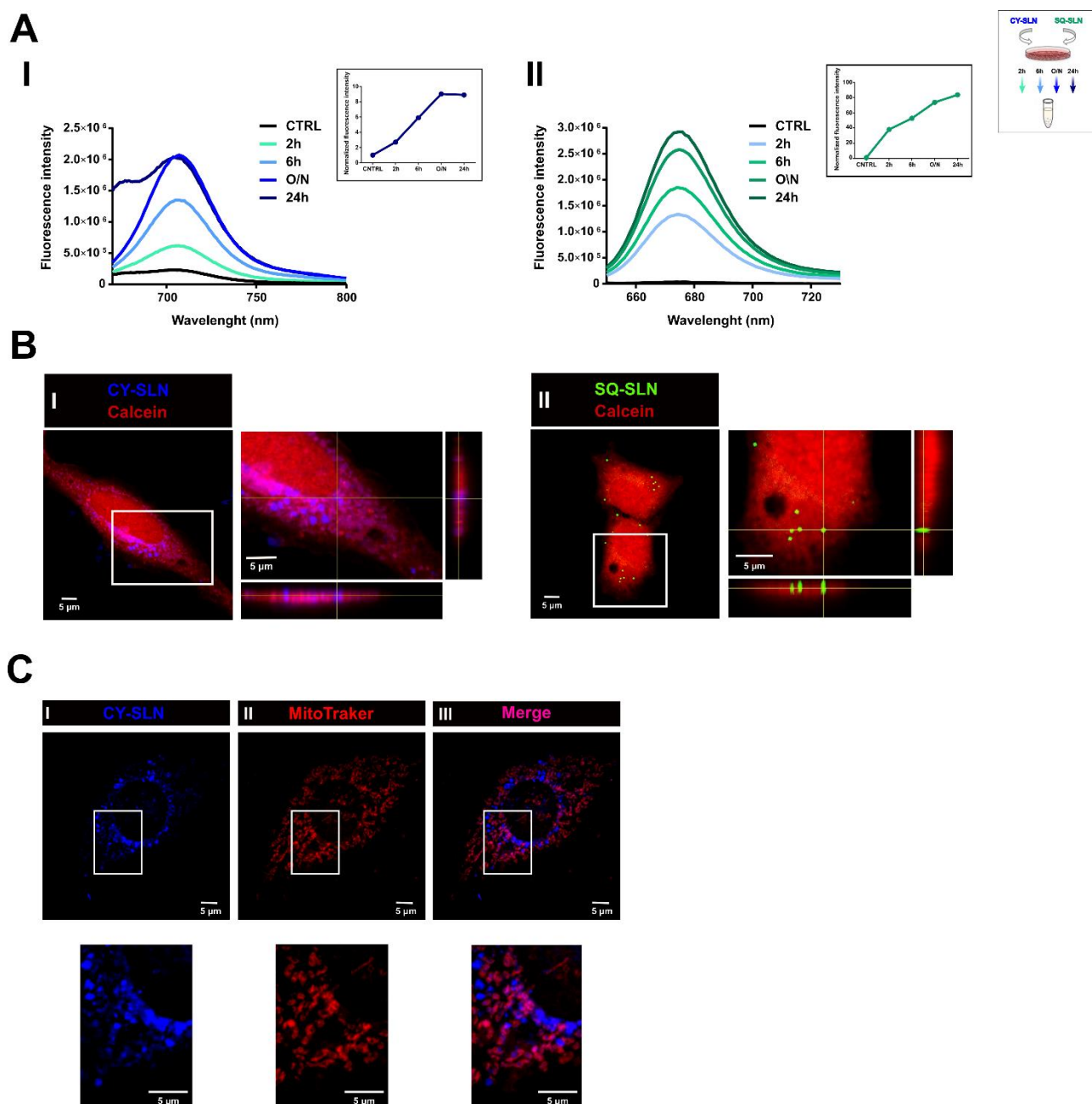
472 As mentioned in the introduction, the development of good diagnostic and therapeutic agents in
 473 biomedicine requires a setup of positive interactions between the molecule and the cellular system. Indeed, the

474 nanosystem must be internalized efficiently and with reasonable timing by the target cell to reach a good drug
475 delivery performance. Therefore, we investigated the cellular uptake and the intracellular localization of SLN.
476 The cell uptake timings of the synthesized fluorescent nanosystems were analyzed by measuring the
477 fluorescence intensity relative to cell lysates after incubation of MCF-7 cells with CY-SLN and SQ-SLN (dyes
478 concentration: 100 nM) for four different incubation times (2 h, 6 h, O / N and 24 h). An efficient time-dependent
479 accumulation of the dyes was observed in cells treated with dye-loaded SLN, with an appreciable uptake starting
480 from 2 hours in both samples (Fig. 6 A-I: CY-SLN, $\lambda_{em} = 708$ nm; Fig. 6 A-II: SQ-SLN, $\lambda_{em} = 674$ nm). CY-SLN uptake
481 reaches a plateau after O/N incubation (Fig. 6 A-I). The tailing of the peak observed after 24 h incubation is most
482 likely due to the presence of phenol red ($\lambda_{em} = 674$ nm at pH 7) in the culture medium. In order to compare the
483 cell uptake of CY in the free form and CY encapsulated into SLN, we perform the same assay on MCF-7 cells
484 treated with 100 nM of CY in culture media for four different incubation times (Fig. S3). At 100 nM, it seems that
485 the presence of the nanocarrier slows down the internalization of the dye. Indeed, comparing Figure 6 A-I and
486 Figure S3 A it results evident that the fluorescence intensities related to the free dye are strongly more intense
487 than those relating to the incorporated dye. However, it is difficult to establish a replicable ratio between the
488 two signals because it changes with sample and biological variability (n=3).

489 In order to verify the effective internalization of the nanosystem into the cell, excluding the possibility that
490 fluorescence signals recorded from cell lysates came from particles attached on the cell surface, we performed
491 confocal laser scan microscopy experiments on MCF-7 cells treated O/N with 100 nM of dyes incorporated into
492 SLN. The whole cellular volume was labeled using Calcein (red signals in Fig. 6 B and S3 B) and images were
493 acquired on the three coordinates of the space (XYZ), allowing to reconstruct the 3D cellular volume and
494 therefore to check whether dyes fluorescent signals were included within the cellular volume or not. Figure 6 B
495 clearly shows that both types of loaded-SLN are internalized by MCF-7 after O/N incubation. Indeed, in cells
496 treated with CY-SLN (Fig. 6 B-I) or SQ-SLN (Fig. 6 B-II) several fluorescent spots were detected ($\lambda_{ex} = 633$ nm)
497 within the cell volume labeled with Calcein. Results obtained from samples treated with cyanine in the free form
498 show, instead, a greater amount of intracellular labeling and a more widespread and delocalized signal of the
499 dye on the entire cell volume as compared to CY-SLN (Fig. S3 B), suggesting higher internalization of the free CY.

500 This result may be explained on one hand by the cationic nature of CY, which may promote its interaction with
501 the cell surface and on the other hand by the overexpression of organic anion-transporting polypeptide (OATP)
502 channels in tumor cells, which may increase its internalisation [3]. On the contrary, the incorporation of the
503 cyanine derivative into the lipid nanoparticle not only may limit its interaction with the plasma membrane,
504 partially masking the positive charge associated with the dye, but also alters its molecular internalization
505 mechanism, which, in fact, in this case, is mediated by an endocytotic mechanism [52]. Finally, comparing CY (Fig.
506 S3 B) and CY-SLN (Fig. 6 B-I) signals it is also possible to detect the greater compartmentalization of cyanine signal
507 when incorporated within the nanoparticle system: compared to the non-specific and diffuse signal given by the
508 dye in its free form, incorporation into a solid lipid matrix allows to obtain a more localized signal.

509 It has been suggested that mitochondria are a major subcellular site for photosensitizer localization and
510 that both cyanine and squaraine dyes localize in mitochondria organelles [4,24]. We, therefore, decided to
511 further investigate the subcellular localization of CY-SLN, staining MCF-7 mitochondria with MitoTracker Red (red
512 signals in Fig. 6 C and S3 C) and to evaluate its co-localization with the dye. Figure 6 C shows that CY-SLN is mainly
513 located in close proximity to mitochondrial regions. A partial co-localization with mitochondria was observed
514 also for CY in the free form as shown in Figure S3 C, although a more widespread signal of the dye on the entire
515 cell volume was observed. These data suggest that the cyanine derivative, both in its free form and encapsulated
516 into SLN, partially associates with the mitochondria once it has penetrated into the cells and this could be linked
517 to its photodynamic activity (Fig. 5 A-II and [38]).



518

519 **Figure 6. Dye-loaded SLN uptake and intracellular localization.**

520 **A)** Fluorescence intensity relative to MCF-7 cellular lysates after incubation with 100 nM CY-SLN (I) or SQ-SLN (II) for
 521 increasing time intervals (2 h, 6 h, O/N and 24 h). Emission spectra were recorded exciting CY-SLN and SQ-SLN at 620 and
 522 640 nm, respectively. Insets show the trend over time of fluorescence intensity normalized on the control (untreated cells).
 523 Data refer to one representative experiment of at least three. On the right a scheme representing cell uptake assay.

524 **B)** Representative confocal fluorescence images of MCF-7 cells incubated O/N with either CY-SLN (I) or SQ-SLN (II) at the
 525 same concentration (100 nM). Red signal refers to calcein (excitation at 561 nm) and blue/green signal refers to CY-SLN and
 526 SQ-SLN respectively (excitation at 633 nm). For each image zoom on a region of interest (indicated by white box) with
 527 orthogonal views are reported. Scale bar: 5 μm.

528 **C)** Representative confocal fluorescence images of MCF-7 cells incubated O/N with CY-SLN (100 nM). Blue signal in panel I
 529 refers to CY-SLN (excitation at 633 nm) and red signal in panel II refers to MitoTracker Red (excitation at 561); panel III:

530 merged image of panel I and II (pink for overlapped regions); below: zoom on a region of interest indicated by white box.
531 Scale bar: 5 μm .

532

533 5 CONCLUSIONS

534

535 We successfully incorporated two polymethine dyes (a cyanine CY and a squaraine SQ) based on bromo
536 benzoindolenine ring into SLN in order to overcome their solubility issues in aqueous solutions. Dye-loaded SLN
537 displayed a homogeneous size of <200 nm and high entrapment efficiency, preserving dyes' excellent
538 spectroscopic properties. This study is the first example in the literature of incorporation of a squaraine
539 derivative into SLN, which not only permits the solubilization of this dye (completely insoluble in water), but even
540 enhances its spectroscopic performances with higher ϕ_{fl} . This data, together with the low cytotoxicity of the
541 system and its efficient cellular uptake, suggest that SQ-SLN may be a suitable and appealing candidate as
542 diagnostic agent in *in vivo* optical imaging. On the other hand, CY-SLN, beyond the good optical properties shown,
543 led to a photoactivity on MCF-7 cells. Moreover, CY-SLN seem to have a good uptake and a partial mitochondrial
544 localization, suggesting their potential application as PS for photodynamic anticancer treatment.

545 In summary, SLN are a valuable delivery strategy for PMD in biomedical applications, although further
546 investigation on *in vivo* models is needed in order to assess the real applicability of these nanosystems in both
547 diagnostic and therapeutic fields.

548

549 6 AUTHOR CONTRIBUTIONS

550

551 SV, AFP and GC conceived the study; GC synthesized nanoparticles and performed chemical characterization and
552 cell culture experiments; GC wrote the paper and designed the figures; AG performed immunofluorescence and
553 confocal acquisitions; NB synthesized PMD; AGP supervised SLN's synthesis and characterization, performed A4F
554 experiments and wrote A4F results; SV, AFP, CB and PG supervised experiments. All authors revised the
555 manuscript.

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557

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560

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562

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565

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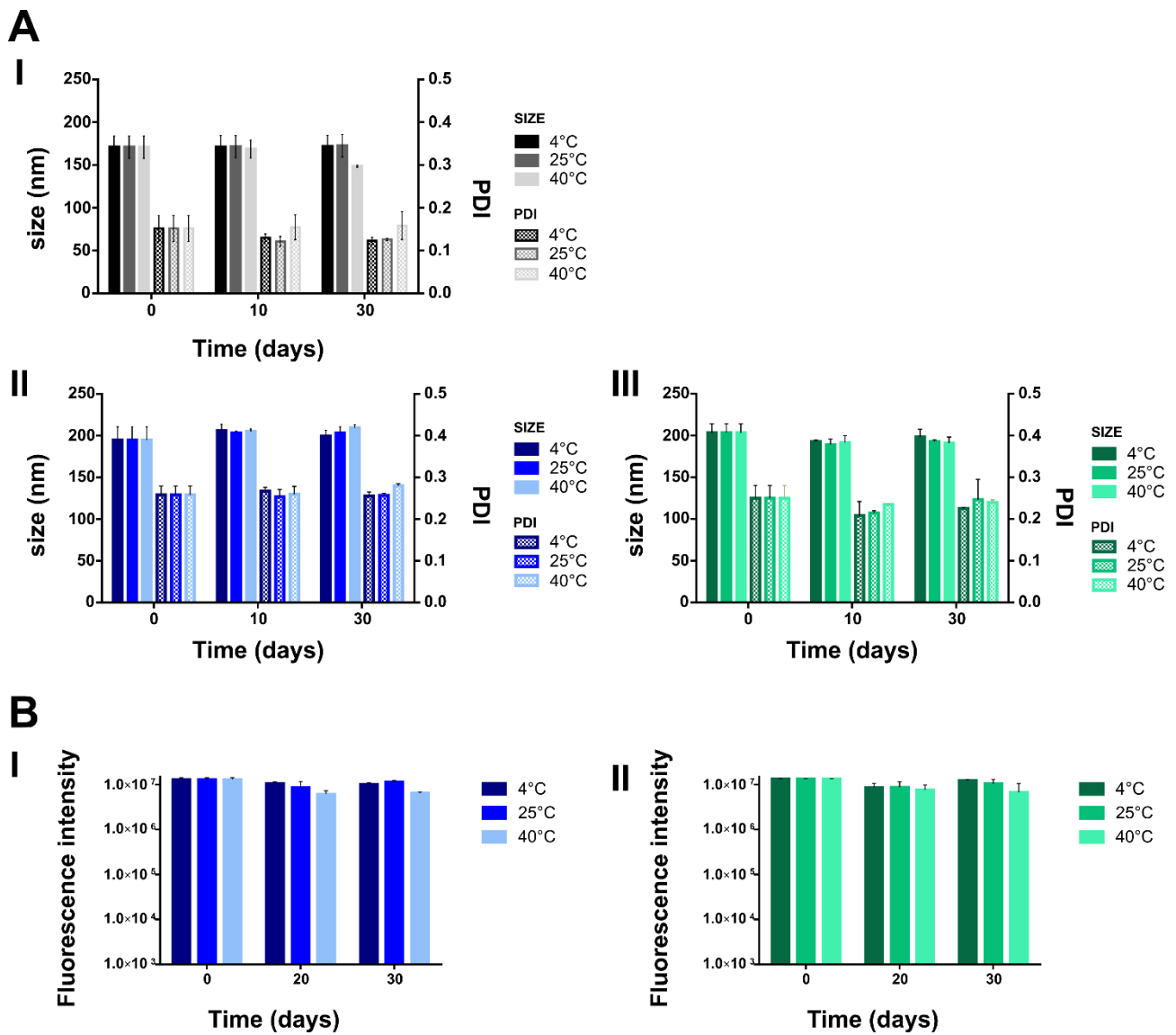
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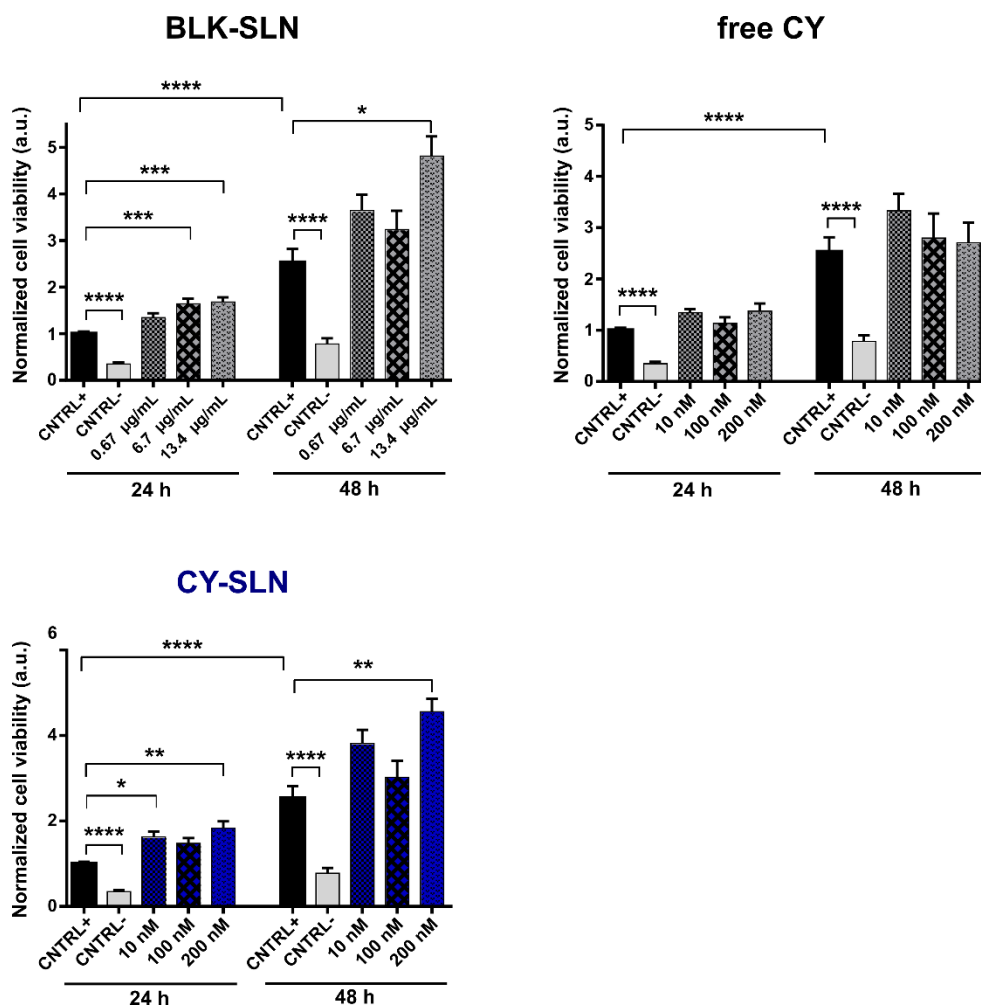


713

714 **Figure S1. SLN stability**

715 A) Change overtime of size and PDI of BLK-SLN (I), CY-SLN (II) and SQ-SLN (III) at three different storage temperature (4°C,
 716 25°C and 40°C). B) Change overtime of fluorescence intensity of CY-SLN (I) and SQ-SLN (II). Data are expressed as mean ±
 717 SEM (n=3).

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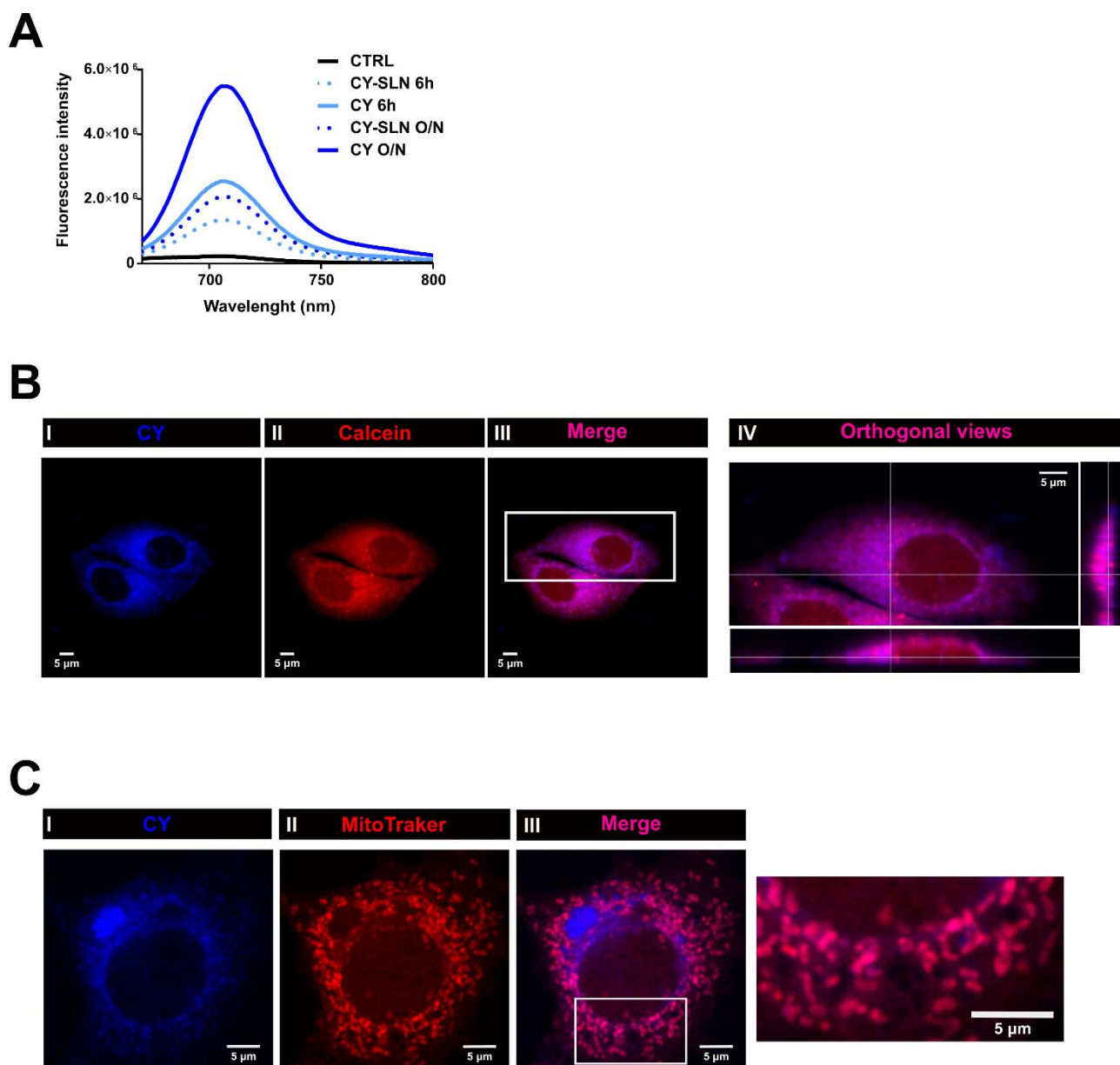


720

721 **Figure S2. In vitro cytotoxicity of CY-SLN and SQ-SLN on HMEC.**

722 Cell viability assays on HMEC 24 h and 48 h after treatment with different concentrations of BLK-SLN, free CY and CY-SLN.
 723 For dye-loaded SLN concentrations refer to dyes incorporated into SLN (from 10 to 200 nM) whereas for BLK-SLN
 724 concentrations refer to phosphatidylcholine content ([PC] from 0.67 to 13.4 µg/mL corresponding to that of loaded-SLN
 725 tested. Data are normalized on CNTRL+ at 24 h and are represented as mean ± SEM. Data refer to at least 3 independent
 726 experiments (eight replicates for each experiment). Statistical significance versus CNTRL+ (HMEC untreated with dyes): ***
 727 P < 0.001, **** P < 0.0001 (Kruskal-Wallis test with *post-hoc* Dunn's test).

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731

732 **Figure S3. CY uptake and intracellular localization.**

733 **A)** Fluorescence intensity relative to MCF-7 cellular lysates after incubation with either CY (continuous lines) or CY-SLN (dot
 734 lines) at the same concentration (100 nM) for two different time intervals (6 h and O/N). Data refer to one representative
 735 experiment of three.

736 **B)** Representative confocal fluorescence images of MCF-7 cells incubated O/N with CY (100 nM). Blue signal in panel I refers to dye-loaded SLN (excitation at 633 nm) and red signal in panel II refers to Calcein (excitation at 561 nm); panel III: merged
 737 image of panel I and II (pink for overlapped regions); panel IV: zoom on panel III with orthogonal views. Scale bar: 5 μ m.

739 **C)** Representative confocal fluorescence images of MCF-7 cells incubated O/N with CY (100 nM). Blue signal in panel I refers to CY (excitation at 633 nm) and red signal in panel II refers to MitoTracker Red (excitation at 561); panel III: merged
 740 image of panel I and II (pink for overlapped regions) with zoom on a region of interest (indicated by white box). Scale bar: 5 μ m.
 741

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: