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1 **Assessment of various formulation approaches for the application of beta-lapachone in**
2 **prostate cancer therapy**

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4

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10

11 **Abstract**

12 Beta-lapachone (β -Lap) is an anticancer drug activated by the NAD(P)H quinone acceptor
13 oxidoreductase (quinone) (NQO1), an enzyme over-expressed in a large variety of tumors. B-
14 Lap is poorly soluble in water and in most biocompatible solvents. Micellar systems,
15 liposomes and cyclodextrins (CDs) have been proposed for its solubilization. In this work, we
16 analyzed the properties and *in vitro* efficacy of β -Lap loaded in polymer nanoparticles,
17 liposome bilayers, complexed with sulfobutyl-ether (SBE)- and hydroxy-propyl (HP)- β -
18 cyclodextrins, or double loaded in phospholipid vesicles. Nanoparticles led to the lowest drug
19 loading. Encapsulation of [β -Lap:CD] complexes in vesicles made it possible to slightly
20 increase the encapsulation rate of the drug in liposomes, however at the cost of poor
21 encapsulation efficiency. Cytotoxicity tests generally showed a higher sensitivity of NIH 3T3
22 and PNT2 cells to the treatment compared to PC-3 cells, but also a slight resistance at high β -
23 Lap concentrations. None of the studied β -Lap delivery systems showed significant enhanced
24 cytotoxicity against PC-3 cells compared to the free drug. Cyclodextrins and double loaded
25 vesicles, however, appeared more efficient drug delivery systems than liposomes and
26 nanoparticles, combining both good solubilizing and cytotoxic properties. Ligand-
27 functionalized double loaded liposomes might allow overcoming the lack of selectivity of the
28 drug.

29

30 **Keywords:** beta-lapachone, double loaded liposome, cyclodextrin, poly-lactide, PC-3 cell,
31 NQO1.

32

33 1. Introduction

34 Beta-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione, β -Lap) is an
35 *ortho*-naphthoquinone extracted from the bark of *Tabebuia avellanedae*. It has drawn strong
36 interest for its wide variety of potential pharmacological effects including anti-tumor activity
37 (Abreu et al. 2005; Kung et al. 2008; Tseng et al. 2013; Ma et al. 2015; Moreno et al. 2015).
38 It has been suggested that β -Lap enters the cancer cells in its oxidized form and is activated
39 after reduction by the NAD(P)H quinone acceptor oxidoreductase (quinone) (NQO1), a
40 multifunctional antioxidant enzyme predominately located in the cytoplasm. This enzyme is
41 overexpressed in a variety of human cancers. Its activity is considered as a key determinant
42 for β -Lap-mediated apoptosis and cytotoxicity in prostate cancer cells, and its expression can
43 be stimulated by the drug itself (Planchon et al. 2001; Kumi-Diaka 2002; Lee et al. 2005;
44 Blanco et al. 2007). The reduced form of β -Lap is unstable leading to the formation of
45 reactive oxygen species (ROS). ROS attack DNA causing cellular stress capable of activating
46 the p53 protein and the expression of the Bax gene. The Bax protein triggers the release of
47 intracellular calcium from the endoplasmic reticulum by interacting with the cytosolic
48 domains of calcium channels. The release of intracellular calcium is also provoked by the
49 depletion of NADH/NADP and ATP due to the overconsumption of the enzyme NQO1.
50 Intracellular calcium release induces apoptosis *via* permeabilization of mitochondrial
51 membranes and release of cytochrome c. It follows the activation of the calpain and caspase
52 pathways leading to cell death. The oxidative pathway to apoptosis also includes mechanisms
53 leading to cell cycle arrest (Kung et al. 2008). Despite its long known therapeutic potential, β -
54 Lap has still no clinical application today. This is mainly due to its low bioavailability,
55 photodecomposition and rapid metabolism once in the blood. Some clinical studies also
56 revealed a certain toxicity. B-lap is poorly soluble in water (0.16 mM or 0.038 mg/mL)
57 (Nasongkla et al. 2003) and in most biocompatible solvents. Approaches that could improve

58 its solubility and stability include the use of micellar systems and cyclodextrins (CD).
59 Whereas β -Lap solubilization in polymer micelles proved insufficiently effective (Blanco et
60 al. 2007), formation of inclusion complexes with cyclodextrins increased β -Lap stability and
61 bioavailability (Nasongkla et al. 2003; Cunha-Filho et al. 2007; Mangas-Sanjuan et al. 2016;
62 Xavier-Junior et al. 2017). It was shown that the hydrophobic interaction between β -Lap and
63 the apolar cavity of a CD molecule was the main driving force for the formation of inclusion
64 complexes. The complex formed by β -Lap and 2-hydroxypropyl- β -cyclodextrin (HP- β -CD)
65 allowed increasing 413-fold β -Lap water solubility from 0.16 mM to 66.0 mM (Nasongkla et
66 al. 2003), but the most effective candidates for β -Lap solubilization appeared to be the
67 SulfoButyl-Ether (SBE) and Randomly Methylated (RM) β -cyclodextrins (Cunha-Filho et al.
68 2007; Mangas-Sanjuan et al. 2016; Xavier-Junior et al. 2017). However, inclusion of β -Lap in
69 cyclodextrins does not limit its degradation, although Cunha-Filho et al. (2013) reported the
70 protection of the drug by RM- β -CD against hydrolytic process in darkness. Also,
71 methemoglobinemia and hemolysis were observed with [β -Lap:hydroxypropyl- β -
72 cyclodextrin] complex formulations used in clinical trials (Hartner et al. 2007). Encapsulation
73 of a lipophilic and unstable drug into liposomes is another way to improve its water solubility
74 and limit its degradation. Liposomes can encapsulate hydrophilic drugs in their aqueous core
75 and lipophilic drugs in their phospholipid bilayers. Due to its hydrophobicity, β -Lap
76 intercalates between phospholipid acyl chains in vesicle bilayers (Wu et al. 2019). B-Lap was
77 encapsulated in liposomes made of a mixture of epikuron[®]200, cholesterol and stearylamine
78 (7:2:1) with 97% encapsulation efficiency (EE) (Cavalcanti et al. 2011). Epikuron[®]200 mostly
79 consists of 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLOPC), a poly-unsaturated
80 phospholipid. Stearylamine (SA) is expected to limit vesicles aggregation thanks to its
81 positive charge, destabilize endosomes (Roseanu et al. 2010), produce deformable vesicles
82 and increase drug permeability (Maestrelli et al. 2010). When β -Lap was complexed with HP-

83 β -CD and encapsulated into the liposomes, the EE was similar as without cyclodextrin
84 (Calvacanti et al. 2011), as if encapsulation efficiencies in the lipid bilayer or the aqueous
85 core of vesicle could be identical. This was intriguing. In this work, we studied the interfacial
86 behavior of β -Lap in mixtures with lipids, its insertion in liposome bilayers and polymer
87 nanoparticles, its complexation with cyclodextrins and the encapsulation of [β -Lap:CD]
88 complexes in lipid vesicles. We analyzed the properties of the various studied systems and
89 their *in vitro* cytotoxicity on prostate epithelial and cancer cells.

90

91 **2. Materials and methods**

92 2.1. Materials

93 Beta-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione, $M_w = 242.27$
94 g/mol) was a gift of Prof. Nereide Santos-Magalhaes (Federal University of Pernambuco,
95 Recife, Brazil) (Figure 1). 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine PC(18:2/18:2)
96 (DLOPC, $M_w = 782.08$ g/mol) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
97 (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and poly
98 (D,L)-lactic acid (PLA) (Resomer R202H[®], $M_w = 10,000-18,000$ g/mol) from Boehringer
99 Ingelheim (Ingelheim am Rhein, Germany). Cholesterol, stearylamine, 4-(2-hydroxyethyl)-1-
100 piperazine-ethanesulfonic acid (HEPES, 99.5% pure), sodium chloride (NaCl, 99% pure),
101 tetrahydrofuran ($\geq 99.9\%$ pure), ammonium molybdate (VI) tetrahydrate (81-83% pure), L-
102 ascorbic acid (99% pure), phosphorus standard solution and hydrogen peroxide (30 wt%)
103 were provided by Sigma Aldrich (St. Louis, MI., USA). The reagents for quantification of the
104 enzyme NQO1 in prostate cell lines included: Mini-PROTEAN[®] TGX[™] Gel, 12 well, 20 μ L,
105 Clarity[™] Western ECL Substrate, 2X Laemmli sample buffer and 2-mercaptoethanol (Bio-
106 Rad, USA), RIPA buffer R0278-50mL and protease inhibitor cocktail P8340-1mL (Sigma-

107 Aldrich), BCA reagent A and B, Anticorps NQO1 (A180): sc-32793(Santa Cruz Animal
108 Health) and Goat anti-mouse IgG human ads-HRP (Southern Biotech, Birmingham, USA),
109 protein ladder plus prestained, Tris glycine SDS buffer solution 10X and Tris glycine buffer
110 solution 10X (Euromedex, Strasbourg), PVDF Transfer membrane (Immobilon[®]-P, USA)
111 with 0.45 μ m pore size, Pierce[™] BCA protein Assay kit (Thermo scientific, Rockford, USA),
112 and skimmed milk powder (Régilait, France). Sulfobutyl ether- β -cyclodextrin (SBE- β -CD,
113 DS = 6.4) was a gift from Cyclolab (Budapest, Hungary), and 2-hydroxypropyl- β -
114 cyclodextrin (HP- β -CD) was obtained from Sigma-Aldrich. Spectroscopic grade chloroform
115 and methanol (> 99.80% pure) were Carlo Erba reagents. The ultrapure water obtained from
116 the Millipore Milli-Q[®] Direct 8 water purification System was used for preparing buffer
117 solutions (10 mM HEPES, 150 mM NaCl, pH 7.4).

118

119 2.2. Methods

120 2.2.1. Surface pressure measurements

121 Surface pressure-surface area (π -A) measurements of lipid monolayers in the absence or
122 presence of the drug were performed using a thermostated Langmuir film trough (775.75 cm²,
123 Biolin Scientific, Finland) enclosed in a plexiglas box, protected from light. All experiments
124 were performed at 21 \pm 1 $^{\circ}$ C. Prior to monolayer deposition, the buffer subphase was cleaned
125 by suction. Phospholipids and the drug in a chloroform and methanol (9:1 v/v) mixture were
126 spread at the air/buffer interface, and the system was left for 15 min to allow complete
127 evaporation of the organic solvents. Monolayer compression was then performed at 5
128 $\text{\AA}^2 \cdot \text{molecule}^{-1} \cdot \text{min}^{-1}$ speed. Compression cycles included a first compression of monolayers up
129 to 30 mN/m followed by a full expansion and a second complete compression. Results are

130 mean values of at least 3 measurements. The surface compressional moduli (C_s^{-1}) of
131 monolayers were calculated from the equation (1):

$$132 \quad C_s^{-1} = -A \frac{d\pi}{dA} \quad (1)$$

133 where A is the molecular area, and $d\pi$ the surface pressure change. Excess free energies of
134 mixing were calculated from equation (2):

$$135 \quad \Delta G^{exc} = \int_0^\pi (A_{12}^{exp} - X_1 A_1 - X_2 A_2) d\pi \quad (2)$$

137
138 with $A_{12}^{exp} - X_1 A_1 - X_2 A_2$ the excess molecular area (ΔA^{exc}), A_{12}^{exp} the experimental mean
139 molecular area in the mixed monolayer, A_1 and A_2 the molecular areas of the pure
140 components 1 and 2, and X_1 and X_2 the molar fractions of the two components in the mixed
141 film.

142

143 2.2.2. Liposome preparation and characterization

144 Liposomes were prepared by the thin film hydration method (Bangham 1965). Beta-
145 lapachone (10 mol% of the total mixture amount) and the lipids (pure POPC or POPC-CHOL-
146 SA 7:2:1 molar ratio) were dissolved in a mixture of chloroform and methanol (9:1 v/v). The
147 solvents were evaporated under reduced pressure for 2 h at 45°C yielding a thin lipid- β -Lap
148 film. Then 1 mL buffer, containing or not the cyclodextrins, was added to the thin film to
149 obtain 4 to 10 mM lipid suspension. Vesicles were formed by submitting the lipid- β -Lap
150 suspension to 5 min heating at 45°C, 5 min vortexing, and 5 min sonication at 40°C.
151 Downsizing was performed either by extrusion 21 times through a 200 nm pore-sized
152 polycarbonate membrane by means of a mini-extruder (Avanti Polar Lipids, Inc.) at room
153 temperature, or tip sonication in ice bath using a probe sonicator Vibracell 75041 (750 W, 20

154 kHz, Bioblock Scientific, Aalst, Belgium) at 20% amplitude. A series of 10 sec-on/5 sec-off
155 cycles were applied for 30 min. All obtained β -Lap-loaded vesicle suspensions were stored at
156 4°C, in darkness.

157 The hydrodynamic diameter and zeta potential (ζ) of the obtained vesicles were measured by
158 dynamic light scattering (DLS), and laser doppler electrophoresis, respectively, using a
159 zetasizer Nano ZS90 (Malvern), after dilution of the liposome suspension. Measurements
160 were carried out in triplicate at 21 \pm 1°C. The morphology of the vesicles was analyzed by
161 cryogenic transmission electron microscopy (cryo-TEM) using a JEOL 2200FS (JEOL USA,
162 Inc., Peabody, MA, U.S.A.) equipped with a Gatan Ultrascan 2K camera (Gatan, Evry,
163 France). Samples were diluted to 1 mg/mL in 1 mM NaCl solution and 5 μ L of the
164 suspensions were deposited onto a perforated carbon-coated, copper grid (TedPella, Inc.).
165 After removal of the excess liquid with a filter paper, the grid was quickly frozen in a liquid
166 ethane bath at -180°C and mounted on the cryo holder for microscopic analysis.

167

168 *2.2.3. Formation of [β -lapachone:cyclodextrin] inclusion complexes and double loaded*
169 *vesicles*

170 Based on the study by Xavier-Junior et al. (2017), [β -Lap:CD] inclusion complexes were
171 prepared using HP- β -CD and SBE- β -CD in a 1:8 and 1:4 β -lapachone:CD molar ratio,
172 respectively. The β -Lap solution in methanol went through a 30 min nitrogen purging and was
173 freeze-dried to form a thin film. This film was then hydrated with 2.5 mL of aqueous CD
174 solution. The obtained dispersion was sonicated for 30 min, and then submitted to 16 h
175 magnetic stirring at 1.5 g, at 25 \pm 1°C. The obtained [β -Lap:CD] solution was filtered through
176 a 0.2 μ m membrane filter (Millipore) to remove the non-solubilized drug. The samples were
177 freeze-dried prior to β -Lap assay and characterization of the inclusion complexes.

178 Encapsulation of [β -Lap:CD] complexes was performed following two protocols: (i) free CDs
179 were added to a POPC film containing 10 mol% β -Lap; (ii) freeze-dried [β -Lap:CD]
180 complexes were dissolved in buffer and added to a POPC film containing 10 mol% β -Lap.
181 Free β -Lap was removed by ultracentrifugation.

182

183 2.2.4. Encapsulation of β -Lap in PLA nanoparticles

184 Poly (D, L)-lactic acid nanoparticles (PLA NPs) were prepared by nanoprecipitation. A
185 solution of PLA and β -Lap in acetone was added dropwise to ultrapure water under moderate
186 magnetic stirring. Acetone was evaporated under reduced pressure. The final suspension was
187 filtered through a 1 μ m pore-sized membrane (Glass acrodisc®, Waters) and centrifuged 3
188 times at 3,000 g for 15 min at 15 °C to separate free β -Lap. The pellet was suspended in water
189 at the final concentration of 2 mg/mL.

190

191 2.2.5. B-lapachone and lipid assays

192 B-lapachone assay was performed at 256 nm by UV-visible spectroscopy using a CARY 300
193 Bio UV-Visible spectrophotometer (Varian, USA). All samples were freeze-dried (for
194 weighing) then dissolved in a mixture of buffer/methanol/THF (0.2:0.8:1). At the studied
195 wavelength, neither the polymer nor the lipids interfered with drug absorbance. Standard
196 calibration curves were obtained with β -Lap concentrations ranging from 5×10^{-6} to 3.5×10^{-5}
197 M in a mixture of methanol and buffer. POPC was quantified using the phosphorus
198 quantitative method described by Chen et al. (1956). All assays were performed in triplicate.

199

200 2.2.6. Determination of the loading efficiency of liposomes and cyclodextrins

201 The freeze-dried [β -lap:CD] complexes were accurately weighed and dissolved in methanol
202 before β -Lap assay. The encapsulation rate (ER) and encapsulation efficiency (EE) of β -Lap
203 (Equations 3 and 4) were determined after centrifugation of vesicle or nanoparticle
204 suspensions at 100,000 *g*, at 4°C for 1h to remove the untrapped drug. The pellet was
205 disrupted by a HEPES buffer/methanol/THF (0.2:0.8:1 v/v/v) solution for nanoparticles and
206 liposomes without CDs, and by HEPES buffer/methanol (1:25 v:v) for liposomes containing
207 CDs, because THF was incompatible with CDs (a white precipitate was formed). The
208 absorbance of the pellet solution and of pure β -Lap diluted in the same solvent mixture was
209 measured at 256 nm. The encapsulation rate (ER) and encapsulation efficiency (EE) were
210 calculated using Equations (3) and (4):

211
$$ER = \frac{[\beta-Lap]_{pellet}}{[excipient + \beta-Lap]} \times 100 \quad (3)$$

212
$$EE = \frac{[\beta-Lap]_{pellet}}{[\beta-Lap]_{initial}} \times 100 \quad (4)$$

213

214 2.2.7. Cell culture

215 Prostate adenocarcinoma cells PC-3 derived from metastatic bone were purchased from
216 ATCC® CRL-1435™, and SV40 immortalized-normal human prostate epithelium cells
217 (PTN2) from Sigma-Aldrich. Fibroblast from mouse embryo (NIH 3T3) were obtained from
218 ATCC® CRL-1658™. PNT2 et PC-3 cells were cultured in RPMI 1640 medium (Roswell
219 Park Memorial Institute medium, Sigma-Aldrich) and NIH 3T3 in DMEM (Dulbecco's
220 Modified Eagle Medium, Sigma-Aldrich), supplemented with 10% fetal bovine serum
221 (Gibco® by Life Technologies™) and 0.5% penicillin-streptomycin (Sigma-Aldrich) in
222 humidified atmosphere containing 5% CO₂ at 37°C.

223

224 *2.2.8. Expression of NQO1 in prostate cells*

225 Western blot analysis of NQO1 expression was performed as described by Lamberti et al.
226 (2013) with some modifications. About 2 million cells per line were treated with 100 μ L of a
227 mixture of RIPA buffer and protease inhibitor cocktail (9:1 v/v). After 30 minutes incubation
228 in ice (vortexing every 10 min), non-soluble materials were eliminated by centrifugation
229 (5000 g, 5 min). Aliquots of soluble fractions containing 30 μ g of total proteins (quantified by
230 bicinchoninic acid assay, Thermo Scientific) were combined with Laemmli-2-
231 mercaptoethanol (95:5 v/v) sample buffer (1:1 v/v), heated at 80 °C for 10 min, before
232 electrophoresis on Mini-PROTEAN® TGX™ gels at 180 V for 45 min and electro-transfer
233 onto PVDF membrane at 100 V for 45 min. The membrane was blocked in 5% skim milk in
234 Tris buffered saline-Tween 20 (0.1% TBS-Tween) for 1 hour at room temperature with
235 agitation, incubated with the anti-NQO1 antibody (1:200 dilution) in blocking buffer (TBS-
236 Tween milk-5%) overnight at 4°C. Blot was rinsed in TBS-T (1h incubation with replacement
237 of the TBS-Tween buffer by fresh medium every 10 min) and then incubated with the
238 secondary antibody (2 h, room temperature, Goat anti-mouse IgG, 1:2000 dilution in blocking
239 buffer), followed by rinsing. Bands were visualized with enhanced chemiluminescence (ECL)
240 substrate and then imaged using MFChemibIS 3.2 (DNR Bio-Imaging Systems Ltd.,
241 Jerusalem, Israel). Molecular weight of NQO1 bands was determined by comparison to the
242 Euromedex protein ladder plus prestained (France). The content of soluble NQO1 assemblies
243 was quantified by measuring the intensity of the chemiluminescent bands using GelCapture
244 Software. All samples were analyzed in triplicate.

245

246 2.2.9. Cytotoxicity tests

247 4000 cells in 100 μ L cell culture medium per well were seeded in 96-well plates and left
248 overnight in the humidified incubator at 37°C with 5% CO₂. On the following day, the
249 treatments, namely free β -Lap (in dimethyl sulfoxide, DMSO), [β -Lap:CD] complexes, β -
250 Lap-loaded nanoparticles, β -Lap-loaded liposomes or [β -Lap:CD] complex-loaded liposomes
251 (in buffer) were added to the cells at different concentrations. Each well contained a final
252 volume of 200 μ l of full medium. Final β -Lap concentrations ranged from 0 to 4 μ M. Cells
253 were incubated again for 24, 48 or 72 hours in the same conditions. Cell viability was then
254 determined by the MTT test (Labtech LT-5000 Plate Reader). Briefly, 20 μ l of 3-(4,5-
255 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide were added to each well at the
256 final concentration of 0.5 mg/mL in full medium and incubated at 37°C with 5% CO₂ for 2 h.
257 The medium was then aspirated, taking care not to remove any cell, and the purple formazan
258 product formed was dissolved in 200 μ L DMSO. After 10 min shaking, the optical density
259 (OD) at 570 nm of each well was measured using a microplate reader (LT-5000 MS,
260 Labtech). For each plate, each concentration was analyzed in triplicate.

261

262 3. Results and discussion

263

264 3.1. Characterization of the interfacial behavior of β -Lap-lipid mixtures

265 Cavalcanti et al. (2011, 2015) prepared β -Lap-loaded liposomes using Epikuron[®]200
266 (DLOPC), cholesterol and stearylamine. Since DLOPC is highly sensitive to oxidation, we
267 envisaged to replace it by other phospholipids. In a previous work we showed that β -Lap
268 interacts differently with phospholipids, depending on the presence and number of double-
269 bonds. The drug inserts in the lipid bilayer close to the polar head groups in dipalmitoyl

270 phosphatidylcholine (DPPC), between the polar head groups and double bond in unsaturated
271 palmitoyl-oleoyl-phosphatidylcholine (POPC) or dioleoyl phosphatidylcholine (DOPC) (Wu
272 et al. 2019). POPC could be a good alternative to DLOPC because it is partially saturated. In
273 this work, we compared the interfacial behavior of the two phospholipids alone or mixed with
274 CHOL and SA, in the presence or absence of β -Lap. The results are presented in Figures 2
275 and 3, and in Tables 1 and 2.

276 For both drug-lipid mixtures, the π -A isotherms of the mixed β -Lap-lipid monolayers barely
277 differ from those of lipid mixtures without the drug (Figure 2). When looking more closely, a
278 slight change in the shape of the π -A isotherms is observed for monolayers containing
279 DLOPC. This is confirmed by the characteristic values summarized in Table 1. The decrease
280 in the maximal compressibility modulus (C_s^{-1}), although limited, accounts for a
281 disorganization of the monolayers in the presence of β -Lap. Moreover, the compression
282 cycles (Figure 3) show that the profiles of the compression isotherms for POPC and DLOPC
283 monolayers are affected by the presence of β -Lap, especially those of POPC. From the
284 isotherms in Figure 2, free energies of mixing were calculated (Table 2). For both the POPC-
285 CHOL-SA and DLOPC-CHOL-SA mixtures, the ΔG^{exc} values were positive at all surface
286 pressures accounting for an unfavorable interaction between the three components in the two
287 mixtures. ΔG^{mix} values were positive at 30 mN/m, a surface pressure considered to be close to
288 the lateral surface pressure in liposome membranes. The addition of CHOL and SA to the two
289 phospholipids may thus affect the stability of prepared vesicles. Surface pressure
290 measurements also showed that β -Lap tends to disorganize phospholipid-CHOL-SA
291 monolayers. Previous results suggested that CHOL and β -Lap may compete for the same
292 location in vesicles (Wu et al. 2019). As a result, β -Lap could insert less deeply in liposome
293 bilayers than in pure phospholipid vesicles. Aloisio et al. (2017) showed that the
294 encapsulation rate of lipophilic drugs decreases with the cholesterol/phosphatidylcholine (PC)

295 ratio. The presence of cholesterol could thus be detrimental to β -Lap stability in the bilayers
 296 and accelerate its release, as reported for vesicles containing paclitaxel and CHOL (Steffes et
 297 al. 2017).

298

299

300 **Table 1.** Main characteristics of the π -A isotherms of lipids spread with 3.5 mol% β -Lap onto
 301 HEPES buffer at $21^\circ\text{C} \pm 1^\circ\text{C}$. A_c : molecular area at collapse; π_c : surface pressure at collapse;
 302 $C_s^{-1}_{\max}$: maximal compressibility modulus.

303

Film forming components	A_c (\AA^2)	π_c (mN/m)	$C_s^{-1}_{\max}$ (mN/m)
Pure POPC	60.0 ± 0.5	44.0 ± 0.2	100.95 ± 3.02
POPC- β -Lap	59.3 ± 1.2	43.5 ± 0.5	101.04 ± 0.39
Pure DLOPC	57.1 ± 0.8	42.8 ± 0.3	104.78 ± 4.40
DLOPC- β -Lap	53.3 ± 1.2	41.6 ± 0.7	95.52 ± 5.08
POPC-CHOL-SA	49.5 ± 0.5	47.8 ± 0.3	169.72 ± 6.02
(POPC-CHOL-SA)- β -Lap	47.0 ± 0.2	47.5 ± 0.0	152.98 ± 4.46
DLOPC-CHOL-SA	50.2 ± 0.8	44.6 ± 0.3	124.23 ± 5.53
(DLOPC-CHOL-SA)- β -Lap	47.8 ± 0.7	43.9 ± 0.6	115.22 ± 5.79

304

305

306 **Table 2:** ΔG^{exc} and ΔG^{mix} values for (a) POPC-CHOL-SA and (b) DLOPC-CHOL-SA mixed
 307 monolayers.

308

	POPC-CHOL-SA		DLOPC-CHOL-SA	
Surface	ΔG^{exc}	ΔG^{mix}	ΔG^{exc}	ΔG^{mix}
pressure	(kJ/mol)	(kJ/mol)	(kJ/mol)	(kJ/mol)
(mN/m)				
5	0.1773	-0.6282	0.3461	- 0.4594
10	0.3791	-0.4263	0.6851	- 0.1204
15	0.5500	- 0.2554	1.0010	0.1955
20	0.7302	- 0.0752	1.2794	0.4740
25	0.9333	0.1279	1.5453	0.7398
30	1.1860	0.3806	1.8056	1.0002
35	1.3401	0.5346	1.9945	1.1890
40	1.5435	0.7380	2.1243	1.3189
45	1.7443	0.9388	2.2464	1.4409

309

310

311 3.2. Characterization of β -Lap-loaded nanoparticles and liposomes

312 UV-visible spectroscopy at 256 nm was used to assay β -Lap in nanoparticles and liposomes.
313 Standard calibration curves were obtained with drug concentrations ranging from 5×10^{-6} to
314 3.5×10^{-5} M in a mixture of methanol and buffer (Figure S1, *in supplementary information*).

315 The hydrodynamic diameter of PLA nanoparticles was 170 ± 3 nm, with a PDI of 0.08 ± 0.02
316 and a negative zeta potential value of -46 ± 1 mV. The encapsulation of β -Lap did not
317 significantly change these characteristics (size: 176 ± 9 nm, PDI: 0.06 ± 0.03 , ζ -potential: -50
318 ± 2 mV). Drug EE and ER values were 12.82 and 1.97 % respectively (N'Diaye et al. 2019).

319 When measuring the absorbance of liposome samples containing SA, the absorption bands of
320 β -Lap appeared always shifted towards lower wavelengths (Figure S2, *in supplementary*
321 *information*). Since POPC does not display any absorbance peak at wavelengths higher than
322 203 nm (McHowat et al. 1996), it is possible that β -Lap interacted with SA (Ci et al. 1989).
323 We observed that when β -Lap was encapsulated in liposomes made of POPC only, or
324 solubilized by CDs, its UV-visible absorption spectrum remained unchanged.

325 In light of these results and those obtained by surface pressure measurements, SA and CHOL
326 were both excluded from liposome composition. Considering the strong sensitivity of DLOPC
327 to oxidation, we encapsulated β -Lap in liposomes made of POPC only. MLVs were prepared,
328 in order to increase the encapsulation rate of the lipophilic drug per vesicle by greater
329 lamellarity. Figure 4 shows the morphology of liposomes and Table 3 summarizes their
330 characteristics. Both extrusion and tip sonication yielded mixtures of mono-, oligo- and
331 multilamellar liposomes.

332 B-lapachone loaded and unloaded vesicles exhibited monodisperse narrow size distribution
333 characterized by a polydispersity index (PDI) < 0.2 and a mean hydrodynamic diameter
334 around 190 nm (Table 3). Sonicated β -Lap-loaded vesicles appeared smaller than extruded
335 ones, with larger polydispersity index (Figure 4C). The EE values, calculated from direct drug

336 and phospholipid assays in the vesicle pellets collected after ultracentrifugation, varied
 337 between 27 and 34%.

338

339

340 **Table 3.** Characteristics of unloaded and β -Lap-loaded POPC vesicles. HD: hydrodynamic
 341 diameter. E: extrusion. S: tip sonication. Nd: not determined. SD: standard deviation, PDI:
 342 polydispersity index.

Composition	Method	HD (nm) (\pm SD)	PDI (\pm SD)	ζ -potential (\pm SD) (mV)	ER (%) (\pm SD)	EE (%) (\pm SD)
POPC	E	186.1 \pm 20.2	0.083 \pm 0.067	nd	-	-
POPC- β -Lap	E	193.4 \pm 6.2	0.076 \pm 0.005	nd	3.42 \pm 0.29	34.2 \pm 2.9
POPC- β -Lap	1h-S	167.2 \pm 1.2	0.108 \pm 0.038	-5.75 \pm 0.07	2.90 \pm 0.50	29.0 \pm 5.0
POPC- β -Lap	0.5h-S	179.9 \pm 1.4	0.115 \pm 0.016	-2.12 \pm 0.13	2.73 \pm 0.21	27.3 \pm 2.1

343

344 The presence of β -Lap did not change the morphology of liposome bilayers. This is not
 345 surprising considering the small payload of β -Lap in these vesicles and its shallow insertion in
 346 lipid bilayers demonstrated by physicochemical and simulation experiments (Wu et al. 2019).
 347 For hydrophobic drugs such as β -Lap, the poor loading capacity of liposomes is related to the
 348 limited space offered by a lipid bilayer, which leads to low drug-to-lipid ratio (Deo et al.
 349 2004). Excess loading may compromise bilayer integrity, by alteration of the packing of
 350 phospholipid molecules. It has been demonstrated that, above a critical saturation
 351 concentration, lipophilic drugs such as ibuprofen increase the permeability of the lipid bilayer
 352 by formation of transient pores and membrane defects (Angelini et al. 2017). They may even
 353 lead to the formation of mixed micelles (Deo et al. 2004). In the case of β -Lap, we did not

354 observe any apparent instability related to the presence of the drug, although no long-term
355 stability trial was performed.

356 Increasing the initial drug-to-lipid ratio invariably led to the same final encapsulation rate. For
357 extruded vesicles, drug-to-lipid ratio and ER were equal to 3.54 (\pm 0.31) and 3.42 mol (\pm
358 0.29) %, respectively, and for sonicated vesicles, they were equal to 2.99 and 2.90 mol%,
359 respectively. Compared to the initial drug-to-lipid ratio (10 mol%) in the dried lipid film,
360 there was obviously a significant loss of drug during vesicles preparation. However, the
361 loading rate obtained by sonication or extrusion was very similar to that achieved with other
362 lipophilic drugs. For example, for paclitaxel which is much more lipophilic than β -lapachone,
363 the solubility limit in lipid bilayers was reported to be 3 mol% only (Steffes et al 2017; Bhatt
364 et al. 2018). Aloisio et al. (2017) prepared vesicles by the thin film hydration and
365 dehydration-rehydration methods and compared the encapsulation rate of lipophilic drugs
366 depending on the method used. The thin hydrated film method led to vesicles with only 1 mol%
367 maximal encapsulation rate for pure indomethacin, whereas it was 43 times higher using the
368 dehydration-rehydration method with drug-cyclodextrin complexes entrapped in the aqueous
369 core of vesicles. Obviously, encapsulation of the drug was dramatically enhanced by
370 complexation with cyclodextrins. This is an interesting result because Cavalcanti et al. (2011;
371 2015) also reported on the encapsulation of [β -Lap:CD] complexes in vesicles. However, they
372 did not study the change in β -Lap ER in these conditions.

373

374 3.3. Formation of [β -Lap:CD] complexes

375 As previously mentioned, a number of researchers have attempted to overcome the low
376 solubility and bioavailability of β -Lap by forming inclusion complexes with cyclodextrins.
377 The association constants were generally determined by the phase solubility method, ^1H -

378 NMR, and fluorescence spectroscopy. Beta-CD and HP- β -CD showed higher binding affinity
379 to β -Lap ($K_c = 0.9-1.2 \times 10^3 \text{ M}^{-1}$) than α -CD (20 M^{-1}) and γ -CD (160 M^{-1}) (Nasongkla et al.
380 2003). RM- β -CD and SBE- β -CD both significantly enhanced β -Lap apparent solubility
381 (Cunha-Filho et al. 2007). However, RM- β -CD showed some renal toxicity. Conversely,
382 SBE- β -CD and HP- β -CD appeared well tolerated in humans and did not provoke any
383 significant adverse effects following oral or intravenous administration (Stella and He 2008).
384 Therefore, we chose SBE- β -CD and HP- β -CD and used the drug-to-CD molar ratios already
385 successfully tested by Xavier-Junior et al. (2017) *i.e.*, 1:8 and 1:4 for [β -Lap:HP- β -CD] and
386 [β -Lap:SBE- β -CD] complexes, respectively.

387 Our solubilization tests indicated that the actual drug-to-CD molar ratios were 1:8.4 (± 0.3)
388 and 1:5.0 (± 0.1) for HP- β -CD and SBE- β -CD, respectively. The obtained solutions of [β -
389 Lap:CD] complexes were filtered and lyophilized. Freeze-drying was necessary to weigh the
390 samples and calculate CD and β -Lap molar ratios. However, after lyophilization of [β -
391 Lap:SBE- β -CD] samples partial separation and crystallization of β -Lap was observed.
392 Apparently, the drug was partially expelled from SBE- β -CD cavities during drying. This
393 separation was not observed for [β -Lap:HP- β -CD] complexes. The interaction of β -Lap with
394 the two cyclodextrin derivatives was analyzed by UV-visible spectroscopy. The absorption
395 spectrum of β -Lap was not modified by complexation with them (Figure S3, *in supplementary*
396 *information*).

397

398 3.4. Formation and characterization of [β -Lap:CD] complexes-loaded liposomes

399 3.4.1. Formation of double loaded liposomes and determination of the drug loading

400 Double loaded liposomes are vesicles containing both the drug in their bilayers and [drug:CD]
401 complexes in their aqueous core. During the last two decades, many double loaded liposomes

402 have been proposed and investigated (Maestrelli et al. 2010; Angelini et al. 2017; Fatouros et
403 al. 2001; Gillet et al. 2009; Gharib et al. 2015; Gharib et al. 2017; Gharib et al. 2018; Zhang et
404 al. 2015a; Zhang et al. 2015b; Zhang et al. 2015c; Soo et al. 2016; Azzi et al. 2018). These
405 systems may allow fast and sustained release. The encapsulation of the [drug:CD] complexes
406 into liposomes combines the advantages offered by both the CDs and the liposomes (Gharib
407 et al. 2015). Liposomes prevent CD complex dissociation due to dilution in the plasma
408 (McCormack and Gregoriadis 1996), and they reduce renal excretion of CD inclusion
409 complexes (Frank et al. 1976). Cyclodextrins increase drug water solubility and, therefore,
410 drug availability. In principle, they can preserve the integrity of the lipid bilayer from the
411 perturbation due to drug insertion in the lipid membrane. However, Ohtani et al. (1989) have
412 shown *in vitro* that certain cyclodextrins can remove some components of human erythrocyte
413 membranes (*e.g.*, phospholipids, cholesterol, and proteins) forming inclusion complexes with
414 them and destabilizing the cell bilayer. For example, HP- β -CD extracts cholesterol from lung
415 epithelial cells (Dos Santos et al. (2017). Also, CDs can participate to the transfer and
416 redistribution of phospholipids in a bilayer, interact with the inner lipid leaflet of the
417 liposomes and transfer their cargo to the bilayer (Zidovetzki and Levitan 2007).
418 Destabilization may follow (Angelini et al. 2017). Methyl- β -cyclodextrin can induce the
419 disruption of vesicles and the formation of micelles (Anderson et al. 2004; Hatzi et al. 2007).
420 At high concentrations, the random methylated β -cyclodextrin adsorbs to
421 dimyristoylphosphatidylcholine vesicles and is able to extract phospholipid molecules (Joset
422 et al. 2015). Many cyclodextrins, including HP- β -CD and SBE- β -CD, alter the permeability of
423 soybean phosphatidylcholine bilayers (Piel et at. 2007). HP- β -cyclodextrin, especially, affects
424 the fluidity and stability of liposomes (Gharib et al. 2018). It interacts with the polar head
425 groups of all phospholipids and with the acyl chains of saturated ones, while disorganizing the
426 unsaturated phospholipids (soybean PC). Considering that POPC has both saturated and

427 unsaturated chains, the effect of the cyclodextrin could be significant and lead to the release
428 of β -Lap molecules inserted in phospholipid leaflets. The possible interaction of cyclodextrin
429 derivatives with the vesicle bilayer is thus an important point to consider when encapsulating
430 CDs in the aqueous core of liposomes.

431 In order to better understand the interactions between the three components (*i.e.*, β -Lap, CD
432 and POPC), two protocols were followed: (i) free CDs were added to a POPC film already
433 containing 10 mol% β -Lap and (ii) a solution of [β -Lap : CD] complexes was used to hydrate
434 a POPC film containing 10 mol% β -Lap. The characteristics of the various samples are
435 summarized in Tables 4 and 5.

436 (i) The first protocol was tried to determine whether the addition of β -CDs to a lipid film
437 containing the drug could allow solubilization and encapsulation of the excess free drug that
438 usually precipitates in the aqueous medium surrounding the vesicles. However, it led to a very
439 low encapsulation rate (0.95 mol%) (Table 4), indicating the extraction of β -Lap from the
440 POPC film without much incorporation of [β -Lap:CD] complexes in the vesicles' core. After
441 ultracentrifugation, most of β -Lap complexed by CD molecules was eliminated with the
442 supernatant. Based on the interaction of β -CD derivatives with phospholipids reported by
443 different research groups (Fatouros et al. 2001; Gharib et al. 2018; Anderson et al. 2004;
444 Hatzi et al. 2007), it is likely that free cyclodextrin molecules interacted with phospholipid
445 ones during film hydration.

446

447 **Table 4.** Characteristics of vesicles formed by hydration of a POPC- β -Lap film by free CDs-
448 containing buffer and tip sonication (S). HD: hydrodynamic diameter. SD: standard deviation.

Cyclodextrin	Lipid film	Method	HD (nm)	PDI	ζ -potential	ER	EE
--------------	------------	--------	---------	-----	--------------------	----	----

			(\pm SD)		(\pm SD) (mV)	(%)	(%)
SBE- β -CD	POPC	0.5 h-S	169.3 \pm	0.131	-2.13 \pm 0.13	0.95	9.5
	+10% β - lapachone		1.7	\pm 0.017			

449

450 The absorption spectrum of β -Lap showed a blue shift when the POPC- β -Lap film was
451 hydrated with free SBE- β -CDs (Figure S4, *in supplementary information*). This blue shift was
452 not observed for samples in which the drug was already complexed with SBE- β -CD
453 molecules. This modification in the absorption spectrum indicates a change in β -Lap
454 environment that may be attributed to CD-POPC interaction. There are examples of
455 phosphatidylcholines (DPPC, DOPC) solubilized by β -CD and its derivatives, like HP- β -CD
456 (Irie et al. 1992), or methyl- β -CD (Anderson et al. 2004; Hatzi et al. 2007). Anderson et al.
457 (2004) calculated that 2 methyl- β -CD per acyl chain would be necessary to solubilize POPC.
458 We observed a very low encapsulation rate of β -Lap in double loaded vesicles prepared with
459 SBE- β -CD and POPC (Table 4). Complexation of phospholipid molecules would consume
460 more CDs than β -Lap complexation. Because of the high lipid-to-drug ratio, phospholipid
461 might be preferentially complexed by the CDs leading to β -Lap separation.

462 (ii) In the second protocol, [β -Lap:CD] complexes were dissolved in buffer and free β -Lap
463 was solubilized with POPC in the organic phase. There was both excess β -Lap in the dried
464 film, and excess free cyclodextrin cavities in the aqueous medium, because the CD-to- β -Lap
465 ratio necessary for a 1:1 stoichiometry is high (Table 5) (Xavier-Junior et al. 2017).

466

467 **Table 5.** Characteristics of POPC vesicles formed by hydration of a POPC- β -Lap (10 mol%)
 468 film by [β -Lap:CD] complexes-containing buffer, at optimal [β -Lap:CD] ratio (1:8.4 and
 469 1:5.0 for HP- β -CD and SBE- β -CD, respectively). Nd: not determined.

470

[CD: β -Lap] complex	Method	HD (nm) (\pm SD)	PDI	ζ -potential (\pm SD) (mV)	ER (%) \pm 10%	EE (%) \pm 10%
HP- β -CD	E	202.0 \pm 5.2	0.099 \pm 0.048	Nd	4.30	24.36
SBE- β -CD*	E	159.9 \pm 0.8	0.065 \pm 0.011	Nd	4.94	27.98
HP- β -CD	1h-S	166.5 \pm 1.3	0.113 \pm 0.031	-5.63 \pm 0.27	4.68	19.43
SBE- β -CD	1h-S	163.4 \pm 1.9	0.095 \pm 0.027	-5.20 \pm 0.26	3.96	21.56
HP- β -CD	0.5h-S	183.6 \pm 1.7	0.140 \pm 0.016	-1.74 \pm 0.16	4.30	21.50
SBE- β -CD	0.5h-S	181.2 \pm 1.4	0.122 \pm 0.025	-3.44 \pm 0.23	4.72	23.60

471 *In this sample, the POPC film also contained 2.5% DSPE-PEG₂₀₀₀. A possible explanation
 472 for the higher ER observed for this batch could be the enlargement of the aqueous space
 473 between bilayers in the presence of PEG chains, allowing the encapsulation of more [β -
 474 Lap:CD] complexes.

475

476 For all studied systems, mean encapsulation rates (ER) and encapsulation efficiencies (EE) of
477 β -Lap ranged from 3.96% to 4.94% and 19.43% to 27.98%, respectively. These results may
478 be attributed to factors such as the concentration of the phospholipid, the initial drug-to-lipid
479 molar ratio, the preparation methods as well as the presence or not of PEG chains. Higher ER
480 was obtained when β -Lap was solubilized by CDs prior to its encapsulation into liposomes
481 core. This agrees with most published research works on drug-in-cyclodextrin-in-liposomes
482 (Gharib et al. 2015; Gharib et al. 2017; Azzi et al. 2018). The encapsulation efficiency of β -
483 Lap (EE) was lower in the presence of cyclodextrins than in their absence. This has also been
484 frequently reported (Maestrelli et al. 2010; Wang et al. 2016). The increment in encapsulation
485 rate resulting from the addition of [β -Lap:CD] complexes in buffer for thin film rehydration
486 was low relative to the excess free β -Lap in the medium. This demonstrates the non-
487 proportionality between the initial β -Lap amount (in the lipid film and complexed with CDs)
488 and the amount of successfully encapsulated drug.

489

490 *3.4.2. Morphology of the double loaded vesicles*

491 The morphology of [β -Lap:CD]-liposomes was analyzed by cryoTEM. Mixtures of uni-, and
492 oligo-lamellar vesicles were obtained by both extrusion (Figure 5A,B) and sonication (Figure
493 5C). Lamellarity slightly decreased for the extruded vesicles. Again, sonication yielded more
494 polydisperse vesicles than extrusion, as deduced from DLS measurements.

495

496 *3.4.3. Biological efficiency of the various formulated systems*

497 We studied the effect of β -Lap free in the medium, solubilized in β -cyclodextrin derivatives
498 or encapsulated in vesicles or nanoparticles, on the viability of different cells lines. The first
499 objective was to test the toxicity of β -Lap against cells of different origins, but also on cancer

500 and normal cells that can coexist in a same tissue. The second objective was to evaluate the
501 NQO1-dependent cytotoxicity of β -lapachone in prostate cancer suggested in the literature
502 (Planchon et al 2001; Li et al 2003). If it is well known that PC-3 is NQO1-positive (Thapa et
503 al. 2014) and NIH 3T3 NQO1-negative (Powis et al. 1995), the level of expression of NQO1
504 in PNT2 cells was unknown. We first verified NQO1 expression in both PC-3 and PTN2
505 (Figure 6). The bands located between 28 and 36 kDa correspond to the molecular weight of
506 NQO1 (31 kDa). Both prostate cell lines are thus NQO1+.

507 Cytotoxicity of free β -Lap at 24, 48 and 72 h following addition of the drug in the cell culture
508 medium was evaluated in the three cell lines. For NIH 3T3 cells, the half maximal inhibitory
509 concentration (IC_{50}) decreased from 2.9 to 1.6 and then 0.7 μ M after 24 h, 48h and 72 h of
510 contact with cells, respectively (Figure 7). NIH 3T3 cells grow very fast, which could explain
511 their high sensitivity to the treatment. However, cells seemed resistant to β -Lap above 4 μ M.
512 For PNT2, IC_{50} values ranged between 2.2 and 1 μ M depending on the incubation time, and
513 for PC-3 cells, the obtained IC_{50} values were very similar (2.4 to 1.2 μ M) (Figure 7).

514 According to Li et al. (2003), normal cells are more resistant to β -Lap than tumor cells. This
515 is true for NIH 3T3 cells but only at high β -Lap concentrations. The cytotoxicity of β -Lap
516 against epithelial and cancer cells was almost identical, which calls into question the
517 selectivity of the β -Lap treatment. As previously mentioned, both PC-3 and PNT2 cells are
518 NQO1+. Conversely, NIH 3T3 cells are NQO1 negative but were also affected by β -Lap,
519 although they were less sensitive at high concentrations. This means that drug toxicity against
520 any of these cells could be controlled by another mechanism of action of β -Lap than NQO1-
521 mediated apoptosis, like cell cycle arrest or targeting of other proteins (Kung et al. 2008). The
522 mechanism of β -lapachone cytotoxicity is more complex than previously stated.

523 Further reported results correspond to cell viability after 48h incubation with the drug,
 524 excipients or drug carriers (Table 6 and Figure 8). The cytotoxicity of β -Lap-loaded
 525 nanoparticles against PNT2 was not studied, considering the non-negligible cytotoxicity of
 526 PLA nanoparticles in contact with these cells (Table 6). All IC₅₀ values for PNT2 cells were
 527 lower than those for PC-3 ones confirming that immortalized epithelial prostate cells were
 528 slightly more sensitive to β -Lap than cancer cells. However, Figure 8 (A,B) shows some
 529 resistance of PNT2 cells to the treatment above 1 μ M. This is not the case for PC-3 cells
 530 (Figure 8 (C,D)) for which a dose-response profile was observed with all treatments.

531

532 **Table 6:** IC₅₀ values at 48h for β -Lap free or inserted in various drug delivery systems.

533 Standard deviations did not exceed 10%. Nd: not determined

System	β -Lap	PNT2, IC ₅₀ (μ M)	PC-3, IC ₅₀ (μ M)
POPC vesicles	-	500	>1000
PLA NPs	-	150 (μ g/mL)	> 2200 (μ g/mL)
HP- β -CD	-	7000	>10000
SBE- β -CD	-	2000	1800
Free β -Lap	DMSO	1.0	1.6
[HP- β -CD: β -Lap]	complexed	1.0	1.7
[SBE- β -CD: β -Lap]	complexed	1.0	1.8
β -Lap-loaded NPs	encapsulated	Nd	1.3

β -Lap-loaded vesicles	inserted in bilayers	1.7	3.6
Liposome/HP- β -CD/ β -Lap	Double loaded	0.6	1.9
Liposome/SBE- β -CD/ β -Lap	Double loaded	0.9	2.2

534

535 As expected from the previous work of Nasongkla and coworkers (2003), toxicity of the pure
536 cyclodextrin derivatives was observed at much higher concentrations than those necessary to
537 solubilize β -Lap (Table 6). SBE- β -CD affected more the cell viability than HP- β -CD, and
538 IC₅₀ of both cyclodextrin derivatives were lower for PNT2 cells than for PC3 ones. However,
539 cell viability profiles of β -Lap in CD complexes were similar to those of free β -Lap
540 solubilized in DMSO, which confirms the poor cytotoxicity of cyclodextrins in the conditions
541 used (Figure 8 (A,C), Table 6).

542 Many authors have reported the enhancement of the cytotoxicity of a drug when complexed
543 with cyclodextrins. This was the case for telmisartan, niclosamide and paclitaxel in HP- β -CD
544 complexes (Kaur et al. 2014, Lodagekar et al. 2019, Shen et al. 2020) or chrysin in SBE- β -CD
545 ones (Kulkarni and Belgamwar 2017). However, this enhanced cytotoxicity seemed merely
546 related to the better solubilization of the drugs in cyclodextrins than in the buffer used for
547 experiments.

548 In our system, cyclodextrins did not add to β -Lap toxicity, probably because the free β -Lap
549 was already solubilized in DMSO prior to addition to the cell culture medium. In their
550 experiments, Nasongkla et al. (2003) also observed that the toxic dose killing 50% of MCF-7
551 cells after 4h contact was similar when β -Lap was solubilized in HP- β -CD (2.1 μ M) or in

552 DMSO (1.7 μM). If β -Lap had been dispersed in buffer, the results would certainly have been
553 different.

554 Cyclodextrins contributed to β -Lap solubilization but had no apparent effect on drug
555 permeability nor drug activity. Lioffi et al. (2017) who studied by differential scanning
556 calorimetry measurements the interaction of [irbesartan:HP- β -CD] complexes with
557 dipalmitoylphosphatidylcholine vesicles showed that HP- β -CD adsorbed at the surface of
558 phospholipid bilayers and contributed to the solubilization of the drug in the bilayer. Indeed,
559 when irbesartan was free, crystals formed in the membrane, suggesting that the drug
560 aggregated. In our system, β -Lap would not aggregate in the membrane at concentration
561 below 4 mol% (Wu et al. 2019). In fact, it is reasonable to think that it would be released from
562 cyclodextrin complexes near the cell membrane, which would explain the similar viability
563 profiles observed in the presence and absence of cyclodextrins. Other authors reported a lower
564 or similar cytotoxicity of [drug:cyclodextrin complexes], compared to free drug. This was the
565 case for camptothecin, zerumbone, chlorzoxazone, barbigerone or budesonide complexed
566 with HP- β -CD (Sætern et al. 2004; Eid et al. 2011; Tang et al. 2015; Qiu et al. 2015; Dos
567 Santos et al. 2017).

568 The cytotoxicity of β -Lap-loaded liposomes against PNT2 cells also appeared similar to that
569 of β -Lap solubilized in DMSO ($\text{IC}_{50} = 1.7$ and $1.0 \mu\text{M}$, respectively), but it was lower than the
570 free drug against PC-3 cells (3.6 versus $1.6 \mu\text{M}$). A resistance to the treatment was observed
571 for all studied liposome formulations in contact with PNT2 cells at concentrations exceeding
572 the IC_{50} (Figure 8B). Conversely for PC-3 cells (Figure 8 C,D), typical dose-response
573 relationships were obtained, and the cytotoxicity of the formulated systems can be ranked in
574 the following manner: β -Lap-loaded nanoparticles \geq [CD: β -Lap] complexes \geq double loaded
575 vesicles $>$ β -Lap-loaded vesicles. The difference between the three first systems may be

576 considered as insignificant. Although the cytotoxicity of all formulated systems was not very
577 different from that of the free β -Lap, they allowed drug solubilization, which is mandatory for
578 its injection. When considering the ER values, the [β -Lap:CD] complexes and double loaded
579 vesicles seem the most efficient systems for β -Lap treatment of prostate cancer.

580 The sensitivity of NIH 3T3 and PNT2 cells to β -Lap at low concentration however highlights
581 the necessity to target β -Lap towards cancer cells. Since NQO1-controlled targeting does not
582 seem effective, functionalized double-loaded liposomes could, in the end, be more interesting
583 systems than simple CDs, especially when considering the methemoglobinemia/hemolytic
584 side-effects observed with the [β -Lap:HP- β -cyclodextrin] complex used in clinical trials.

585

586 **4. Conclusion**

587 This study was aimed at evaluating the effect β -lapachone in different formulated systems for
588 the treatment of prostate cancer. Nanoparticles, cyclodextrins and liposomes allowed
589 solubilizing β -Lap. Various parameters of the encapsulation of β -Lap and [CD:drug]
590 complexes in liposomes were compared, namely morphology, encapsulation rate (ER),
591 encapsulation efficiency (EE), and *in vitro* cytotoxicity on PC-3, PNT2 and NIH 3T3 cells.
592 ER and EE values were particularly low for nanoparticles. In agreement with most studies
593 published on double loaded liposomes, the [drug:CD] complexes allowed reaching higher
594 encapsulation rates but with lower encapsulation efficiency than β -Lap-loaded vesicles.
595 Double loading is costly, as more β -Lap is wasted during liposome preparation. The study of
596 the interaction between β -lapachone, the CDs and POPC showed that CDs interacted with
597 both the drug and phospholipid, and this affected the EE and ER values. Viability tests and
598 EE and ER values led to the conclusion that CDs, and to a smaller extent the double loaded
599 vesicles, were more efficient drug delivery systems than liposomes. However, viability tests

600 also highlighted the non-negligible sensitivity of PNT2 and NIH 3T3 cells to the drug. The
601 similar cytotoxicity of β -Lap against NQO1-positive and NQO1-negative cell lines calls into
602 question the NQO1-related selectivity of β -Lap in cancer suggested by some authors.
603 Obviously, β -Lap activity does not solely depend on NQO1 expression and NQO1-induced
604 apoptosis pathways. If β -Lap is not selective then it must be targeted. Ligand-functionalized
605 double loaded liposomes could prove interesting systems for this purpose.

606

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830

Legends to the figures

831 **Figure 1:** Chemical structures of (A) β -Lapachone; (B) PLA; (C) Cholesterol; (D) POPC, (E)
832 DLOPC; (F) Stearylamine; (G) HP and SBE- β -Cyclodextrins.

833 **Figure 2:** Mean π -A isotherms and C_s^{-1} - π relationships for monolayers of DLOPC (A,C),
834 POPC (B,D) and their mixtures with CHOL and SA spread on HEPES buffer in the absence
835 or presence of 3.5 mol% β -Lap.

836 **Figure 3:** Compression cycles for (A) DLOPC, (B) POPC mixed monolayers with CHOL and
837 SA, in the absence or presence of β -Lap.

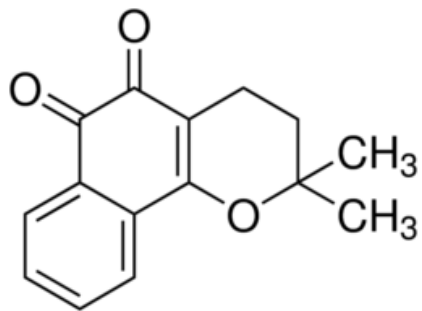
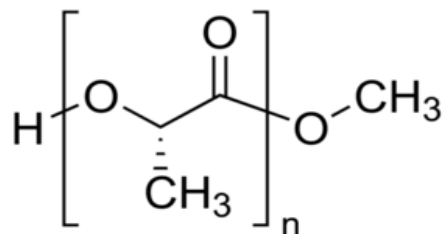
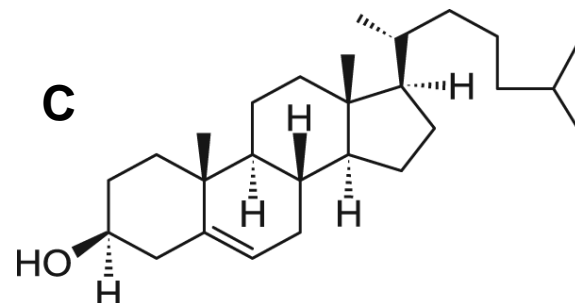
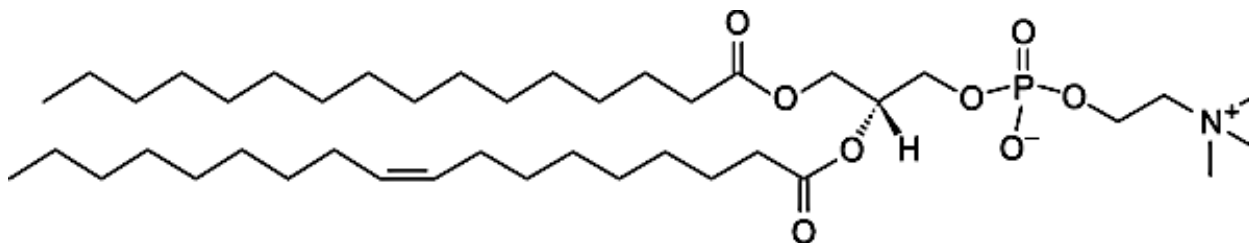
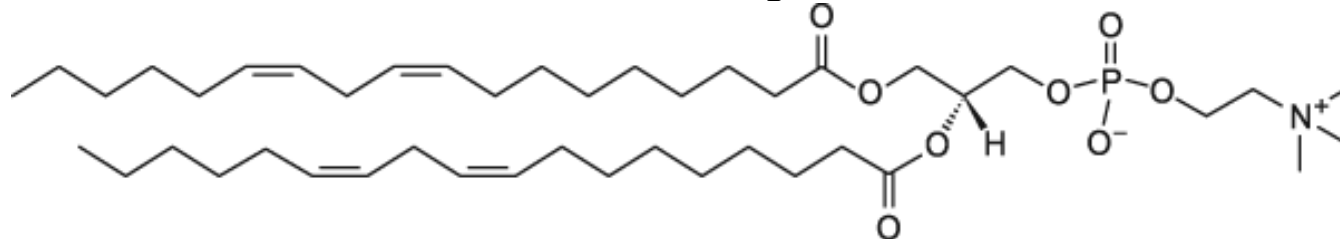
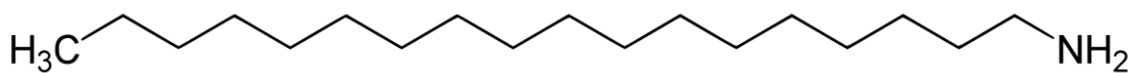
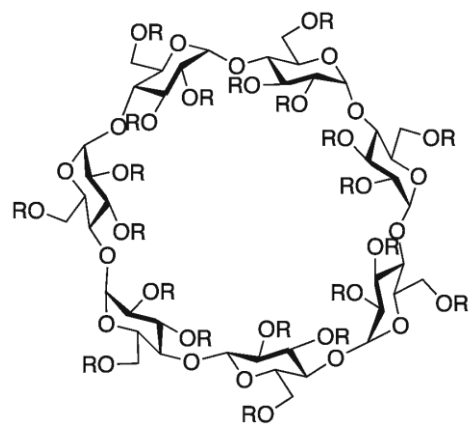
838 **Figure 4.** Cryo-TEM micrographs of unloaded (A) and β -lapachone-loaded (B,) POPC
839 vesicles prepared by extrusion; (C) β -lapachone-loaded POPC vesicles prepared by tip
840 sonication.

841 **Figure 5.** Cryo-TEM micrographs of liposomes obtained from a film of POPC- β -Lap
842 hydrated by (A) [β -Lap:HP- β -CD] and (B,C) [β -Lap:SBE- β -CD] complexes. (A) and (B)
843 were prepared by extrusion and (C) by tip sonication.

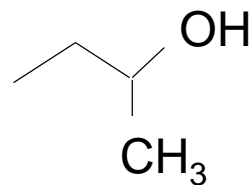
844 **Figure 6:** Western blot for NQO1 expression in PC-3 and PNT2 cells (n = 3).

845 **Figure 7:** Effect of free β -Lap on the viability of (A) mouse fibroblasts, (B) PNT2 and (C)
846 PC-3 cells.

847 **Figure 8.** Cytotoxic effect of free β -Lap, β -Lap loaded vesicles, inclusion complexes and
848 double loaded vesicles on (A, B) PNT2 and (C, D) PC-3 cells after 48 h contact, relative to
849 control.

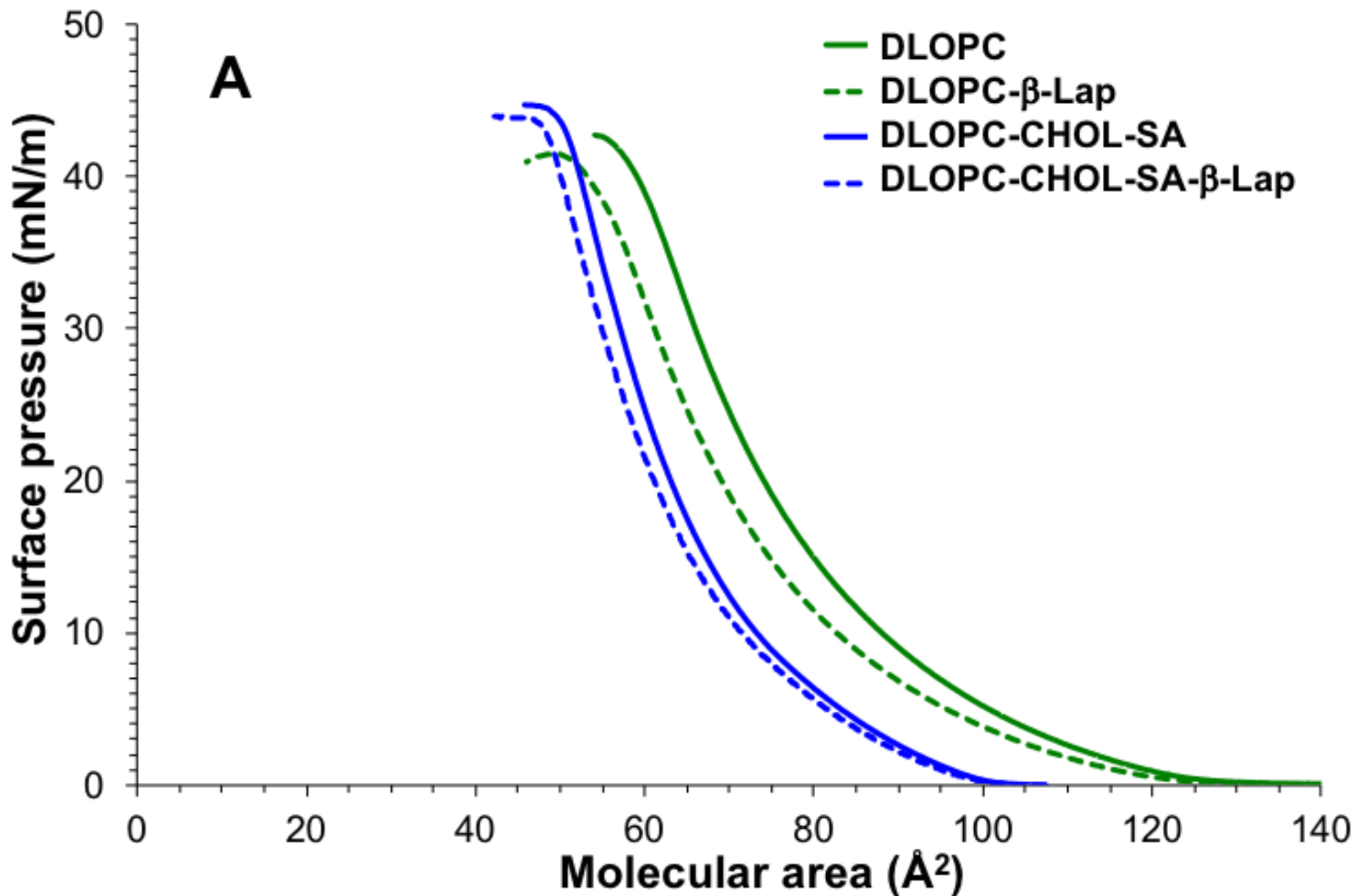
A**B****C****D****E****F****G**

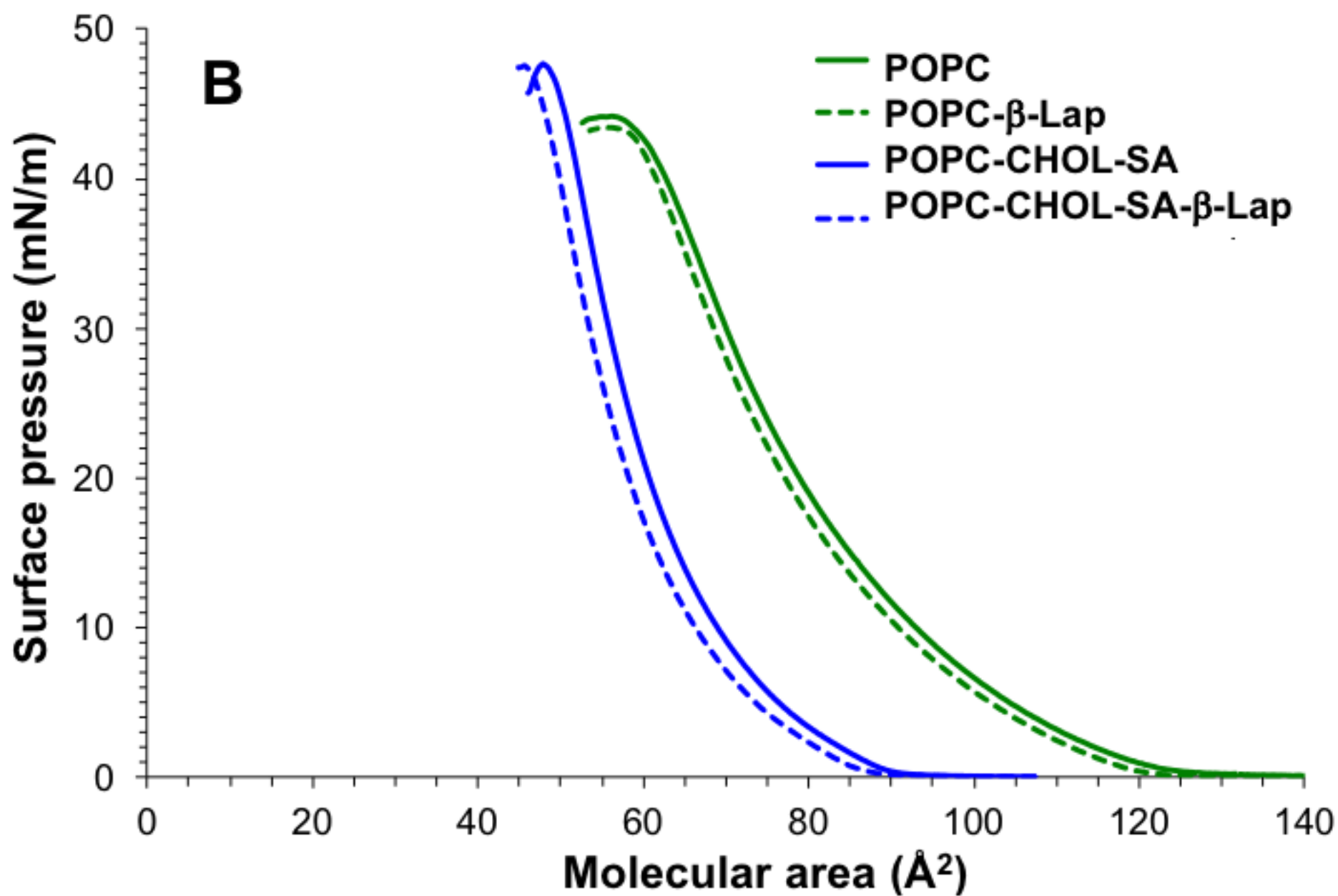
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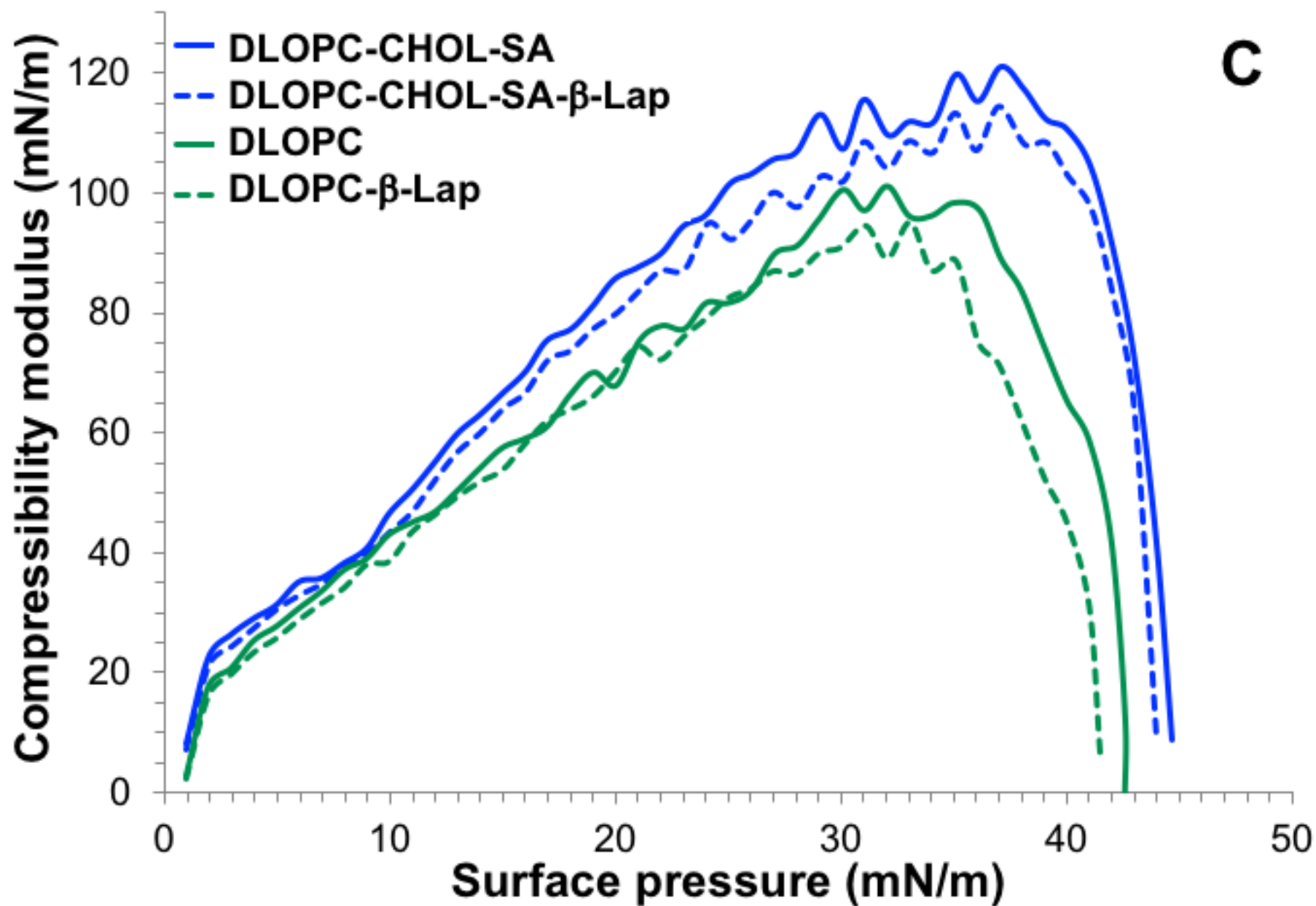


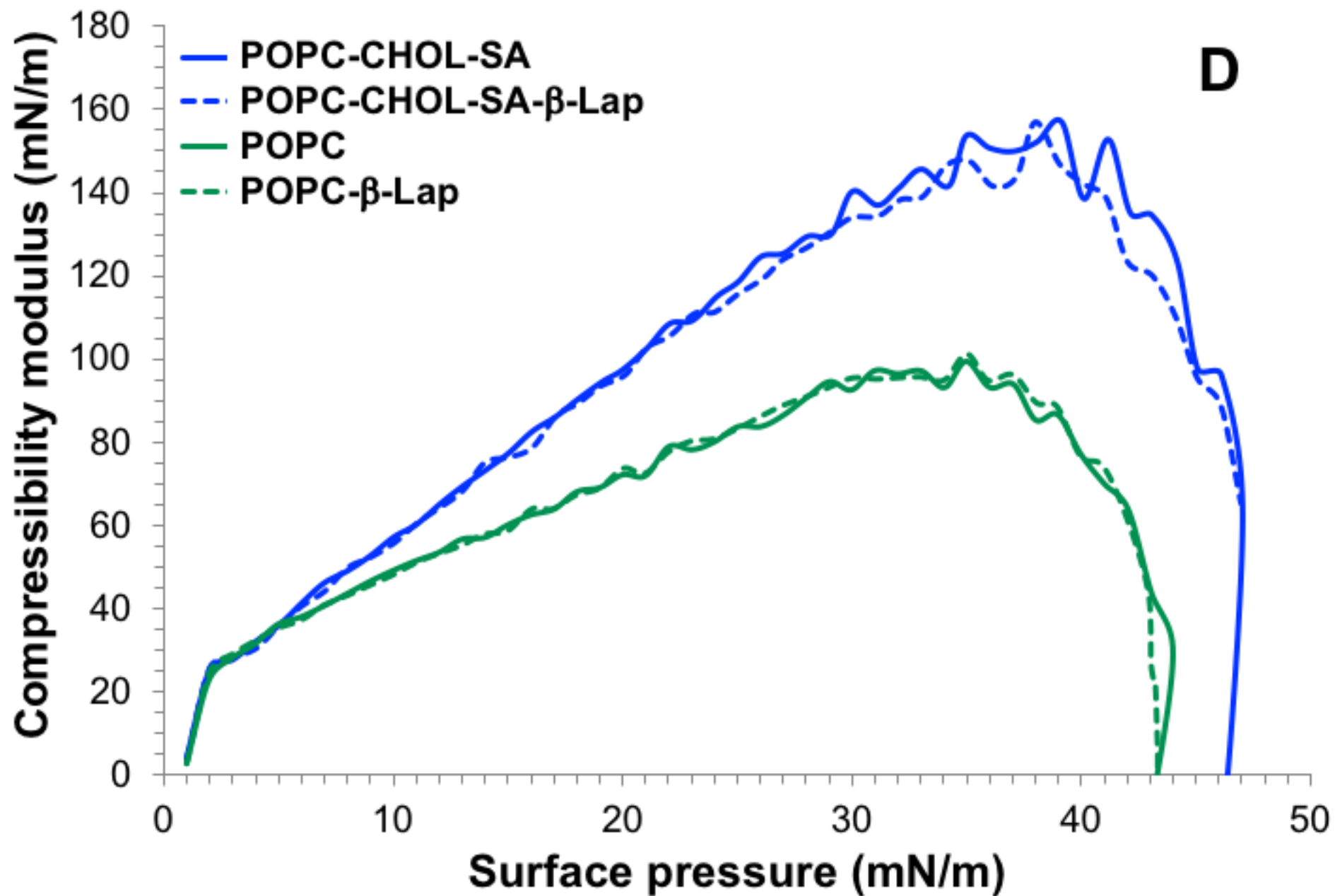
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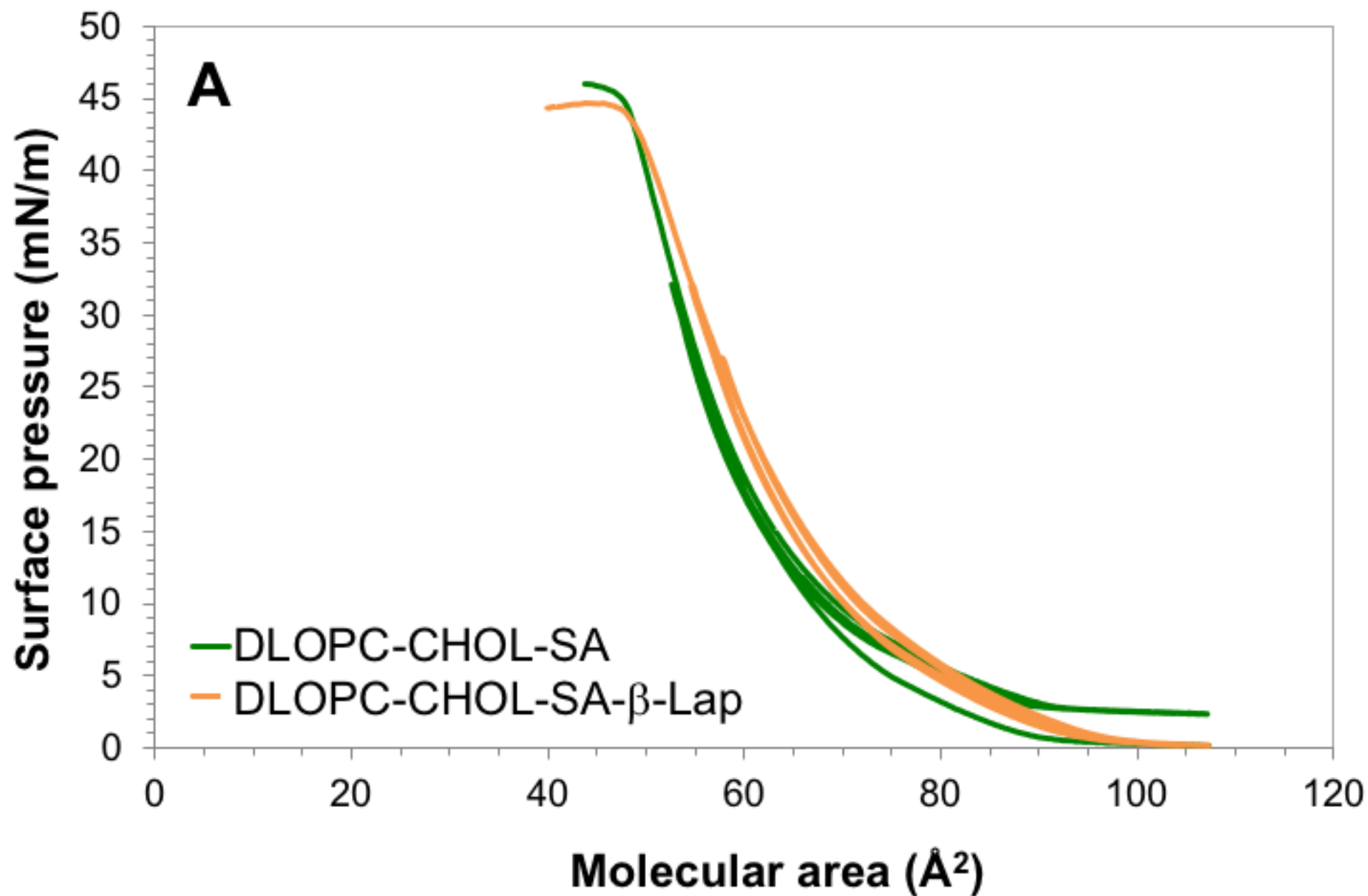
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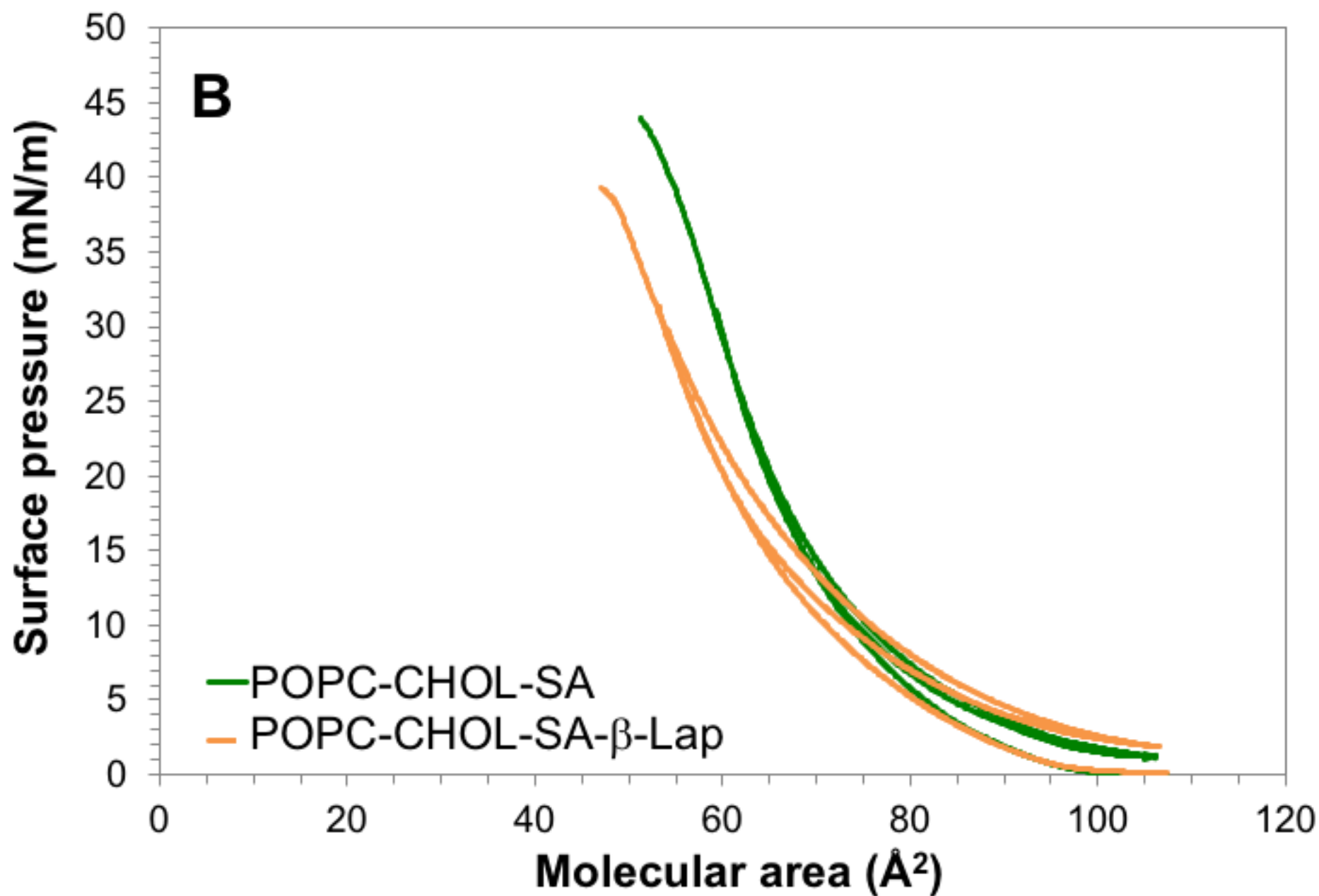
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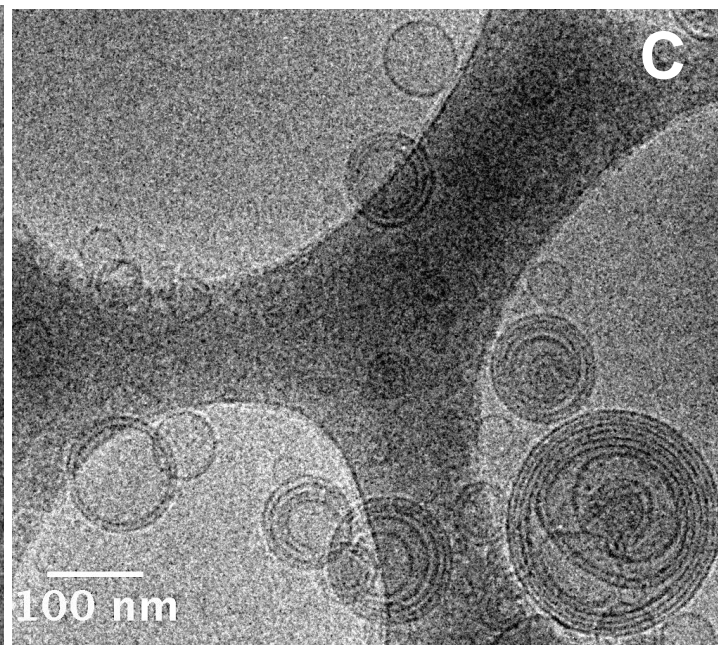
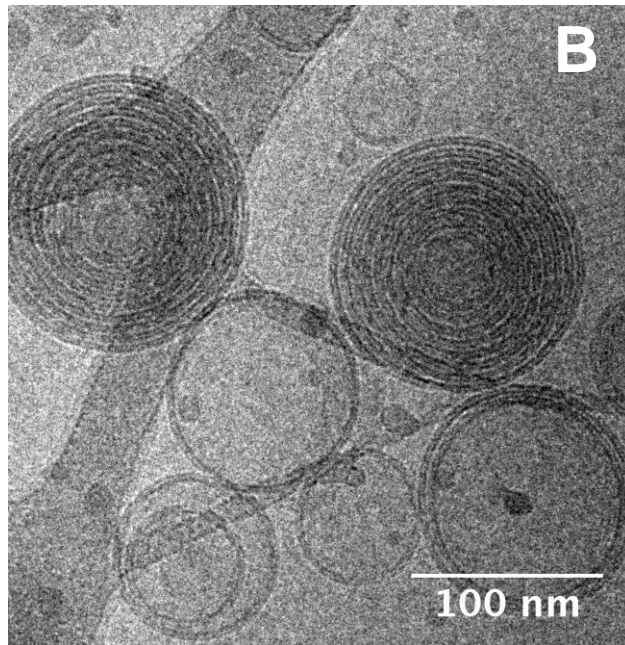
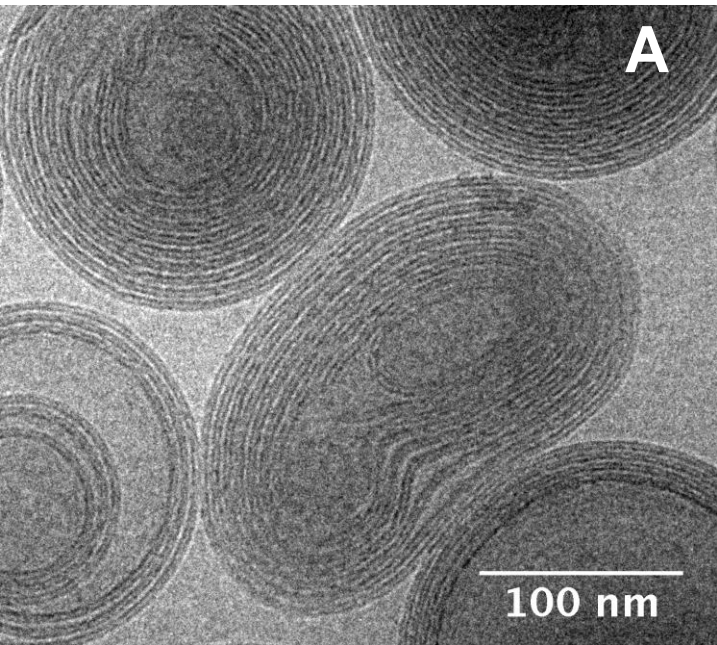


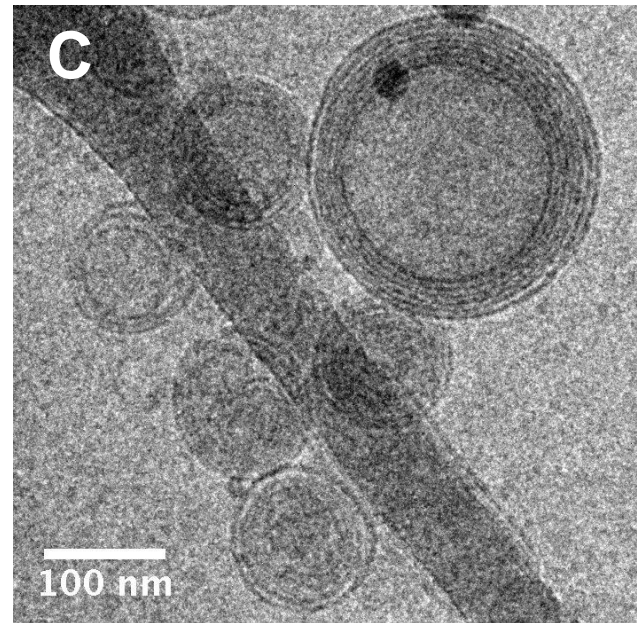
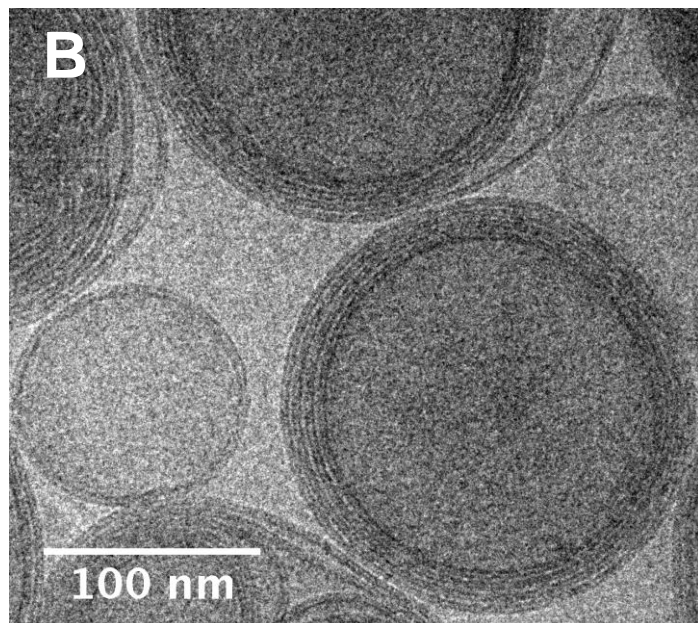
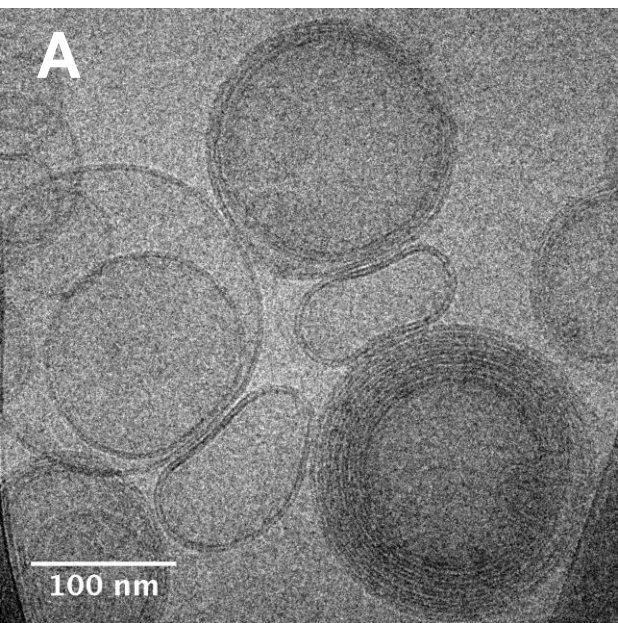












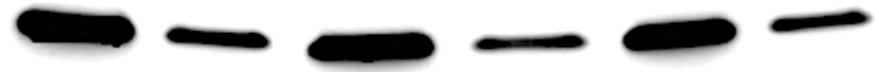
Molecular Weight (kDa)

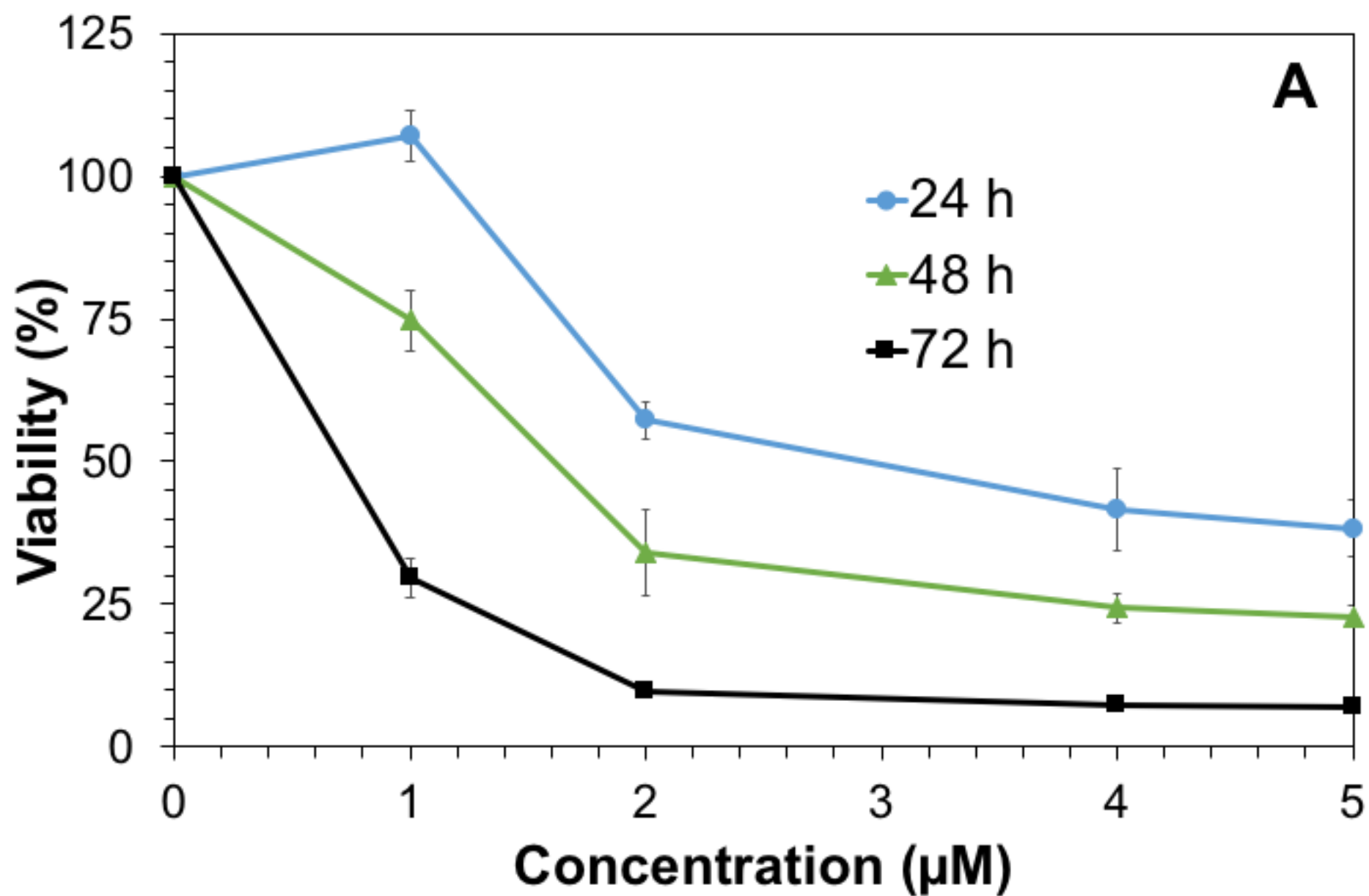
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55
36
28

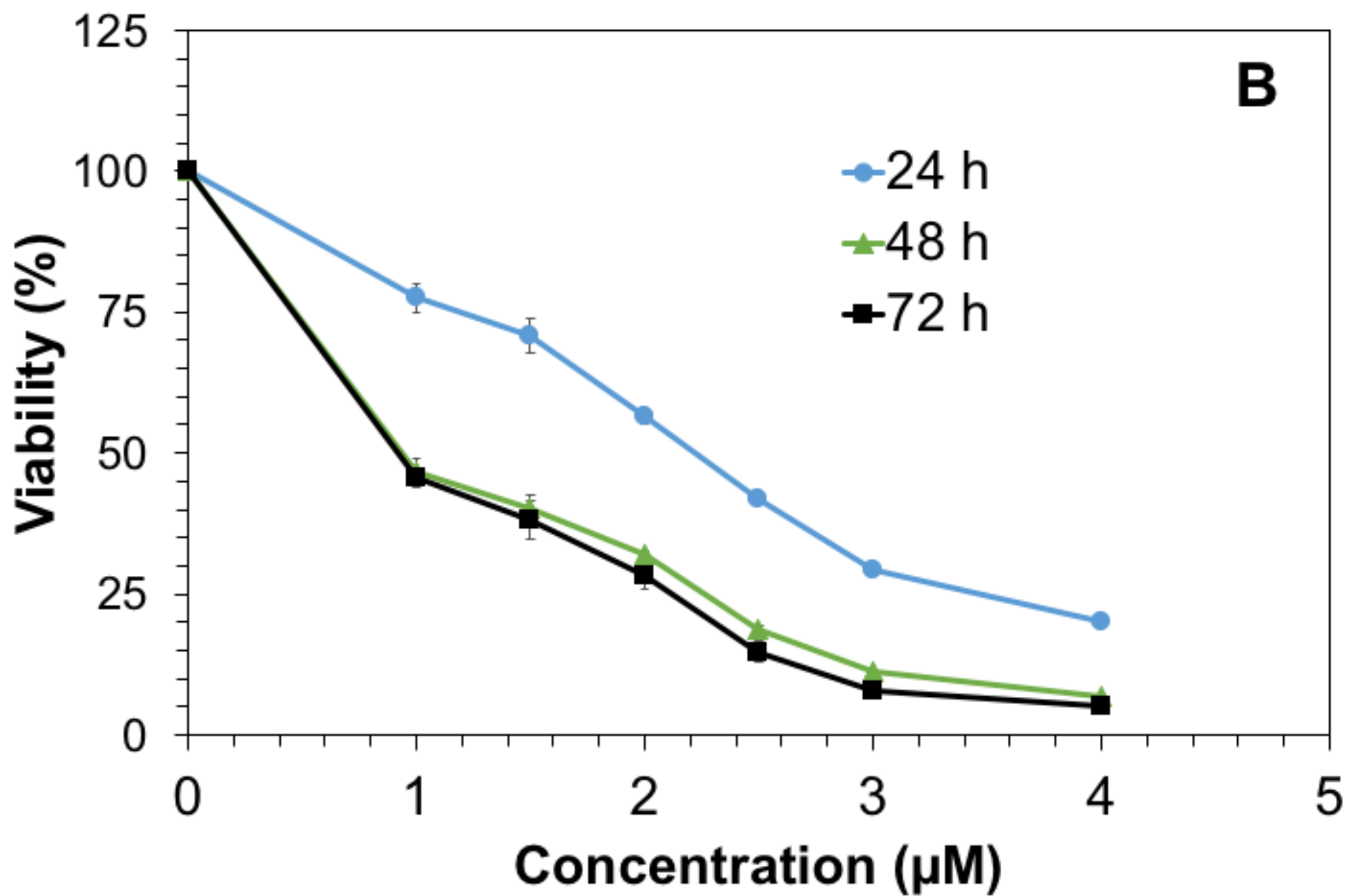


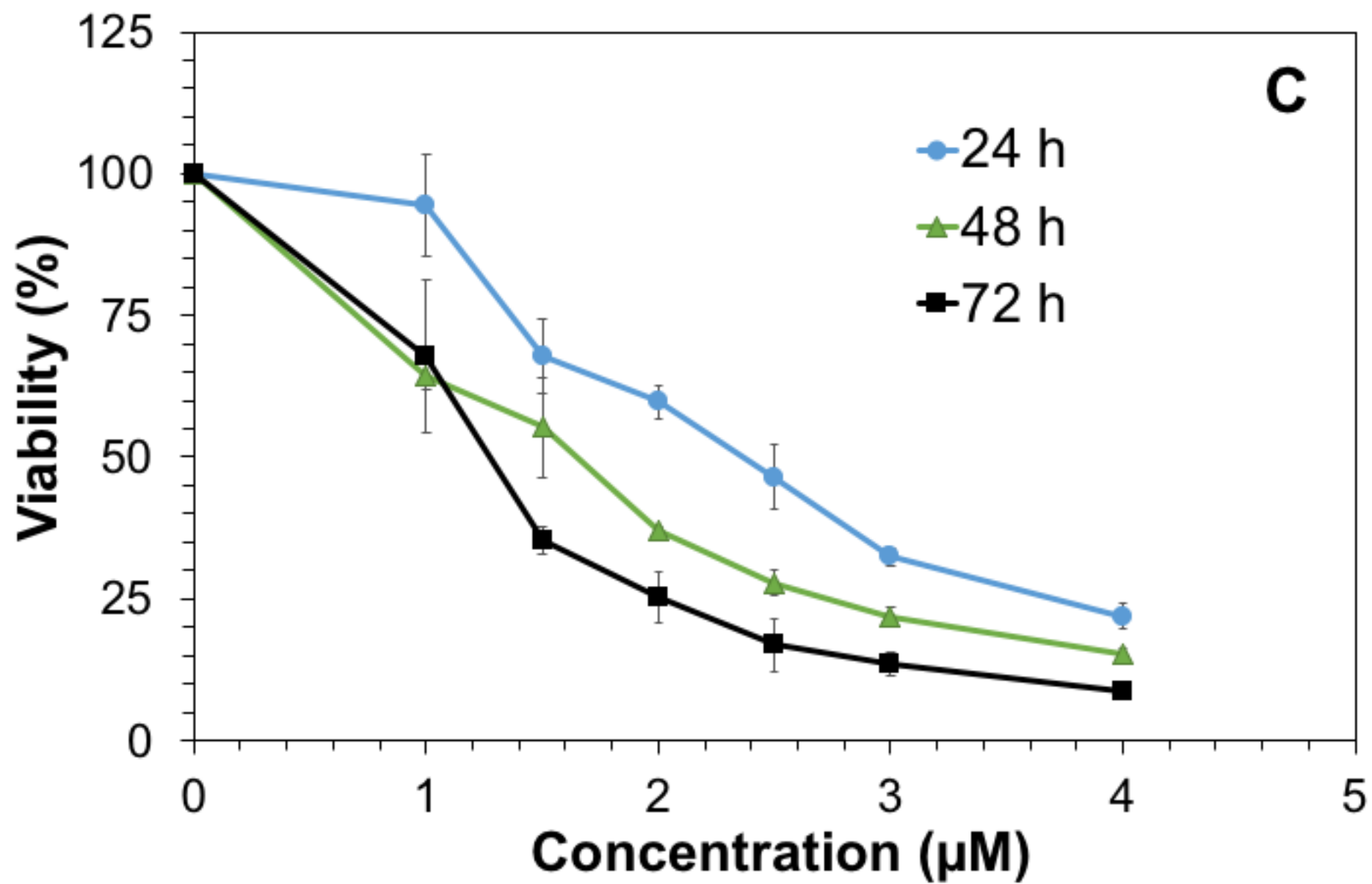
PC3 PNT2 PC3 PNT2 PC3 PNT2

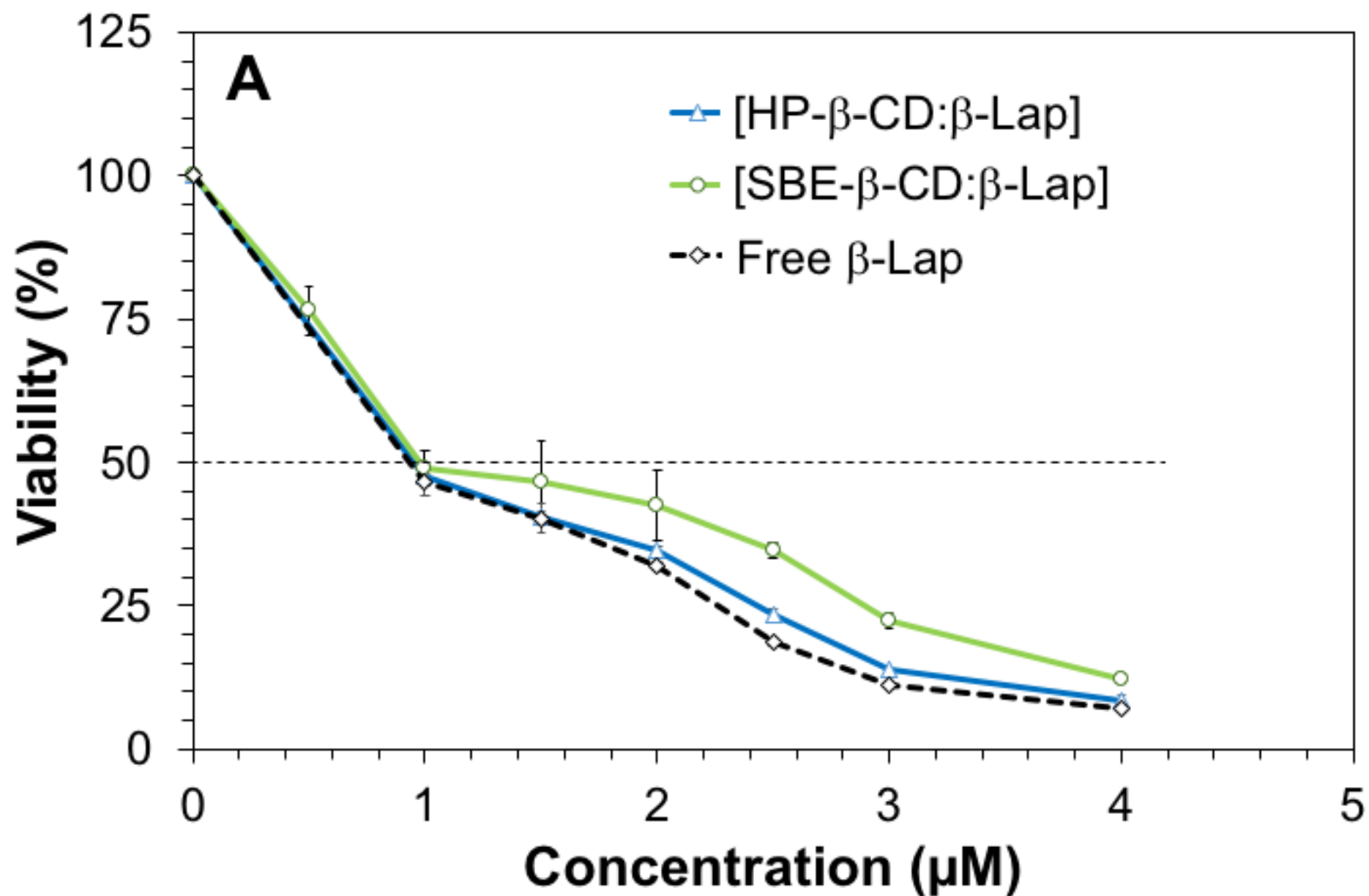
Cell Lines

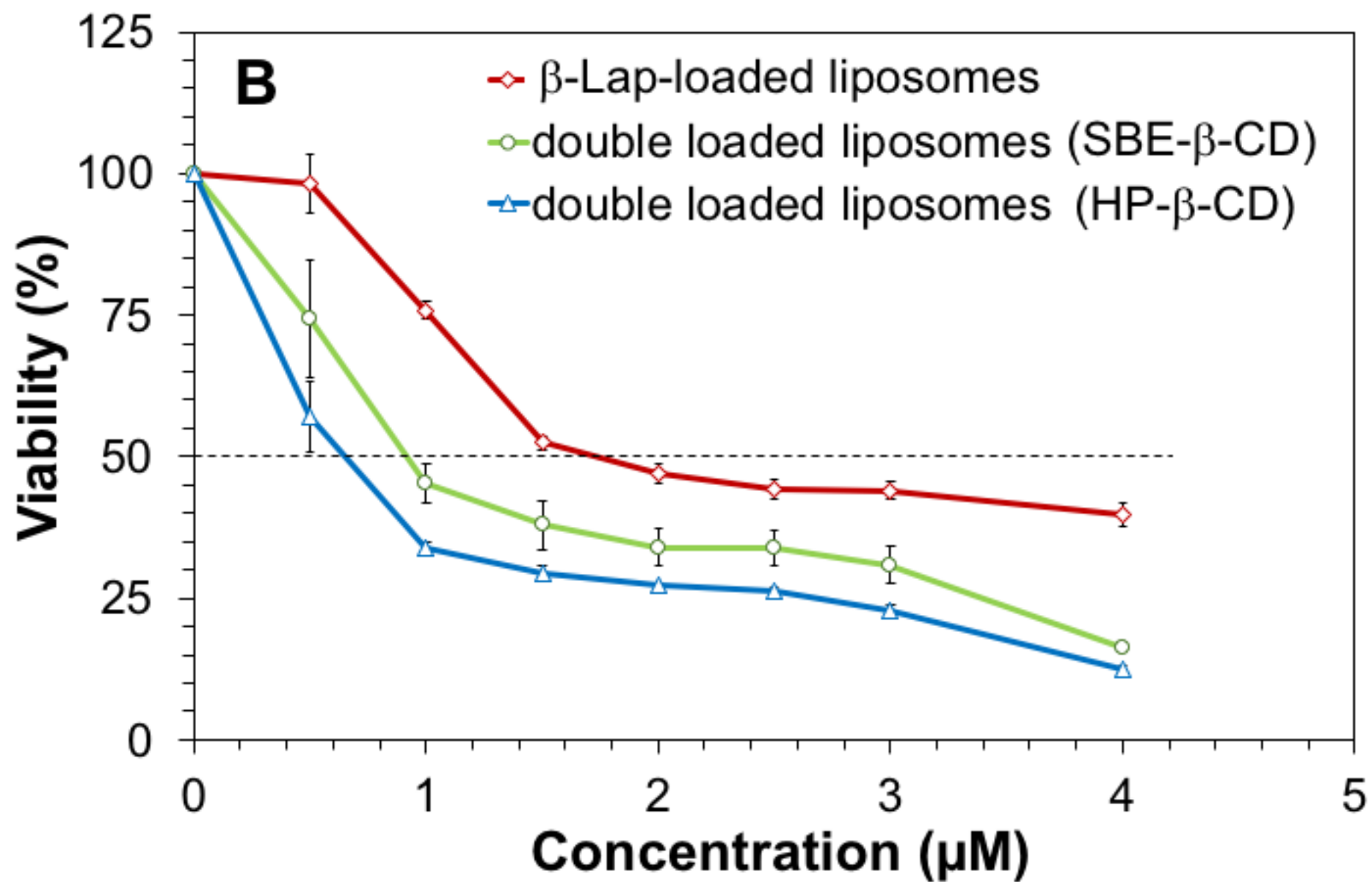


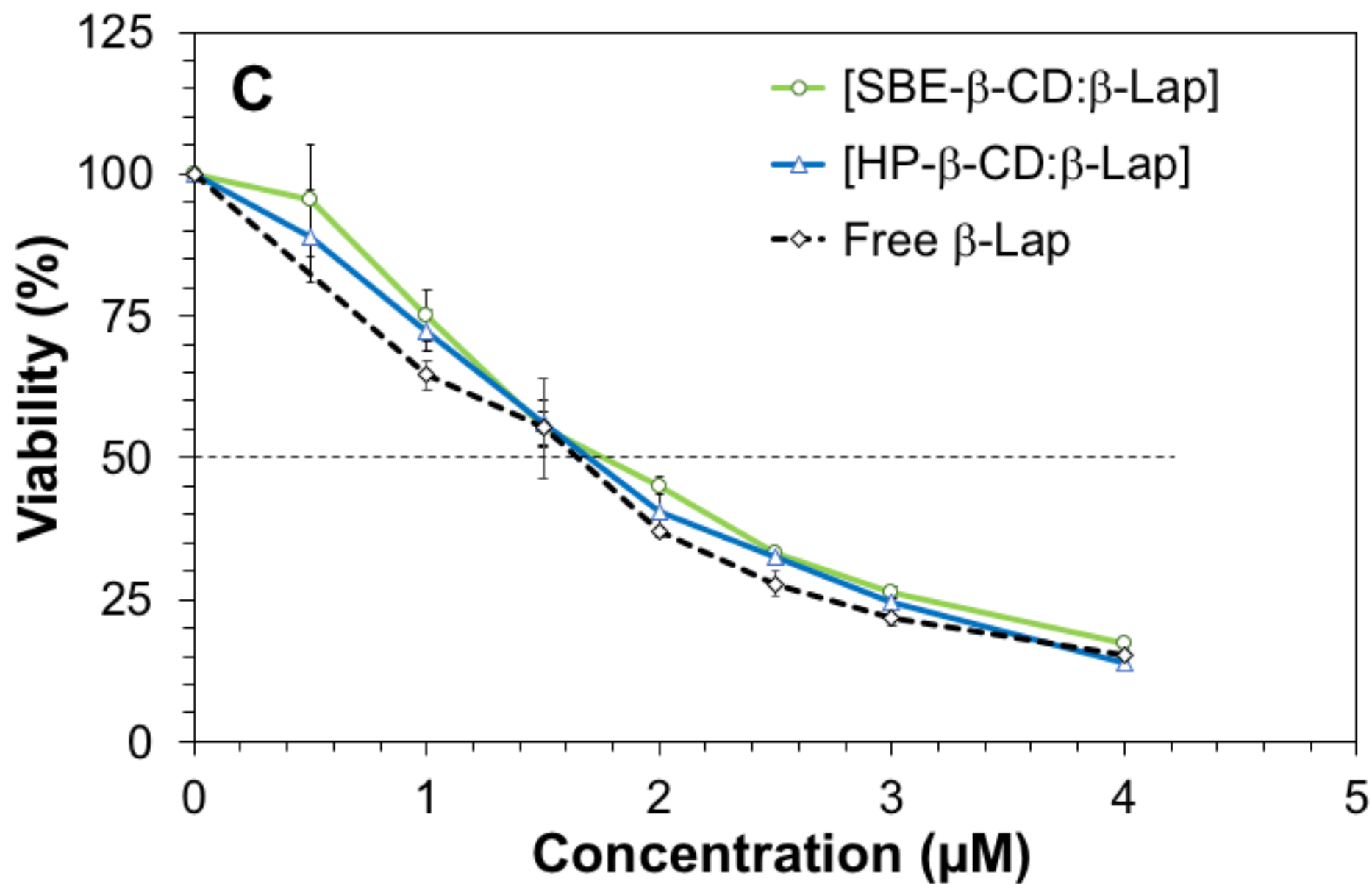


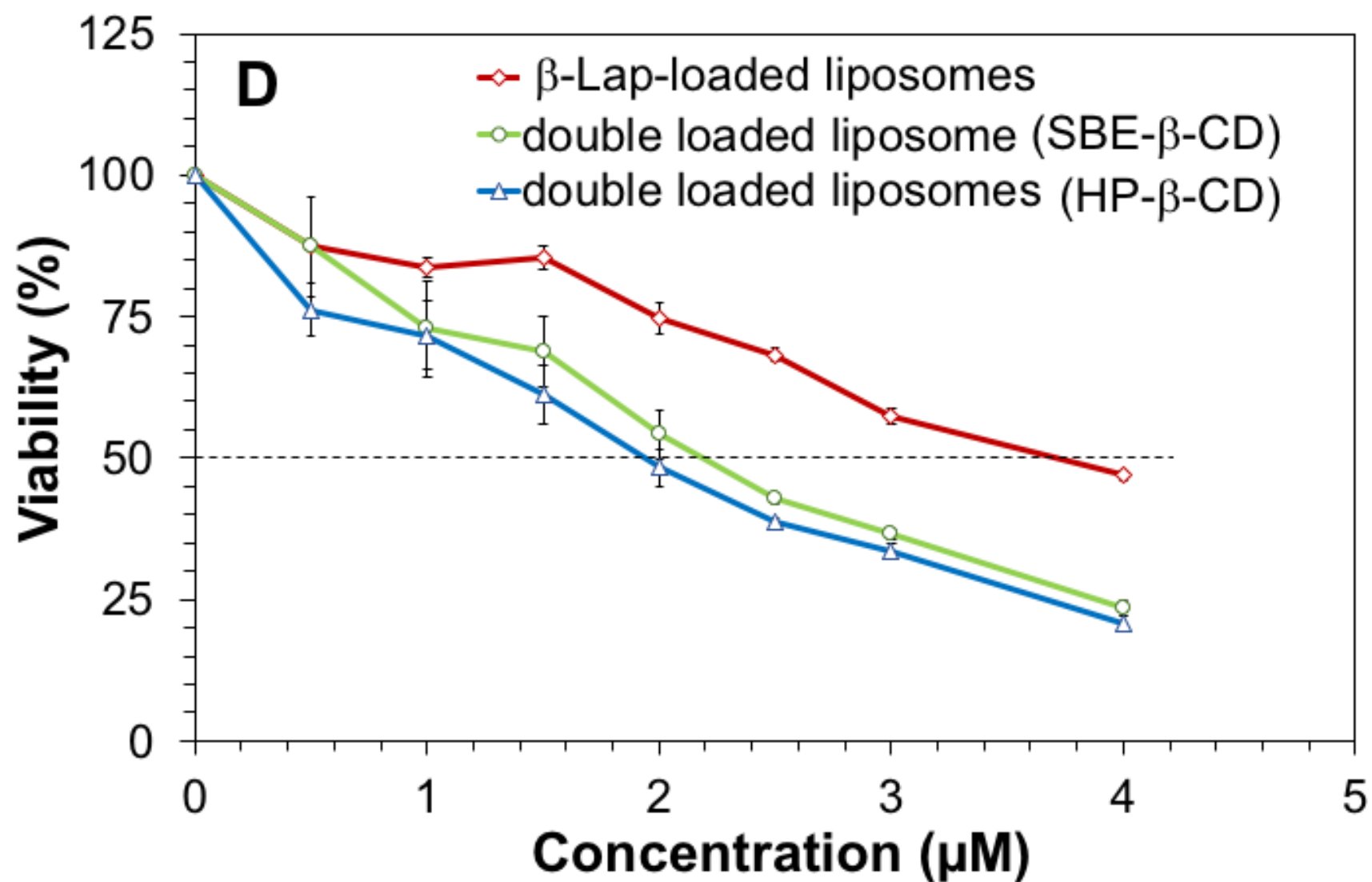




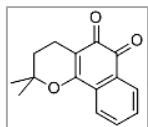




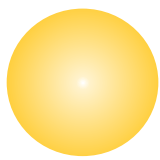




β -Lap

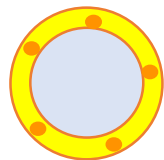


Loading
IC₅₀ (PC-3)



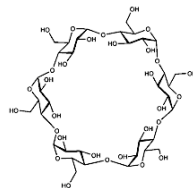
NP

2 %
1.3 μ M



Liposome

2.7 – 3.4 %
3.6 μ M



[β -Lap:CD]

[1:5]-[1:8]
1.7-1.8 μ M



Double loaded vesicle

4.0 – 4.7 %
1.9-2.2 μ M