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# Glycolipid Biosurfactant as Multilamellar Vesicular Drug Carriers

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## Abstract

Microbial amphiphiles, known as biosurfactants, are molecules obtained from fermentation of yeasts or bacteria. Biobased and biodegradable, they have been historically developed for detergency formulations, whereas more recent work has shown their interest as antimicrobials or depollutants. However, their self-assembly properties and their interactions with macromolecules suggest a broader potential of applications. Drug encapsulation for anti-cancer purposes is a well-known application of lipids such as phospholipids. In this study, for the first time, a drug delivery system based on microbial amphiphiles is designed and tested against human cervical carcinoma HeLa cells. For this purpose, multilamellar wall vesicles (MLWV) consisting of microbial glucolipid (GC) amphiphiles and polylysine (PLL), attracted by electrostatic interactions, have been synthesized. Curcumin, a highly lipophilic molecule, has been used as natural drug model to evaluate the GCPLL MLWVs as potential nanocarrier to specifically deliver drugs into cancer cells. The curcumin loaded MLWVs uptake measured by flow cytometry is much higher in HeLa cells (50%) compared to NHDF (35%) and THP-1 derived macrophages (20%). This uptake is correlated to cytotoxicity as cell viability only decreases for HeLa cells (by 50%). A dedicated mechanistic study shows that the cytotoxic effect is based on MLWV fusion with the cell membrane and the curcumin release within the cellular cytoplasm. Taken together, these results demonstrate that

34 microbial amphiphiles can be used to develop engineered drug delivery system to  
35 efficiently target cancer cells.

36

## 37 **Introduction**

38 Biological amphiphiles, sometimes referred to as biosurfactants, are molecules  
39 produced by microorganisms and developed for their high ecosustainable profile.<sup>1</sup>  
40 Among the different families of biosurfactants available (glycolipids, lipoproteins or  
41 lipopeptides, polymeric), glycolipids are certainly the most relevant one for their high-  
42 throughput production process and broadness of applications. In particular, their use in  
43 the biomedical field<sup>2</sup> has long been reported, but is mainly focused on the development  
44 of antibiotics.<sup>2</sup> In the pharmacological field, there have been reports<sup>3,4</sup> on the anticancer  
45 properties of specific microbial glycolipids (e.g., sophorolipids) since 2008,<sup>5</sup> but these  
46 results are still under debate.<sup>6</sup> Biosurfactants-based carriers have been proposed in the  
47 past years,<sup>7</sup> but in the best case scenario, the main lipidic vehicle is generally constituted  
48 by a classical phospholipid liposome.<sup>8,9</sup> Hence, a better understanding of the effects of  
49 biosurfactants on pre-formed bilayer membranes,<sup>10-12</sup> thus making the liposomal vector  
50 is necessary to obtain more complex particles. In the several cases, the lipid particle  
51 formation leads to an uncontrolled structure.<sup>13</sup>

52 The analysis of the self-assembly properties of microbial glycolipids<sup>14,15</sup> has  
53 opened the opportunity to conceive phospholipid-free stimuli-responsive complex  
54 colloidal structures, only composed of bioamphiphiles. Multilamellar wall vesicles  
55 (MLWV), belonging to the family of polyelectrolyte-surfactant complexes (PESC), are  
56 interesting colloids, originally employed in gene transfection,<sup>16</sup> for which the stability is  
57 generally considered better than single-wall vesicles. In recent reports, we have  
58 controlled the attractive electrostatic interactions between microbial C18:1-*cis* single-  
59 glucose lipid (G-C18:1, **GC**, Figure 1) and poly-L-lysine (**PLL**) at the micelle-to-vesicle  
60 phase transition of **GC** and shown the formation of phospholipid-free, stable, MLWV.<sup>17,18</sup>  
61 The multilamellar wall structure is composed of alternating layers of GC and PLL and is  
62 prepared from a pH-stimulated phase transition in water around pH 7.<sup>17</sup> Indeed, microbial  
63 glycolipids in general, and **GC** in particular, have a curious asymmetric bolaform  
64 structure with a free-standing COOH group, making the molecule pH-sensitive with a  
65 more complex phase behavior than classical lipids or surfactants.

66 Drug delivery systems are engineered technologies which allow the targeting  
67 and/or the controlled release of active principles. They overcome several drawbacks  
68 related to the systemic administration of free pharmacological molecules such as side  
69 effects, drug solubility, stability in biological environment, rapid clearance or non-specific  
70 delivery, etc.<sup>19</sup> Thus, the therapeutic index of a pharmacological drug can be improved

71 thanks to drug delivery technologies. These systems are often in the form of a drug  
72 carrier, which specifically distributes and protects the active principle from degradation  
73 and removal by the reticuloendothelial system (RES).<sup>19</sup>

74 Several examples of such delivery systems have been extensively reported in the  
75 literature, which include liposomes,<sup>20</sup> polymers<sup>21</sup> and polymeric micelles,<sup>22</sup> peptide based  
76 biomaterials,<sup>23</sup> inorganic nanoparticles,<sup>24</sup> and gels among others.<sup>25</sup> Liposomes are the  
77 most common form, investigated as nanocarriers for drug encapsulation. They have a  
78 characteristic bilayer assembly mimicking the cellular membrane, are easy-to-prepare  
79 and bio-compatible.<sup>20</sup> Despite their benefits, liposomes also face several drawbacks,  
80 such as the reticuloendothelial clearance and/or immune response.<sup>26</sup> A similar behavior  
81 has been reported for PEGylated constructs with an augmented immune response after  
82 several doses.<sup>26</sup> Therefore, there is an emergent and continuous need to discover  
83 alternatives for drug delivery strategies to overcome such issues.

84 In this perspective, it is of particular interest to evaluate engineered MLWV  
85 composed of glycolipid biosurfactant and biocompatible polyelectrolyte as a carrier of  
86 hydrophobic, as well as hydrophilic, drugs. Both small hydrophilic and hydrophobic drugs  
87 have been widely demonstrated that are effective against several diseases, but their  
88 therapeutic effect can be limited by the rapid clearance from the systemic circulation or  
89 a local site of administration, lipophilicity and therefore lower bioavailability.<sup>27,28</sup> In this  
90 study, the drug-loading and targeting potential of MLWV composed of GC and PLL  
91 (**GCPLL** MLWV) is evaluated towards mouse fibroblasts L929, normal human dermal  
92 fibroblasts NHDF, macrophages derived THP-1 and human cervical carcinoma HeLa.  
93 For this purpose, the encapsulation and mechanism of released curcumin, the active  
94 component of *Curcuma longa* plant, which combines lipophilicity, fluorescence but also  
95 anticancer properties, are analyzed.<sup>29,30</sup> In addition **GCPLL** MLWV carriers are also  
96 evaluated to deliver other commercial drugs with different degree of lipophilicity.

97

## 98 **Experimental**

99 The experimental section is given in the Supporting Information file.

100

## 101 **Results and discussion**

102

### 103 *GCPLL MLWVs are stable in cell culture medium*

104

105 Multilamellar wall vesicles only composed of glycolipid biosurfactant **GC** (Figure  
106 1) and polyelectrolyte **PLL** have been previously reported to form in mQ-grade water  
107 below pH ~ 7.5.<sup>17,18</sup> Study of their formation in cell culture medium is then a necessary

108 step to develop carriers for biological applications. A typical cell culture medium contains  
109 a wide variety of compounds such as salts, glucose and amino acids, but also proteins  
110 coming from the supplemented Fetal Bovine Serum (FBS). Such a physicochemical  
111 complexity may alter the charge, composition and/or the pH-range of stability of **GCPLL**  
112 MLWV. This latter is prepared by pH modulation as described elsewhere,<sup>17,18</sup> except for  
113 the replacement of water by FBS supplemented DMEM cell culture medium.

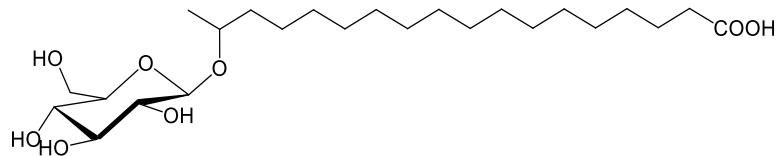
114 SAXS experiments using synchrotron radiation performed during the pH-  
115 controlled synthesis process provide the necessary real-time structural information  
116 proving the existence of multilamellar structure of the nanocarriers.<sup>17</sup> pH-resolved *in*  
117 *situ* synchrotron SAXS is then performed on the GCPLL system from alkaline to acidic  
118 pH in DMEM and the corresponding contour plot between  $q=0.1$  and  $0.4 \text{ \AA}^{-1}$  is shown in  
119 Figure 1a.

120 Below pH 8 and approximately until pH ~5, two sharp diffraction peaks  
121 correspond to the first ( $q_{(001)}$ ) and second ( $q_{(002)}$ ) order reflections ( $q_{(001)}=0.171 \text{ \AA}^{-1}$ ,  $q_{(002)}=$   
122  $0.341 \text{ \AA}^{-1}$ ) of the multilamellar wall vesicles MLWV phase (Figure 1a), evolving during pH  
123 decrease. This is in full agreement with our previous observations in water.<sup>17,18</sup> Below  
124 pH ~5, a structural gap at  $q_{(001)}$  reveals the previously-reported transition between MLWV  
125 and a PLL-free lamellar phase composed of **GC** only, characterized by a peak at  $q=$   
126  $0.169 \text{ \AA}^{-1}$ .<sup>17</sup> Previously, it was shown that **GCPLL** MLWVs synthesized in H<sub>2</sub>O are stable  
127 at a pH ranging from 4 to ~7.5 (2.5 mg/mL), the exact extreme pH values however  
128 depending on experimental conditions, like the **GC-to-PLL** ratio and possibly the salt  
129 content.<sup>17,18</sup> The present experiment shows that the use of DMEM cell culture medium  
130 does not influence at all the formation of **GCPLL** MLWV and it even seems that the  
131 domain of pH stability may even be increased to higher pH values (~8) than in pure  
132 water (~7.5) (Figure 1a). Variations in the limits of the pH transition are not unexpected  
133 and they can be qualitatively explained as follows. **GCPLL** MLWV are stabilized by  
134 attractive electrostatic forces between negatively-charged **GC** and positively-charged  
135 **PLL**. If both NMR and ITC experiments have shown that most negative charges are  
136 compensated by positive charges,<sup>17</sup> the exact amount of both negative and positive  
137 charges varies with pH and ionic strength for both **GC** and **PLL**. In pure water and  
138 absence of added salt, the optimal balance of charges for the MLWV phase starts at pH  
139 ~7.5; in DMEM, rich in salt, one expects that the optimal charge balance occurs at higher  
140 pH, when part of the higher content of negative charges are counterbalanced by the free  
141 cations in solution.

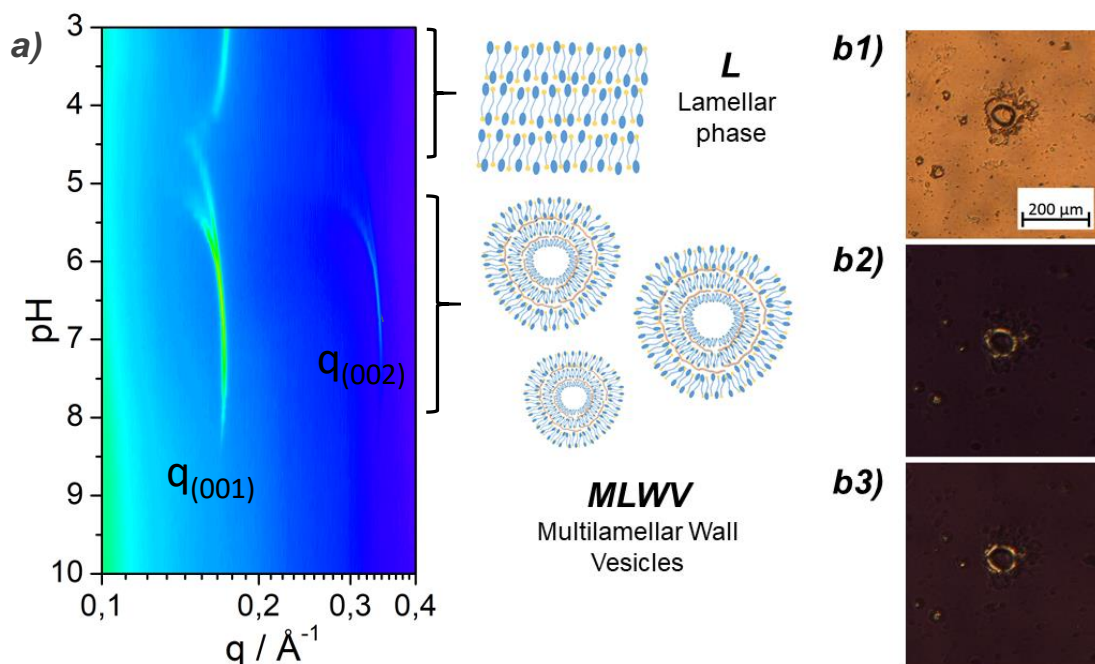
142 Being multilamellar systems, MLWVs are birefringent under polarized light.<sup>18</sup>  
143 Polarized light microscopy (PLM, Figure 1b and Figure S1) images are obtained under  
144 white (b1) and polarized light (b2 and b3) with polarizers at 0°-90° and 45°-135°

145 respectively. PLM reveals the presence of vesicular structures displaying optical  
 146 birefringence in the shape of typical maltese cross, colocalized with the vesicle, in  
 147 agreement with previous work.<sup>18</sup> PLM thereby confirms both the vesicular shape and  
 148 multilamellar wall structure of **GCPLL** colloids in solution.

149



150



151

152 **Figure 1. a) Graphical representation of SAXS at different pH values of 2.5 mg/ml of G-**  
 153 **C18:1PLL (1:1) in DMEM cell culture medium, where L: Lamellar phase and MLWV:**  
 154 **multilamellar wall vesicles. b1) PLM images of GCPLL 2.5 mg/mL in DMEM cell culture**  
 155 **medium containing birefringent patterns on the surface evidenced by rotation of the**  
 156 **polarizers from 45°–135° (b2) to 0–90°(b3)**

157

158 Quantitative <sup>1</sup>H solution NMR, using methanol-d<sub>4</sub> as common solvent, is  
 159 employed to determine the content of **GC** and **PLL** within MLWVs compared to a  
 160 reference standard TMSD-d<sub>4</sub>. <sup>1</sup>H NMR spectra show that **GCPLL** MLWVs in H<sub>2</sub>O (pH=  
 161 5.5) have a molar ratio of molecules consisting of 6.5 % of the initial content of **PLL** and  
 162 70% of the initial content of **GC**. Otherwise, when **GCPLL** MLWVs are prepared in DMEM

163 cell culture medium (pH = 7.4), one observes a proportional decrease of both **PLL** and  
 164 **GC** molar ratios, 4 and 45 % respectively (Table 1 and Figures S2-S5). A reduced  
 165 content of both **GC** and **PLL** in DMEM with respect to water samples agrees well with  
 166 the higher pH at which MLWV formation occurs. As previously measured by SAXS  
 167 (Figure 1a), MLWVs in DMEM are formed in the pH range 4.5-8, whereas pH 8  
 168 constitutes the upper pH limit above which MLWV start to disassemble. It is reasonable  
 169 to suppose that for pH values close to the limit of pH 8, part of the MLWV have started  
 170 to disassemble and the composition decreases. This issue could be easily solved by  
 171 adapting the initial content of **GC** and **PLL**, but this was out of the scope of this work. All  
 172 in all, this structural analysis shows that the **GCPLL** MLWV prepared as such can be  
 173 used for biological applications at physiological pH in culture medium. In addition, the  
 174 final molar ratio  $GC_f/PLL_f$  in H<sub>2</sub>O and DMEM after preparation of MLWV remains  
 175 practically constant (57 and 60 respectively). The ratio between COOH and NH<sub>2</sub>  
 176 functional groups, partially reflecting the charge ratio, is given in Table 1 as  
 177  $[COOH]/[NH_2]$  and one respectively finds 2.8 and 3 for H<sub>2</sub>O and DMEM, concluding that  
 178 the composition of MLWVs is comparable independently of the nature of the aqueous  
 179 medium.

180

181 **Table 1. Quantitative evaluation of G-C18:1 (GC) and PLL in MLWV by <sup>1</sup>H Solution NMR.**  
 182 **Explanation of the calculation is described in Table S1.**

	C <sub>initial</sub> (mM)		C <sub>final</sub> (mM)		C <sub>F</sub> /C <sub>In</sub> (%)		Molar ratio		Functional group
	[GC] <sub>in</sub>	[PLL] <sub>in</sub>	[GC] <sub>f</sub>	[PLL] <sub>f</sub>	GC <sub>f/in</sub>	PLL <sub>f/in</sub>	GC <sub>in</sub> / PLL <sub>in</sub>	GC <sub>f</sub> / PLL <sub>f</sub>	[COOH]/ [NH <sub>2</sub> ]
H <sub>2</sub> O	5.4	1	3.7	0.065	70	6.5	5.4	57	2.8
DMEM	5.4	1	2.4	0.04	45	4	5.4	60	3

183

184

185 *GCPLL MLWVs less cytotoxic than GC alone*

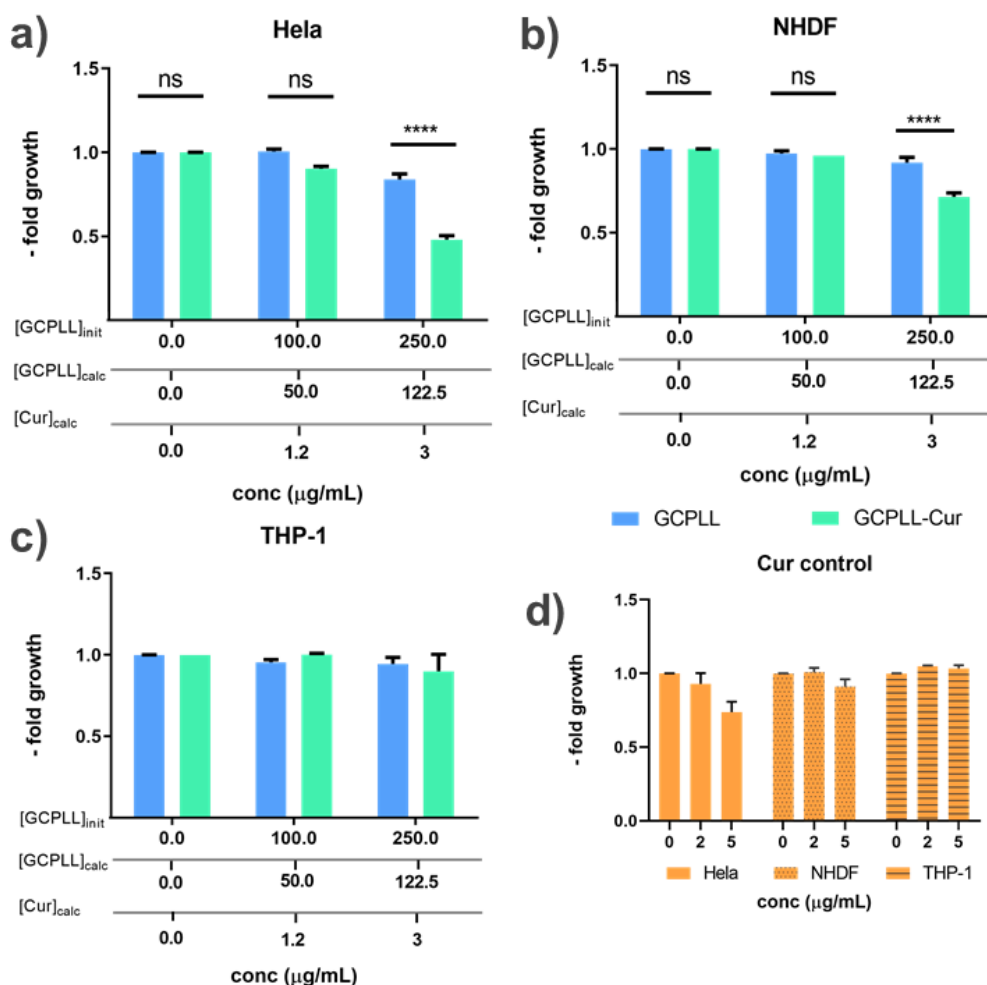
186 The cytotoxicity of **GCPLL** MLWVs is not known. This parameter is evaluated in  
 187 mouse fibroblasts L929 cell line by dilution of the initial 2.5 mg/mL mixture to obtain  
 188 different concentrations (range from 0 to 1 mg/ml). All the concentrations are referred to  
 189 as the initial quantity employed in the preparation. As controls, the viability of cells is  
 190 evaluated after incubation with **GC** and **PLL** independently, as well as the medium at the  
 191 same pH. The latter corresponds to a **GC**- and **PLL**-free DMEM medium which has  
 192 undergone the same pH changes required to prepare **GCPLL** MLWVs. These results  
 193 are shown in Figures S6, S7.

194 **PLL** and pH medium controls show no significant cytotoxic effect under the  
195 studied conditions. On the contrary, **GC** (Figure S7) has a significant impact on the L929  
196 viability, as only 80 % of the control metabolic activity is measured when  $C_{GC}= 250 \mu\text{g/mL}$   
197 is used and dramatically decreases to less than 10% from 500  $\mu\text{g/mL}$  on. Regarding  
198 **GCPLL** MLWVs, a cytotoxic effect is observed from 250  $\mu\text{g/mL}$  as the metabolic activity  
199 drops to 60%. From 500  $\mu\text{g/mL}$ , only 20% of cells are alive (Figure S6). However, no  
200 significant cytotoxicity is observed with  $C_{GCPLL}= 100 \mu\text{g/mL}$ .

201 According to the  $^1\text{H}$  solution NMR analysis (Figure S2-5 and Table S1), about 95  
202 % of free PLL and 50% of free GC are detected in a **GCPLL** colloidal solution fabricated  
203 with DMEM cell culture medium. For this reason, we suspect that most of the cytotoxic  
204 effect of the **GCPLL** solution is associated to free **GC** molecules. We then develop an  
205 alternative strategy to evaluate the cell viability without the presence of extra **GC** free  
206 molecules in solution. **GCPLL** MLWVs are prepared in DMEM as described previously,<sup>18</sup>  
207 but they are centrifuged at 3000 rpm for 5 min, then the supernatant is discarded (thus  
208 eliminating the free forms of **GC** and **PLL**) and replaced by the same volume of fresh  
209 DMEM, prior to their analysis and further use. The **GCPLL** pellet is resuspended by  
210 vortexing and sonicating for few seconds, recovering its initial colloidal stability.<sup>18</sup> The  
211 latter is most likely explained by surface charge arguments, an important  
212 physicochemical parameter that influences the colloidal stability of the suspensions. The  
213 zeta-potential of **GCPLL** MLWVs in cell culture medium is found to be of  $-11.9 \pm 0.4 \text{ mV}$ ,  
214 meaning that **GCPLL** MLWVs have a slight negative surface charge, which may prevent  
215 aggregation.<sup>31,32</sup>

216 The cell viability measured after incubation with **GCPLL** MLWVs after  
217 centrifugation is presented in Figure S6, now showing no significant cytotoxicity of **GCPLL**  
218 MLWVs up to 250  $\mu\text{g/mL}$  in L929 mouse fibroblast cell line, while the as-prepared  
219 MLWVs containing free **GC** exhibits a viability of about 50%. This result confirms that the  
220 cytotoxic effect previously found for **GCPLL** MLWVs is essentially attributable to the free  
221 **GC** in solution. Finally, Figure S7 shows no cytotoxicity associated to the controls, that  
222 is **PLL** and DMEM, the latter undergone with pH changes as described above. The  
223 cytotoxicity of **GCPLL** MLWVs is eventually assessed on several cell lines (blue bars on  
224 Figure 2): macrophages derived THP-1, Normal Human Dermal fibroblasts and Hela  
225 cells. The concentration range settles within the range contained between 20 and 1000  
226  $\mu\text{g/mL}$ , comparable to other drug delivery systems, such as loaded and blank  
227 liposomes.<sup>33-35</sup>





228

229 **Figure 2. a-c) Cell viability of GCPLL (blue), GCPLL-Cur (green) in a) HeLa cells; b)**  
 230 **NHDF cells; and c) THP-1 cells. Three abscissa are displayed from a) to c) to show**  
 231 **the initial concentration employed in the MLWVs preparation, the calculated**  
 232 **GCPLL content and the Cur encapsulated. In particular, [GCPLL]<sub>init</sub> refers to**  
 233 **concentrations employed initially in the preparation of GCPLL; [GCPLL]<sub>calc</sub> refers**  
 234 **to concentrations calculated by <sup>1</sup>H NMR (Table 1) after centrifugation and**  
 235 **resuspension of the MLWVs pellet; and [Cur]<sub>calc</sub> refers to the encapsulated Cur in**  
 236 **the MLWVs measured by UV-Vis (Figure 3). d) Cell viability of Curcumin control in**  
 237 **the three different cell types tested.**

238

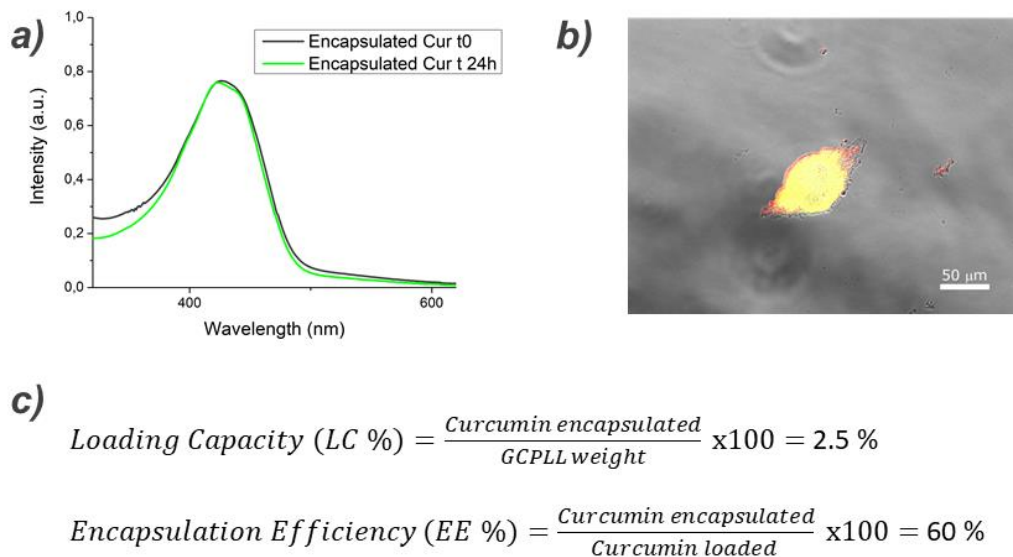
239 *Curcumin is efficiently encapsulated within MLWVs*

240 The multilamellar lipid structure, the stability of **GCPLL** MLWVs in physiological  
 241 culture medium and their absence of cytotoxicity make them ideal candidates as  
 242 phospholipid-free drug delivery system based on biological amphiphiles alone. MLWVs  
 243 may combine the advantages of drug loading capacity, biodegradability, and  
 244 biocompatibility based on biosurfactants obtained from microbial source.

245 Curcumin, the active component of *Curcuma longa* plant, is a molecule widely  
 246 used as drug due to its antioxidant, anti-inflammatory and anticancer properties.<sup>29,36</sup>  
 247 Curcumin (**Cur**) is highly lipophilic, with a water-octanol partition coefficient, logP, in the  
 248 order of 2.6 and a membrane partition constant above  $10^4$  M<sup>-1</sup>.<sup>30</sup> Similarly to other  
 249 hydrophobic drugs,<sup>37</sup> curcumin has limited applicability due to its poor oral bioavailability,  
 250 low chemical stability<sup>38</sup> as well as its weak cellular uptake.<sup>29</sup> As a consequence, the  
 251 accumulation of curcumin is low within the cytoplasm.<sup>39,40</sup> The cell uptake process of  
 252 curcumin has been reported to penetrate the cell membrane and interact with the lipids  
 253 of the membrane through H-bonding and hydrophobic interactions. Different strategies  
 254 could be followed to overcome these limitations, such as the synthesis of curcumin  
 255 derivatives,<sup>29</sup> or the development of drug delivery systems to enhance the stability and  
 256 increase its cellular uptake. <sup>41</sup>

257 Therefore, curcumin was chosen as model natural drug to load the **GCPLL**  
 258 system with the aim of probing the encapsulation capacity of MLWVs and to show their  
 259 potential to enhance the therapeutic index of the encapsulated drug curcumin.

260



261

262 **Figure 3. a) UV-Vis spectra of Curcumin encapsulated in GCPLL at t= 0 and t= 24h**  
 263 **in DMEM cell culture medium. GCPLL-Cur is centrifuged out and resuspended in**  
 264 **ethanol for analysis. b) Fluorescence microscopy image of GCPLL-Rhod-Cur at**  
 265 **2.5 mg/mL in DMEM cell culture medium. Liss-Rhod (red) and Curcumin (green)**  
 266 **are loaded within particles. c) Calculation of Loading capacity (LC%) and**  
 267 **Encapsulation Efficiency (EE%), where the amount of Cur encapsulated is 30 μg,**  
 268 **the loaded quantity 50 μg and the weight of GCPLL 1225 μg considering the values**  
 269 **obtained from <sup>1</sup>H NMR evaluation (Table1).**

270

271 The process of curcumin (**Cur**) encapsulation is based on a straightforward and  
272 fast mixture and vortexing protocol described in the experimental section in the  
273 Supporting Information. Addition of **Cur** does not modify the zeta potential of **GCPLL-  
274 Cur** MLWVs, which is  $-13.2 \pm 0.3$  mV, thus justifying the colloidal stability after  
275 resuspension.

276 UV-vis absorbance measurements are performed to quantify the **Cur**  
277 encapsulated in **GCPLL** MLWVs just after fabrication and after 24 hours of incubation in  
278 culture medium. (Figure 3a). These experiments involve dissolution of the **GCPLL-Cur**  
279 MLWVs pellet in ethanol, a common solvent for all components. The absorption spectra  
280 are superimposable, thereby showing that **Cur** is stable within the MLWVs aqueous  
281 solution over 24 h. In addition, this result shows that **Cur** is encapsulated in **GCPLL** in  
282 its native form. This can be considered as a crucial advantage compared to free **Cur** in  
283 solution. Indeed, it has been reported that **Cur** decomposes approximately by 50% in  
284 cell culture medium supplemented with 10% serum after 8 hours of incubation.<sup>42</sup> The  
285 encapsulated concentration corresponds to 80  $\mu$ M **Cur**, and this concentration is  
286 calculated using the calibration curve reported in Figure S8.

287 To exclude coprecipitation and to confirm that **Cur** is actually colocalized in the  
288 **GCPLL** MLWVs, we perform fluorescence microscopy on a drop of **GCPLL-Cur** MLWVs  
289 solution. **GCPLL** MLWVs are simultaneously labelled with rhodamine using a  
290 rhodamine-modified C18:1 lipid (Liss-Rhod PE), known to intercalate in the glucolipid  
291 membrane without interfering with the structure for lipid-to-dye molar ratio above 200.<sup>43</sup>  
292 The colocalized fluorescence, red for Liss-Rhod PE and green for **Cur** as well as the DIC  
293 white light for **GCPLL** confirm the encapsulation of **Cur** within MLWVs, rather than  
294 coprecipitation (Figure 3b). Colocalization of **Cur** (green) and Liss-Rhod PE (red) is also  
295 demonstrated within the cellular compartment, as shown and discussed later.

296 A key parameter to characterize drug delivery systems is the encapsulation  
297 efficiency (EE %), defined in the formula given in Figure 3c. EE % is sensitive to different  
298 properties related to each system such as morphology, hydrophobicity, charge of the  
299 surface, permeability, the structure of the encapsulated molecule as well as the  
300 encapsulation process.<sup>44,45</sup> Taking into account the molar concentration of **Cur** obtained  
301 by UV-Vis (80  $\mu$ M) and the total loaded Cur (135  $\mu$ M), one can then calculate the drug  
302 loading of this system and finds EE% = 60%. This value is higher than other EE%  
303 estimated for other vesicular systems, which show a high variability, ranging from 1 to  
304 68% for vesicles and 6 to 31% for multilamellar vesicles (MLV).<sup>46</sup> The broad spectrum of  
305 reported EE % values between unilamellar vesicles (ULVs) and MLVs is commonly  
306 explained by the presence of a lumen in ULVs, allowing a higher loading volume of drug

307 compared to the actual lipid content.<sup>46</sup> Despite the structure differences, we have  
308 reached comparable EE % in MLVs compared to ULVs.

309 The loading capacity (LC %, Figure 3c) is defined as the ratio between the  
310 amount of **Cur** encapsulated (30 µg) and the weight of GCPLL system calculated by <sup>1</sup>H  
311 NMR (1225 µg). The loading capacity that we obtain for **GCPLL-Cur** is about 2.5%  
312 (Figure 3c). In the present case, the two-step preparation protocol of **GCPLL-Cur** may  
313 explain the low LC % value: considering that MLWVs are already formed when **Cur** is  
314 added, encapsulation may occur only in the outer layers of the MLWVs. The strong  
315 discrepancy between LC % and EE % may confirm this hypothesis: the drug is mainly  
316 encapsulated in the outer lipid layer of the MLWVs, which would involve a good  
317 encapsulation process but a low drug-to-lipid content. Different protocols could probably  
318 improve both the EE and LC% of Cur, but this is out of the scope of the present work.

319

320 *Curcumin is selectively delivered to HeLa cells via a membrane-fusion mechanism*

321 The antiproliferative activity of curcumin loaded MLWVs is explored in three  
322 different human cell lines (Figure 2a-c): Normal Dermal Human Fibroblasts (NHDF),  
323 cervical carcinoma HeLa cells and THP-1 monocyte-derived macrophages.

324 HeLa cells are chosen as model to evaluate the **GCPLLs** as drug carriers to  
325 target cancer cells, i.e with high proliferative activities. On the opposite, NHDF are used  
326 as model of normal cells with moderate proliferative activities to assess the effect of  
327 MLWVs with regards to the multiplication potential. Last, THP1 derived macrophages  
328 are used in this study to evaluate the targeting of **GCPLL** toward cancer cells.  
329 Macrophages are members of the reticulo endothelial system (RES) and possess high  
330 phagocytic activities to clear particles from the human body. Concentrations in Figure  
331 2a-c are given as [GCPLL]<sub>init</sub>, i.e the initial GCPLL concentrations, and [GCPLL]<sub>calc</sub> and  
332 [Cur]<sub>calc</sub>, the concentrations of GCPLL and curcumin, respectively, calculated after  
333 removal of free GC by centrifugation.

334 First of all, free **Cur** control (Figure 2d) has no toxic effect on THP-1 derived  
335 macrophages (orange/line bar) and NHDF (orange/dotted bar) and it only slightly  
336 reduces cell viability of HeLa cells for the 5 µg/mL concentration (orange bar). Similarly,  
337 the **GCPLL** (blue bar) control has no cytotoxic effect neither on THP-1 (Figure 2c)  
338 derived macrophages nor on NHDF (Figure 2b), with a slight reduction in cell viability  
339 (>80 %) on the HeLa cells for the highest concentration tested (Figure 2a), that is 250  
340 µg/mL but corresponding to 122.5 µg/mL after centrifugation. Finally, the curcumin-  
341 loaded MLWV system, **GCPLL-Cur** (green bar), has no effect on the THP-1 (Figure 2c),  
342 for 100 and 250 µg/mL (initial concentrations), and NHDF (Figure 2b) up 100 µg/mL,  
343 while a slight cytotoxicity is measured at 250 µg/mL (cell viability at about 75%),

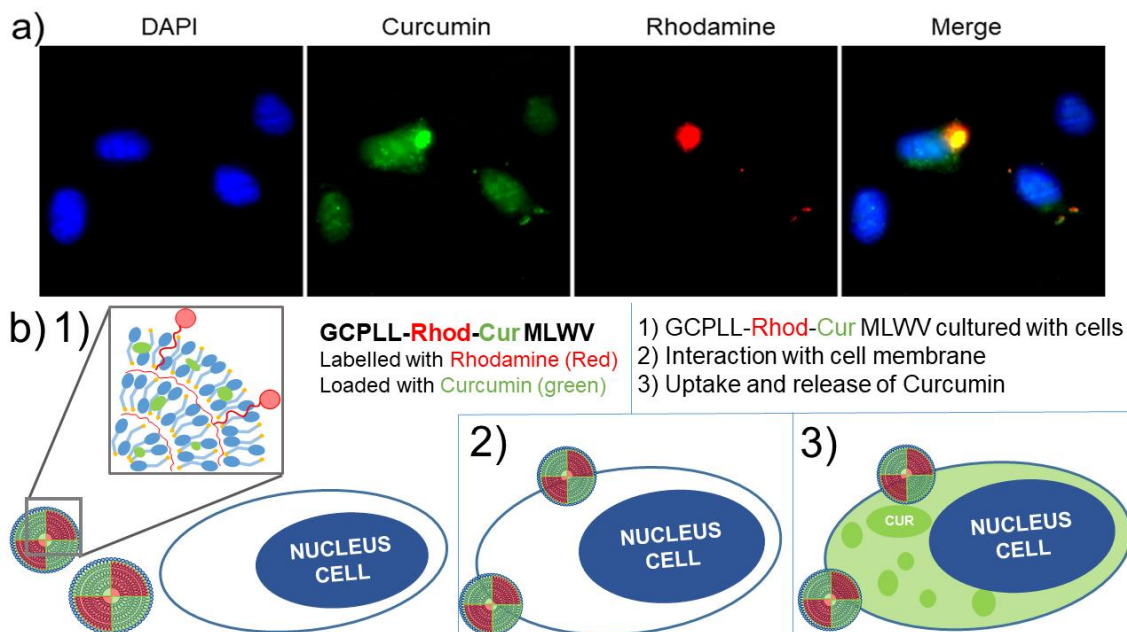
344 respectively of calculated concentrations of 50 and 122.5  $\mu\text{g/mL}$ . Regarding the HeLa  
345 cancer cell line (Figure 2a), a dramatic effect (50% cell viability) is observed for **GCPLL-**  
346 **Cur** at the highest concentration, when compared to the maximum **Cur** content in the  
347 control experiment (Figure 2d, 75% cell viability) and **GCPLL** controls (Figure 2c). The  
348 cytotoxic effect of **GCPLL-Cur** is higher than the one of free curcumin despite a lower  
349 cargo: the calculated curcumin content in the most toxic **GCPLL-Cur** sample (50% cell  
350 viability) is about 3  $\mu\text{g/mL}$ , while the curcumin control displays a 75% viability at curcumin  
351 concentration of 5  $\mu\text{g/mL}$ .

352 As the cytotoxic effect of curcumin encapsulated within **GCPLL** MLWVs is greater in  
353 HeLa cells compared to NHDF and macrophages, it seems to be positively correlated  
354 with the proliferative rate of cells. Indeed, cancer cells such HeLa possess a very short  
355 doubling time, NHDF have moderate proliferative activities and macrophages derived  
356 THP-1 do not proliferate.

357 Liposomal curcumin systems applied in pharmacology,<sup>35</sup> in particular the studies of  
358 Huang *et al.*<sup>47</sup> employed carboxymethyl dextran (CMD) modifying liposomal curcumin  
359 in HeLa cells, reporting an  $\text{IC}_{50}$  of 6.6  $\mu\text{M}$ , and in another study by Saengkrit *et al.*<sup>48</sup>  
360 involving curcumin loaded cationic liposomes showed  $\text{IC}_{50}$  in HeLa and SiHa cells of 21  
361  $\mu\text{M}$  and 16  $\mu\text{M}$ , respectively. In comparison with our results, we employed 8.1  $\mu\text{M}$  loaded  
362 curcumin in MLWVs to reach 50 % cytotoxicity in HeLa cells (Figure 2c), which is  
363 comparable to CMD  $\text{IC}_{50}$  in HeLa cells, but much better than the liposomes based system.

364

365 These results put in evidence the remarkable activity of **GCPLL-Cur** MLWVs towards  
366 cancer cell line HeLa, with little cytotoxic effect in both normal cells, i.e fibroblasts and  
367 no dividing cells such as macrophages, thus avoiding the potential damage in normal  
368 tissue and the clearance by the reticuloendothelial system (RES), which is dedicated  
369 for the foreign particles elimination. Cytotoxicity of curcumin encapsulated within GCPLL  
370 MLWVs seems to be correlated, on one side, with the proliferative rate of cells, and on  
371 the other side, with the encapsulation efficiency. They are highly toxic for cancer cells  
372 which possess a high doubling time but not on no dividing cells such as macrophages.



373

374

375 **Figure 4. a) Fluorescence microscopy images on HeLa cells treated with GCPLL-**  
 376 **Cur 100 µg/ml and nucleus stained in blue with DAPI, green fluorescence from**  
 377 **Curcumin, red from rhodamine and the merged image of the three channels. b)**  
 378 **Schematic representation of the uptake mechanism of GCPLL-Rhod-Cur.**

379

380 To better understand the mechanism of action of the **GCPLL-Cur** MLWVs on  
 381 HeLa cells, we couple fluorescence (Figure 4) and confocal microscopy (Figure 5,  
 382 Figures S9-15) with flow cytometry (Figure 5, Figures S16-18). Figure 4a shows three  
 383 fluorescence microscopy images, each corresponding to different channels: the nucleus  
 384 (stained with DAPI, blue), **Cur** (green) and **GCPLL** (stained with rhodamine, red). All  
 385 channels are eventually combined in a fourth image (merge).

386 Colocalization of curcumin and rhodamine (yellow) in the merge image and  
 387 presence of green curcumin around the blue nucleus demonstrate the mechanism of cell  
 388 uptake occurring in HeLa cells. **GCPLL** MLWVs labelled with rhodamine (Liss-Rhod) and  
 389 loaded with **Cur** penetrate into the cell membrane and **Cur** is eventually released within  
 390 the cytoplasm. At the same time, no red fluorescence signal alone is detected inside the  
 391 cell, meaning that **GCPLL** MLWVs fuse with the cell membrane and deliver **Cur** within  
 392 the the cytoplasm. A schematic representation of the uptake mechanism is illustrated in  
 393 Figure 4b.

394 Figures S9-15 in the supporting information show the fluorescence microscopy  
 395 images of HeLa, NHDF and THP-1 cells with the corresponding images for control, Cur  
 396 alone (with and without Liss-Rhod), **GCPLL** (and **GCPLL-Rhod**) and **GCPLL-Cur** (and  
 397 **GCPLL-Cur-Rhod**). It is worth noticing that fluorescent grains are observed within HeLa

398 cells when they are incubated with **GCPLL-Cur** (Figure S11 and S12, green) and  
399 **GCPLL-Cur-Rhod** (Figure S13, red and overlapped green and red respectively). On the  
400 opposite, a blurry and weak green fluorescence is detected when free curcumin is used  
401 (Figure S11). This shows an accumulation of curcumin within HeLa cells when GCPLL  
402 particles are used, thereby evidencing their efficacy to deliver drugs inside the cancer  
403 cells. This effect is specific as no granny fluorescence is detected in NHDF or  
404 macrophages derived THP-1 (Figure S9, S10, S14, S15).

405 Confocal microscopy imaging in Figure 5a shows images of HeLa, NHDF and  
406 THP-1 cells incubated with GCPLL-Cur-Rhod and nucleus stained with DAPI. In addition  
407 to HeLa cells, both planes yz and zx were included (Figure S19), which confirms the  
408 proposed mechanism: **GCPLL** labelled with rhodamine (red) is localized in the outer part  
409 of the cell while **Cur** (green) is released in the cytoplasm of the HeLa cells (Figure 5a)  
410 and it surrounds the nucleus (blue).

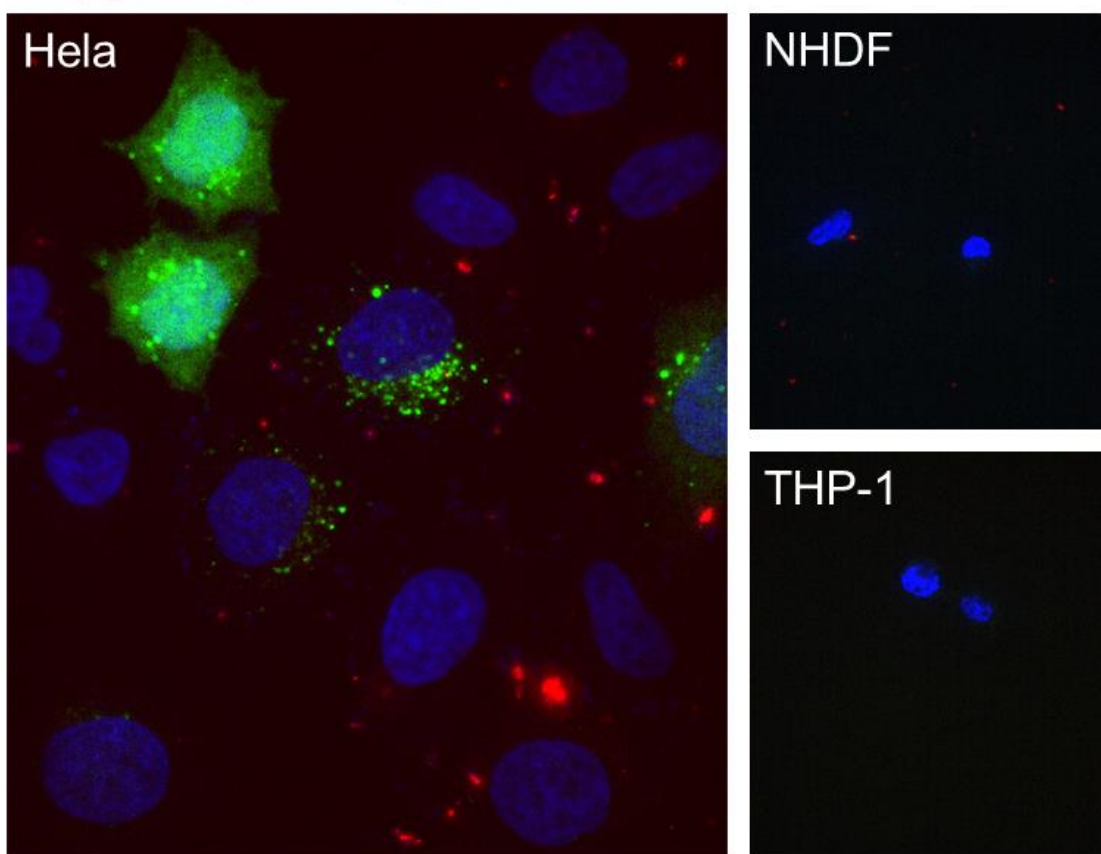
411 Flow cytometry measurements of the three different cell lines tested (THP-1,  
412 NHDF, and HeLa) are presented in Figure 5b and in the Supporting Information (Figure  
413 S16-18) and they evidence different uptake quantities of the MLWVs by measuring the  
414 intensity of rhodamine in cells. The results demonstrate that HeLa cells have the highest  
415 percentage of rhodamine labelling (41% for **GCPLL-Rhod** and 50% for **GCPLL-Rhod-  
416 Cur**), which explains the higher uptake, and therefore higher cytotoxicity, compared to  
417 both THP-1 and NHDF, which respectively show a rhodamine signal of 20% and 34%  
418 for **GCPLL-Rhod-Cur**.

419 The lipid-based particles can be uptaken by the cells following different cellular  
420 mechanisms. Nanoparticles ranging from 50-100 nm can be engulfed by endocytosis,  
421 those less than 400 nm by micropinocytosis and micrometric particles can enter the cells  
422 by phagocytosis.<sup>49</sup> The latter phenomenon is dedicated to immune cells of the  
423 reticuloendothelial system such as macrophages and neutrophils. The curcumin loaded  
424 MLWVs are poorly engulfed by macrophages, thereby showing phagocytosis is not  
425 involved in the intracellular delivery of curcumin. The MLWVs population is quite  
426 polydisperse ranging from 10 nm up to 10  $\mu$ m. A fraction of particles could be uptaken  
427 by macropinocytosis. However, no rhodamine fluorescence, evidencing the presence of  
428 micropinocytosis vesicles, is observed within the cellular cytoplasm of NHDF and HeLa  
429 cells. Hence, the major mechanism of curcumin delivery seems to be based on MLWVs  
430 fuse with the cell membrane. This fusion is much easier in dividing cells as their lipid  
431 membrane is more fluid and favors MLWVs interaction. Particle interaction with dividing  
432 cells does not seem to depend on the cell doubling time as HeLa and NHDF exhibit the  
433 same quantity of cells labeled with rhodamine, as quantified by FACS (Figure 5b).  
434 However, the quantity of curcumin delivered within HeLa cells is much larger than that in

435 normal fibroblasts (Figure 5a). Hence the process of fusion is favored by the proliferative  
436 activities of cells, which is an asset to target and kill cancer cells. Curcumin toxicity has  
437 been improved by its encapsulation in GCPLL MLWVs compared to the free form despite  
438 a smaller quantity as the loading efficiency does not exceed 60%.

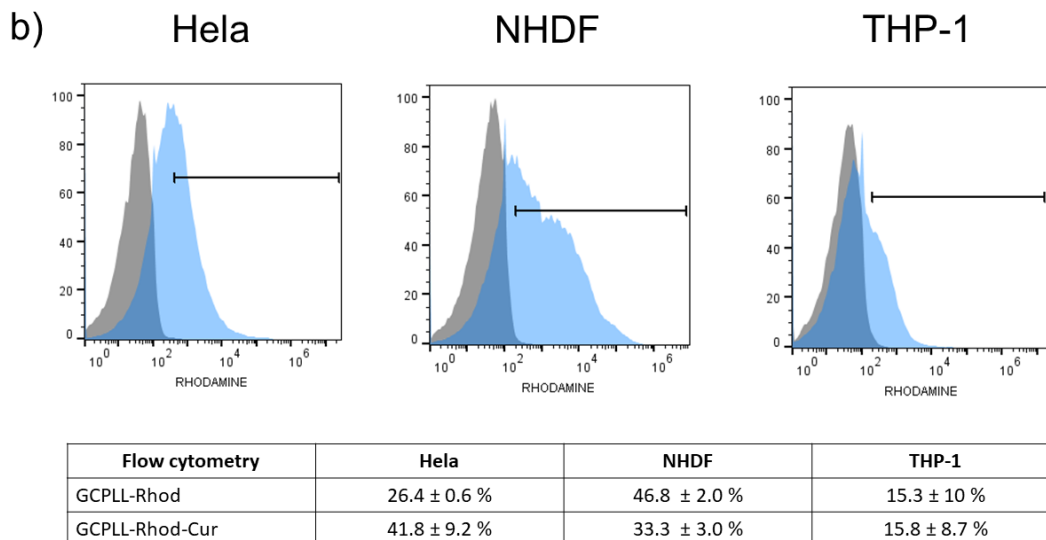
439 In addition to FACS and fluorescence microscopy, cell viability results correlate  
440 to the higher fluorescence detected of the uptaken Curcumin in Hela cells than in NHDF  
441 (Figure 5a), demonstrating a higher toxicity in Hela cells than in NHDF cells (Figure 2).

**a) GCPLL-Cur-Rhod**



442  
443





444

445 **Figure 5. a) Confocal microscopy images of HeLa, NHDF and THP-1 cells treated**  
 446 **with GCPLL-Rhod-Cur 100 µg/mL. Color code: green= Curcumin, red= rhodamine**  
 447 **–stained GCPLL MLWV, blue= cell nucleus. b) Flow cytometry FACS data of HeLa,**  
 448 **NHDF and THP-1 cells of incubated with 100 µg/mL GCPLL and GCPLL-Cur**  
 449 **labelled with Liss-Rhod. Table with mean ± SD values of triplicate of triplicates of**  
 450 **GCPLL and GCPLL-Cur both labelled with Liss-Rhod for the three cell lines tested.**

451

452 In order to extend the encapsulation feasibility of this MLWV system with a wider  
 453 variety of drugs which display a different hydrophobic character, we screened the  
 454 **GCPLL** encapsulation and cell cytotoxicity of Doxorubicin (logP = 1.41),<sup>50</sup> Paclitaxel  
 455 (logP = 3)<sup>51</sup> and Docetaxel (logP = 2.4)<sup>52</sup> in HeLa and NHDF cells (Table 2).

456 Results demonstrated the possibility to exploit the MLWVs with other  
 457 encapsulated small molecules which provoked a higher cytotoxic effect preferentially  
 458 over HeLa cells than in NHDF for all encapsulated drug types tested. In the case of  
 459 Doxorubicin (Dox), there is a great decrease in viability reaching 26.8 ± 1.6% at 250  
 460 µg/mL GCPLL-Dox, compared to empty GCPLL 89 ± 3% in HeLa cells, while in NHDF  
 461 cells there is a slighter decrease in viability being 69.2 ± 0.9% at 250 µg/mL GCPLL-Dox.  
 462 For Paclitaxel and Docetaxel, both GCPLL loaded systems at 250 µg/mL showed a cell  
 463 viability higher than 65% in NHDF cells. On the contrary, in HeLa cells the effect is again  
 464 increased compared with analogous NHDF experiments. GCPLL-Pac at 250 µg/mL is  
 465 59.3 ± 0.5% and GCPLL-Doc at 250 µg/mL is 44.3 ± 1.3%. In these last two systems, it  
 466 is observed a milder effect in the cytotoxicity compared with Doxorubicin experiments.

467

Cell viability ± SEM (%)	Hela cells		NHDF cells	
	100 µg/mL	250 µg/mL	100 µg/mL	250 µg/mL
GCPLL	93.3 ± 6.9	89.5 ± 3.0	95.9 ± 2.9	98.7 ± 1.3
GCPLL-DOX	66.9 ± 1.8	26.8 ± 1.7	81.3 ± 1.4	69.3 ± 0.9
GCPLL-PAC	98.7 ± 1.2	59.3 ± 0.6	91.6 ± 5.6	77.7 ± 6.2
GCPLL-DOC	79.5 ± 8.1	44.3 ± 1.3	85.5 ± 4.2	67.0 ± 2.0

468

469 **Table 2. Cell viability data of three different drug experiment tested in Hela and**  
470 **NHDF cells treated with prepared concentrations 100 µg/mL (50 µg/mL) and 250**  
471 **µg/mL (125 µg/mL) of: GCPLL, GCPLL-drug. Being the drugs tested: Doxorubicin**  
472 **(DOX), Paclitaxel (PAC) and Docetaxel (DOC). Table with mean ± SEM values of**  
473 **triplicate of triplicates.**

474

#### 475 **Conclusions**

476 This work demonstrates that microbial amphiphile biosurfactants can be  
477 used as drug carriers in the absence of phospholipid addition. Control over the  
478 self-assembly properties of microbial glucolipid **GC** in the presence of polylysine  
479 **PLL** shows the possibility to form **GCPLL** vesicle colloids with multilamellar wall  
480 (MLWVs), both in water and DMEM culture medium. **GCPLL** particles are stable  
481 at physiological pH, as their multilamellar structure is probed both by *in situ* SAXS  
482 measurements and polarized light microscopy.

483 With the aim of evaluating **GCPLL** as novel drug delivery system,  
484 curcumin, used as lipophilic drug model have been efficiently encapsulated within  
485 **GCPLL**. Curcumin loaded **GCPLL** (encapsulation efficiency of 60 %) shows a  
486 greater therapeutic effect towards cancer Hela cells compared to free curcumin,  
487 with a decrease of Hela viability by 50 % for an encapsulated content of 3 µg/mL  
488 **GCPLL-Cur**. Beside, this decrease is only of 25% the free curcumin  
489 concentration at 5 µg/mL. In addition, this cytotoxic impact is exclusive to cancer  
490 cells, as no significant effect is observed on normal human dermal fibroblasts  
491 NHDF and THP-1 derived macrophages. This suggests that **GCPLL-Cur** MLWVs  
492 would avoid side effects. In addition, this system would increase the circulation  
493 time of therapeutic drugs in the bloodstream since **GCPLL** are not engulfed by  
494 macrophages, cells in charge of the clearance of foreign particles *in vivo*. Last,  
495 analyses by confocal microscopy and flow cytometry show that curcumin is  
496 delivered within the cell by **GCPLL-Cur** fusion with the Hela membrane.

497 Taken together, these results demonstrate that vesicle-forming  
498 biosurfactant amphiphiles, glucolipids in this work, but possibly rhamnolipids,  
499 sophorolipid-derivatives or surfactin, can be employed as novel multilamellar  
500 vesicular systems for drug delivery, thus broadening the use of this class of  
501 compounds to more high-end applications.

502

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# Supporting Information

## Glycolipid Biosurfactant as Multilamellar Vesicular Drug Carriers

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## Experimental

### *Materials*

Microbial glycolipids G-C18:1 are made of a single  $\beta$ -D-glucose hydrophilic headgroup and a C18 fatty acid tail (monounsaturations in positions 9,10). The synthesis of glycolipid G-C18:1 is described in Ref. <sup>1</sup>, where the typical <sup>1</sup>H NMR spectra and HPLC chromatograms are given. The compound used in this work has a molecular purity of more than 95%. Poly-L-lysine (PLL) hydrobromide ( $M_w \approx 1-5$  kDa,  $pK_a \sim 10-10.5$ )<sup>2</sup> is purchased from Sigma-Aldrich. All other chemicals are of reagent grade and are used without further purification. Curcumin (Cur) is purchased from Sigma-Aldrich without further purification. 18:1 Liss Rhod PE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss-Rhod) ( $M_w = 1301.7$  g/mol,  $\lambda_{abs} = 560$  nm,  $\lambda_{em} = 583$  nm) is purchased from Avanti lipids. Lipopolysaccharides (LPS), Phorbol 12-myristate 13-acetate (PMA), paraformaldehyde (PFA), Docetaxel, Paclitaxel and Doxorubicin are purchased from Sigma Aldrich-Merck. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) is purchased from Life Technologies-ThermoFisher Scientific.

### *Cell culture*

L929 (mouse fibroblast) cells (Merck), HeLa (human cervical carcinoma) cells and NHDF (Normal human dermal fibroblast) (Merck) cells are cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and 1% Amphotericin B. THP-1 (human monocyte) (Promocell) cells are cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and 1% Amphotericin B. Cells are cultivated at 37°C and 5% CO<sub>2</sub> under 100% humidity.

### *Preparation G-C18:1-PLL (GCPLL) multilamellar wall vesicles (MLWVs)*

**GCPLL** MLWVs are prepared according to previous work.<sup>3,4</sup> Stock solutions are prepared by dissolving 5 mg of **GC** or PLL in 1 mL of DMEM cell culture medium supplemented with 10% FBS. Both solutions are raised to pH 10 with NaOH 1 M, a step necessary to solubilize **GC** (micellar phase), and mixed in a 1:1 volume ratio, followed by vortexing. The final concentration of **GC** and **PLL** is 2.5 mg/mL. pH is then lowered to 7 with HCl 1 M to trigger MLWVs formation. The solution is slightly cloudy at pH 7, confirming the presence of MLWVs colloids.<sup>3,4</sup>



### *Encapsulation of curcumin (**Cur**) in GCPLL (**GCPLL-Cur**)*

After the formation of **GCPLL** MLWVs in cell culture medium at pH 7 (once the solution becomes cloudy), an aliquot (10  $\mu$ L) of **Cur** from 13.5 mM stock solution prepared in absolute ethanol is added to 1 mL of GCPLL solution to reach the final concentration 135  $\mu$ M. After vortexing, the suspension is centrifuged at 3000 rpm for 5 min to collect a pellet of **GCPLL-Cur** MLWVs and remove the excess, non-encapsulated, **Cur** in the supernatant. The pellet is resuspended in fresh cell culture medium by vortexing.

### *Encapsulation of Doxorubicin, Paclitaxel and Docetaxel*

As described for Curcumin in the previous paragraph, an aliquot (10  $\mu$ L) of drug (Doxorubicin: 1 mg/ml in DMSO; Paclitaxel: 0.1 mg/ml in ethanol; Docetaxel 0.1 mg/ml in ethanol) were added to **GCPLL** MLWVs following the same protocol of centrifugation and resuspension in fresh cell culture medium by vortexing.

### *Characterization of the drug loading*

The loading capacity (LC %) expressed as a percentage is the ratio: quantity of encapsulated drugs (**Cur**) over the total amount of the delivery vehicles, in this case the weight of **GCPLL** MLWVs.

The encapsulation efficiency (EE %) is calculated by dividing the amount of encapsulated drug by the total amount of drug used during the encapsulation process, expressed as a percentage.

### *Labelling of **GCPLL** and **GCPLL-Cur** with rhodamine (**GCPLL-Rhod** and **GCPLL-Rhod-Cur**)*

Both **GCPLL** and **GCPLL-Cur** MLWVs are prepared as described above. To their corresponding solution, an aliquot of 10  $\mu$ L of 18:1 Liss-Rhod PE in ethanol (4 mg/mL, 3.08 mM) is added to the mixture solution so that the molar **GC**:rhodamine ratio is 200:1. After vortexing, the solution is centrifuged at 3000 rpm for 5 min and the pink pellet is resuspended in fresh cell culture media. 18:1 Liss-Rhod PE is a standard dye labelling lipid bilayers as its lipid backbone intercalates in the lipid bilayer without any perturbation, when the lipid:dye molar ratio  $\geq 100$ .<sup>5,6</sup>

### *Small Angle X-Ray Scattering (SAXS)*

pH-resolved *in situ* SAXS experiments are performed at room temperature on the Swing beamline at Soleil Synchrotron (Saint-Aubin, France) during the proposal N° 20190961. The beam energy is  $E = 12$  keV and the sample-to-detector (Eiger X 4M)

distance is 1.995 m. Silver behenate ( $d(100) = 58.38 \text{ \AA}$ ) is used as standard to calibrate the  $q$ -scale. Raw data collected on the 2D detector are integrated azimuthally using the in-house Foxtrot software, provided at the beamline and so to obtain the typical scattered intensity  $I(q)$  profile, with  $q$  being the wavevector ( $q = (4\pi \sin \theta) / \lambda$ , where  $2\theta$  is the scattering angle and  $\lambda$  is the wavelength). Defective pixels and beam stop shadow are systematically masked before azimuthal integration. Absolute intensity units are determined by measuring the scattering signal of water ( $I_{\text{H}_2\text{O}} = 0.0163 \text{ cm}^{-1}$ ). SAXS profiles are processed with SasView software, version 3.1.2, available at the developer's website (sasview.org).

The experimental setup is reproduced from the Ref. <sup>4</sup> as follows. The sample solution ( $V = 1 \text{ mL}$ ,  $C_{\text{GC}} = C_{\text{PLL}} = 2.5 \text{ mg/mL}$ ) in DMEM and  $\text{pH} \sim 11$  is contained in an external beaker under stirring at room temperature ( $T = 23 \pm 2^\circ\text{C}$ ). The solution is continuously flushed through a 1 mm glass capillary using an external peristaltic pump. The pH of the solution in the beaker is changed using an interfaced push syringe, injecting microliter amounts of a 0.5 M HCl solution. pH is measured using a micro electrode (Mettler-Toledo) and the value of pH is monitored live and manually recorded from the control room via a network camera pointing at the pH-meter located next to the beaker in the experimental hutch. Considering the fast pH change kinetics, the error on the pH value is  $\pm 0.5$ .

#### *Polarized Light Microscopy (PLM)*

PLM images are obtained with a transmission Zeiss AxioImager A2 POL optical microscope. A drop of the given sample solution was deposited on a glass slide covered with a cover slip. The microscope is equipped with a polarized light source, crossed polarizers and an AxioCam CCD camera.

#### *<sup>1</sup>H solution nuclear magnetic resonance (NMR)*

<sup>1</sup>H solution NMR experiments of the various samples are recorded on an AVANCE III Bruker 300 NMR spectrometer using standard pulse programs and a 5 mm <sup>1</sup>H-X BBFO probe. The number of transients is 32 with 7.3 s recycling delay, an acquisition time of 2.73 s, and a receiver gain of 322. Chemical shifts are reported in parts-per-million ( $\delta$ , ppm) and referenced to the 3-(Trimethylsilyl)propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (TMSP-d<sub>4</sub>) (Sigma-Aldrich) peak at 0 ppm at 1 mg/mL (5.8 mM). All samples are prepared by the protocol described above. We have employed a 5 mm NMR tube containing 500  $\mu\text{L}$  of solution. This solution is obtained by solubilizing the pellet of MLWVs GCPLL in MeOD. This pellet is obtained by centrifugation during 5 min at 3000 rpm. The signals that have been used for calculations are:  $\delta (\text{PLL}) = 2.8 \text{ ppm (t)}$  and  $\delta$

(GC) = 2.25 ppm (t). All experiments were performed under the same conditions. (Figures S2-S5).

#### *Dynamic light scattering (DLS) and Zeta potential measurements*

Dynamic light scattering measurements (DLS) DLS experiments are performed using a Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 4 mW He–Ne laser at a wavelength of 633 nm. Measurements were made at 25 °C with a fixed angle of 90° and three acquisitions per sample.

#### *Cell viability assay*

The impact of GCPLL on cell viability is first assessed using the cell line L929 of mouse fibroblasts to determine the optimal dose enabling optimal cell viability. For this purpose,  $5 \times 10^4$  cells/mL are seeded in wells of a 24 well plate and cultivated for 24 hours. Then, different GCPLL MLWV concentrations up to 250 µg/ml (when samples are centrifuged, the maximum concentration administered is 122.5 µg/ml, based on NMR data, as discussed in the text) are added to the fibroblasts L929. Cells are cultivated with GCPLL for another 24-hour period. Besides, fibroblasts are also cultivated in presence of free G-C18:1, PLL and DMEM impacted by pH changes. L929 fibroblasts cultivated in complete medium are used as control samples.

Cell viability is determined by measuring the metabolic activity using Alamar Blue assay. Basically, GCPLL particles are taken out from the wells and L929 cells are rinsed twice with fresh medium. Then, 300 µL of a resazurin solution at 0.01 % (w/v) in colourless fresh DMEM medium is added to cells and incubated for 4 hours. The supernatant in each well is then collected, diluted with 700 µL of fresh medium, and the absorbance measured at  $\lambda = 570$  nm and  $\lambda = 600$  nm. The percentage of resazurin reduction is calculated following the formula provided by the supplier. Cell metabolic activity of the samples is compared to control samples. The arbitrary value 100 % is given to controls.

After determination of the optimal dose, the antiproliferative activity of GCPLL, GCPLL-Cur and Cur is assessed for 3 different human cell lines: HeLa, Normal Human Dermal Fibroblasts (NHDF) and macrophage derived THP-1 cells.

HeLa and NHDF are seeded 24 hours prior to the experiment in 24-well plates with a density of  $5 \times 10^4$  cells/ml and grown under standard conditions (DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% Anphotericin B at 37°C, 5% CO<sub>2</sub> and 90% humidity). Non adherent THP-1 cells are seeded at a density of  $4 \times 10^5$  cells/mL in wells and incubated for 24 hours with PMA 10 µg/mL in deprived RPMI 1640 medium to differentiate them into adherent macrophage

like cells. Then, the cell culture media was replaced by fresh complete RPMI 1640. After 24 hours in culture, the different concentrations of particles are administered to the cells and cells cultured for another 24h. The Alamar Blue colorimetric assay is used for cell viability evaluation for all types of cells as previously described.

#### *Optical fluorescence microscopy*

5 x10<sup>4</sup> cells/well are seeded into a 6 well-plate and grown under standard conditions as previously described. After 24 h, solutions of GCPLL and GCPLL-Cur, analogously GCPLL-Rhod and GCPLL-Rhod-Cur are added at the final concentration of 100 µg/mL and incubated for another 24 h period. Then, samples are rinsed 3 times with PBS, fixed for 1 hour by adding 1 mL of 4% PFA in PBS. After 3 rinses with PBS, cells are incubated for 15 min in a solution of PBS-Tween at 0.2% to permeabilize them. Then, 300 µL/well of DAPI (1/50000) are added and cells incubated for 10 min. Last cells are rinsed 3 times with PBS and kept at 4°C protected from the light until analysis. Cells are observed using a ZEISS fluorescence microscope, equipped with camera AxioCam MRm.

#### *Flow Cytometry Analysis*

1x10<sup>5</sup> cells/well are seeded into a 6 well-plate, cultivated for 24 h, then treated with MLWV particles using a concentration of 100 µg/mL and incubated for another 24 h. Samples for Facs analysis are obtained by detaching cells with 300 µL/well of trypsin for approximately 5 min, and collected within 1 mL of cell culture medium in microfuge tubes. Detached cells are collected in tubes and are centrifuged at 300 g for 5 min. After supernatant removal, the pellet is resuspended in 1 mL of PBS containing 0.5% paraformaldehyde (PFA).

Flow Cytometry is performed on a CELESTA SORP flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Acquisition gate is set to record 10<sup>5</sup> events total for each sample.

#### *Confocal laser scanning microscopy*

Samples are prepared using the same protocol than that for regular fluorescence microscopy, except cells are cultured in a Thermo Scientific™ Nunc™ Lab-Tek™ II Chamber Slide™ with 2 wells for an optimal visualization.

Analyses are performed in a spinning-disk head X1 (Yokogawa) mounted on a Nikon Eclipse Ti inverted microscope. Cells are observed with a 60x/1.4 Plan Apo objective and a Hamamatsu Orca Flash SCMOS camera.

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- 4 C. Seyrig, G. Kignelman, W. Thielemans, P. Le Griel, N. Cowieson, J. Perez and N. Baccile, *Langmuir*, 2020, **36**, 8839–8857.
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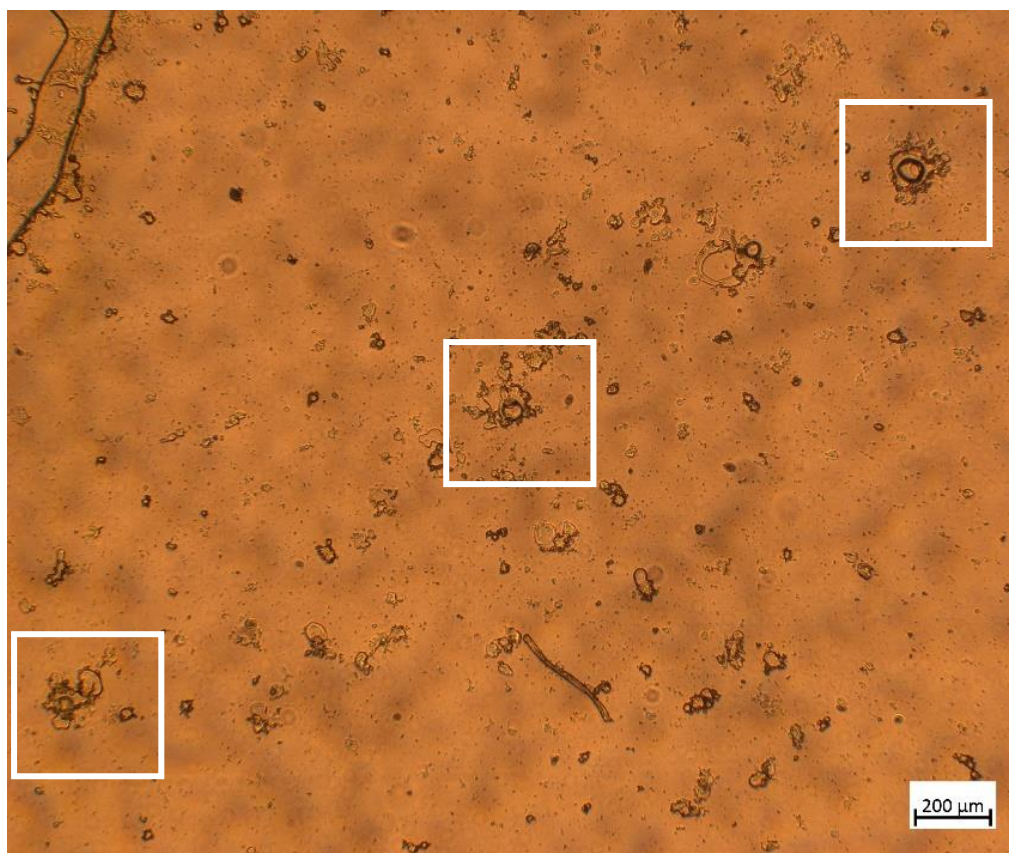


Figure S1. PLM images of GCPLL 2.5 mg/mL in DMEM cell culture media containing birefringent patterns on the surface evidenced by rotating the polarizers from 45°–135° (b) to 0–90°(c).

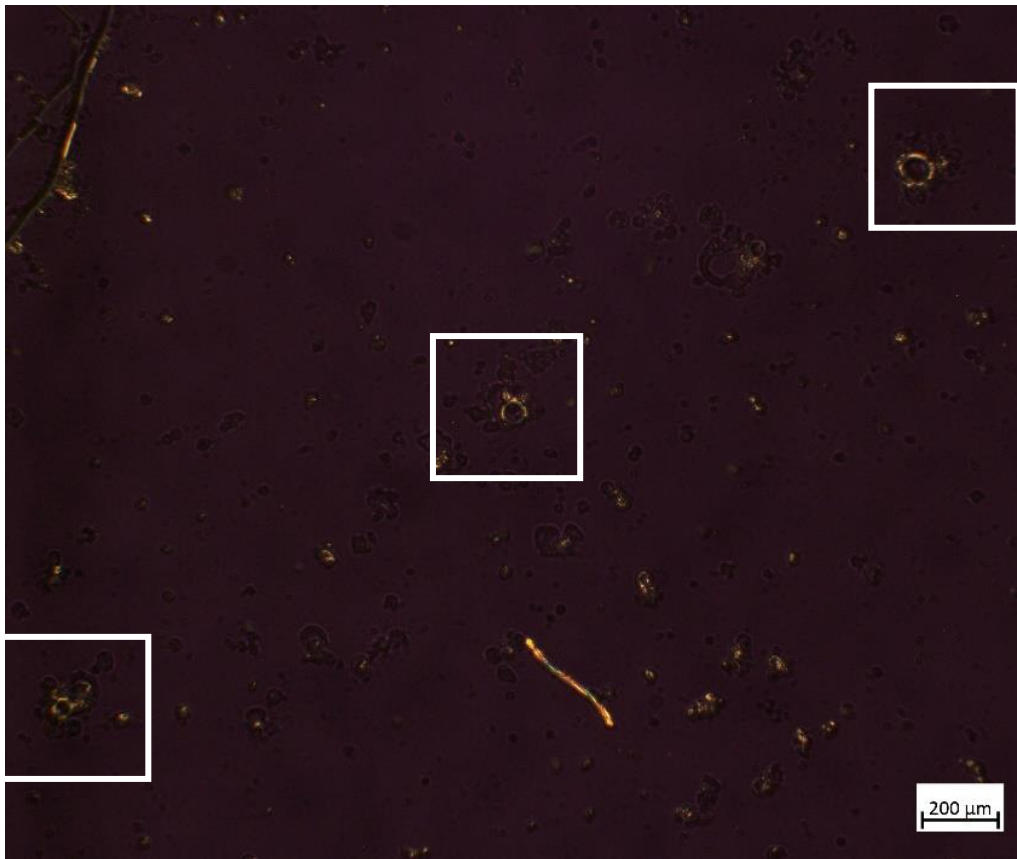
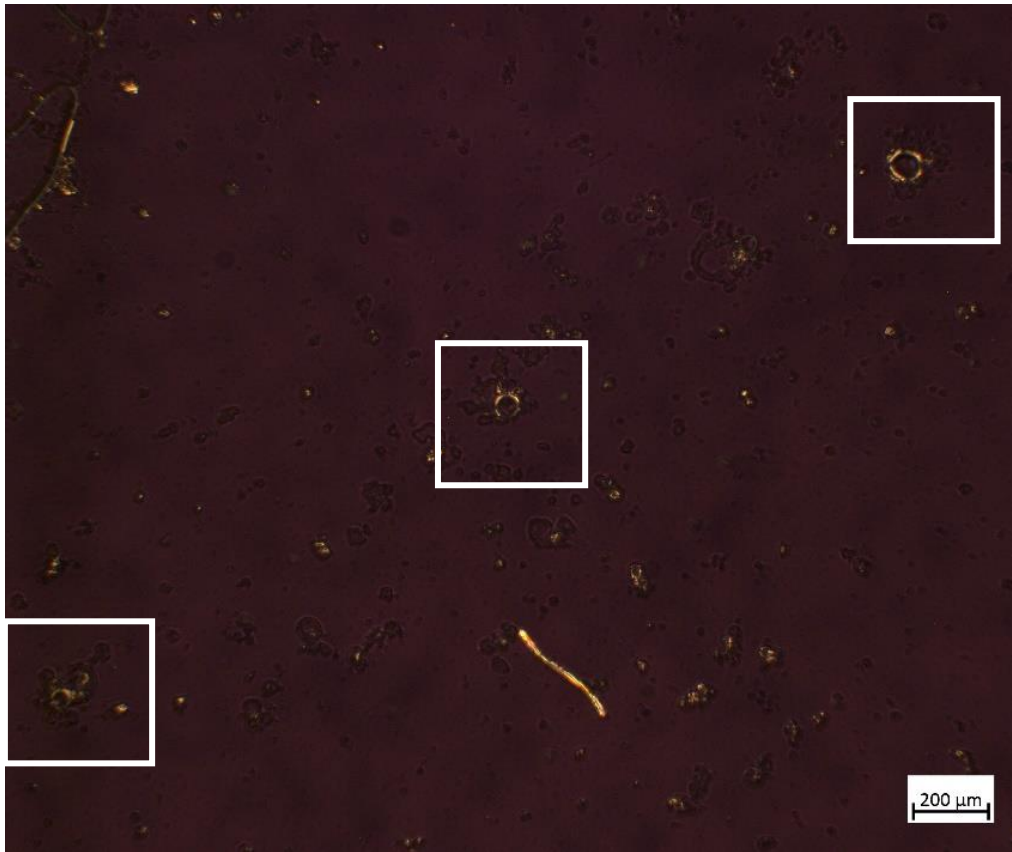


Figure S1 *continued*. PLM images of GCPLL 2.5 mg/mL in DMEM cell culture media containing birefringent patterns on the surface evidenced by rotating the polarizers from 45°–135° (b) to 0–90°(c).

<sup>1</sup>H NMR

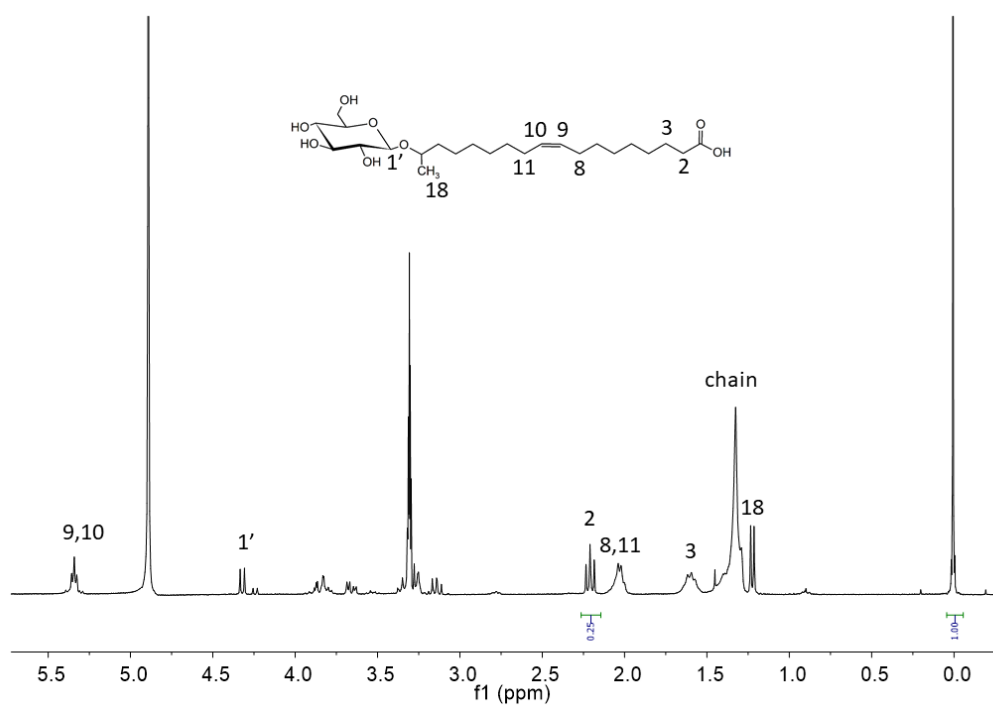


Figure S2. <sup>1</sup>H NMR of control GC in methanol-d<sub>4</sub>.



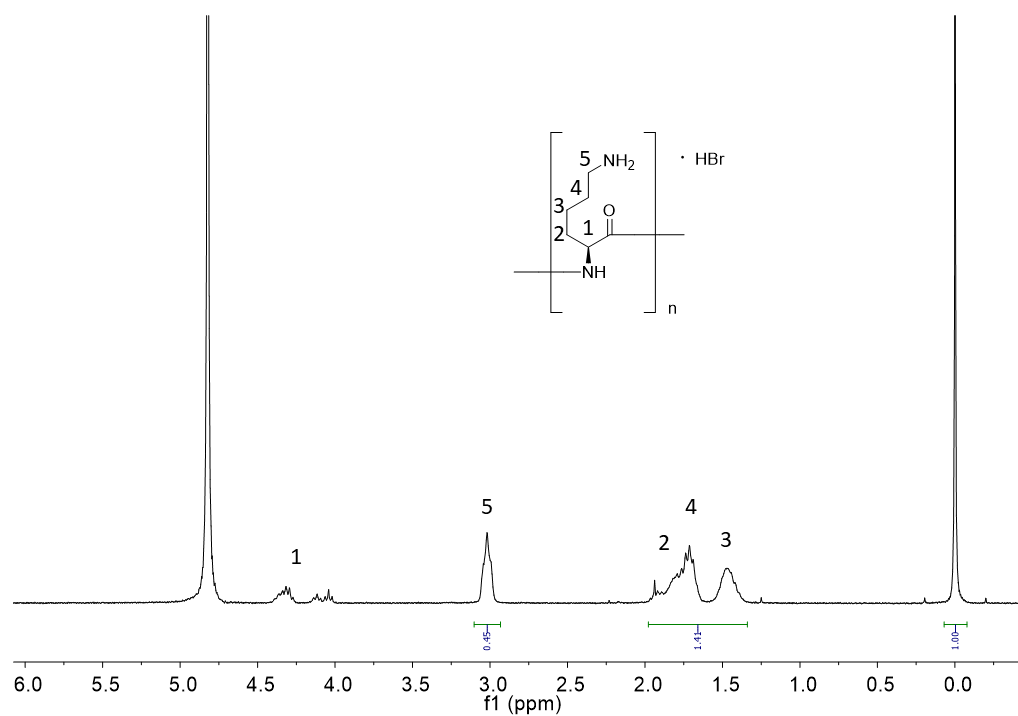


Figure S3. <sup>1</sup>H NMR of control PLL in D<sub>2</sub>O.

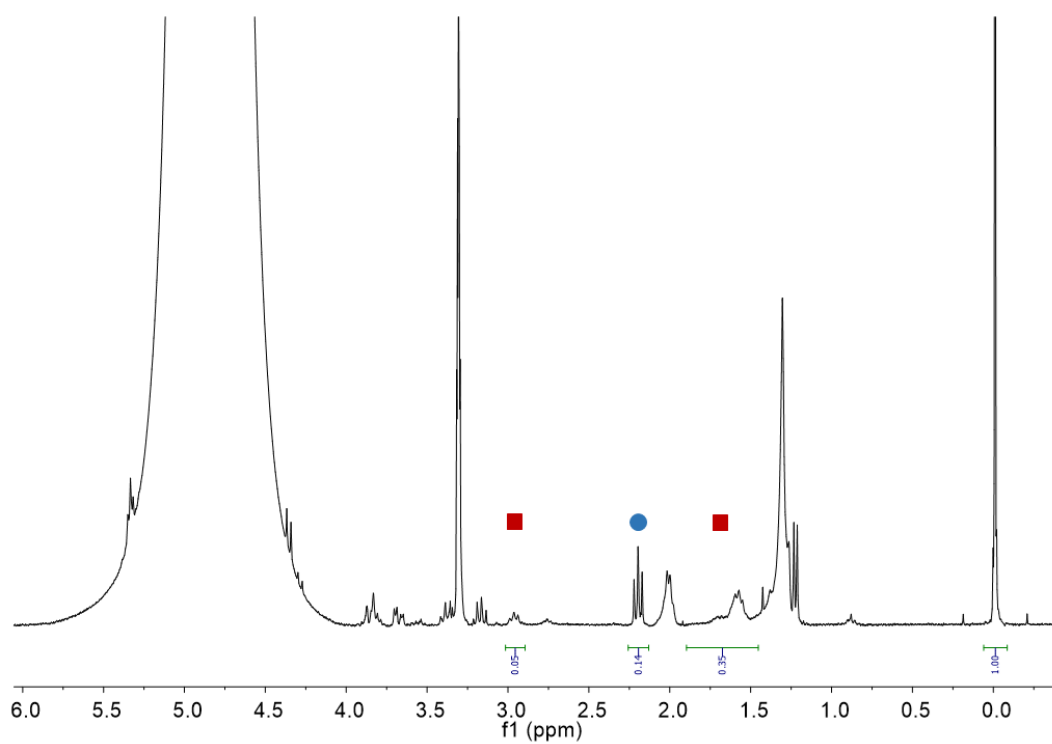


Figure S4. <sup>1</sup>H NMR from sample GCPLL in H<sub>2</sub>O, centrifuged pellet and dissolved in methanol-d<sub>4</sub>, where peaks assigned with ■ and ● correspond to PLL and GC, respectively.

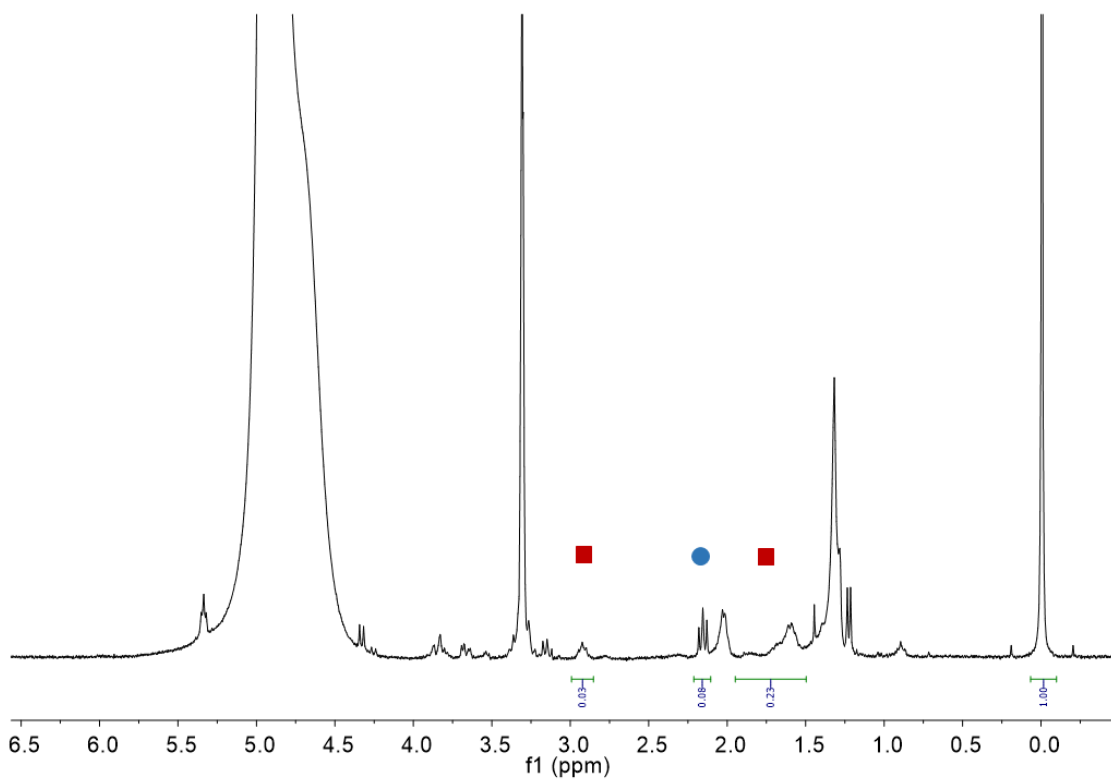


Figure S5  $^1\text{H}$  NMR from sample GCPLL in DMEM, centrifuged and dissolved in methanol- $\text{d}_4$ , where peaks assigned with  $\blacksquare$  and  $\bullet$  correspond to PLL and GC, respectively.

**Table S1.** Quantitative analysis of the integrals corresponding to the  $^1\text{H}$ NMR spectra of the GCPLL prepared in  $\text{H}_2\text{O}$  (pH 5) and DMEM cell culture media (pH 7.5) and the resulting pellet dissolved in  $\text{MeOD-d}_4$ , shown in Figures S2-5. PLL is represented by the  $(\text{RCH}_2\text{NH}_2)_x$  (where  $x \sim 20$ ) peak at  $\delta = 2.8$  ppm. The  $M_w$  (PLL)  $\approx 1\text{-}5\text{ kDa}$ , then we consider an average  $M_w$  (PLL)  $= 2.5\text{ kDa}$ , whereas the  $M_w$  of each monomer is  $128\text{ g/mol}$ , yielding an average of 20 monomers per PLL chain. The valence of the  $(\text{RCH}_2\text{NH}_2)_x$  ( $x \sim 20$ ) peak is then taken as 40. G-C18:1 is represented by the  $\text{RCH}_2\text{C}=\text{O}$  peak at  $\delta = 2.2\text{ ppm}$ . The  $M_w$  (G-C18:1)  $= 460\text{ g/mol}$  and each G-C18:1 bears a single COOH group. The valence of the  $\text{RCH}_2\text{C}=\text{O}$  peak is then taken as 2. The peak at  $\delta = 0$  ppm corresponds to the reference ( $\text{TMSP-d}_4$ ,  $1\text{ mg}\cdot\text{mL}^{-1} \equiv 5.8\text{ mM}$ ), having a valence of 9.

	Integrals			$C_{\text{initial}}$ (mM)		$C_{\text{final}}$ (mM)		$C_{\text{F}}/C_{\text{In}}$ (%)		Molar ratio		Functional group
	GC (2H)	PLL (40H)	TMSP-d4 (9H)	$[\text{GC}]_{\text{in}}$	$[\text{PLL}]_{\text{in}}$	$[\text{GC}]_{\text{f}}$	$[\text{PLL}]_{\text{f}}$	$\text{GC}_{\text{f/in}}$	$\text{PLL}_{\text{f/in}}$	$\text{GC}_{\text{in}}/\text{PLL}_{\text{in}}$	$\text{GC}_{\text{f}}/\text{PLL}_{\text{f}}$	$[\text{COOH}]/[\text{NH}_2]$
$\text{H}_2\text{O}$	0.14	0.05	1	5.4	1	3.7	0.065	70	6.5	5.4	57	2.8
DMEM	0.09	0.03	1	5.4	1	2.4	0.04	45	4	5.4	60	3

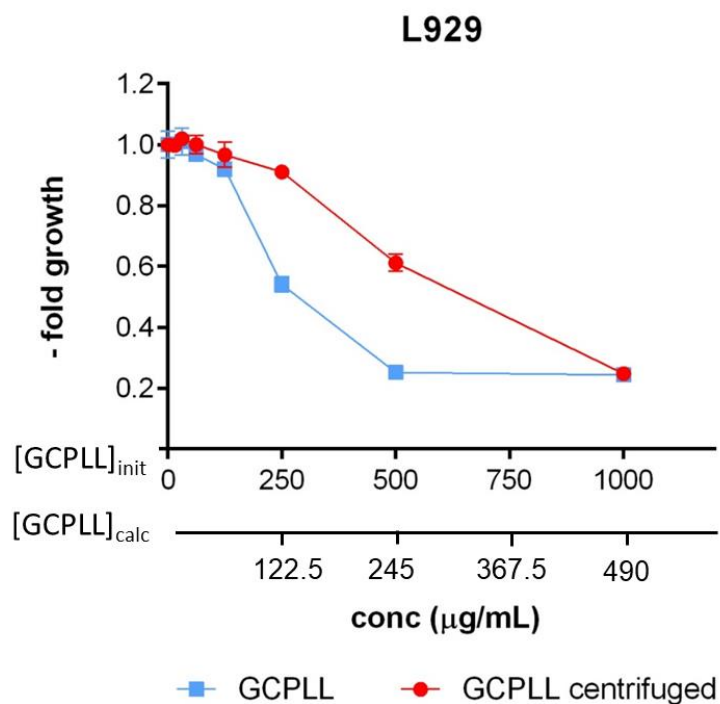


Figure S6. Cell viability comparison of both GCPLL and GCPLL centrifugated at 3000 rpm during 5 min and resuspended with new cell culture media on L929 fibroblasts mouse cell line. [GCPLL]<sub>calc</sub> refers to the concentration of GCPLL centrifuged calculated by 1H NMR described in Table S1.

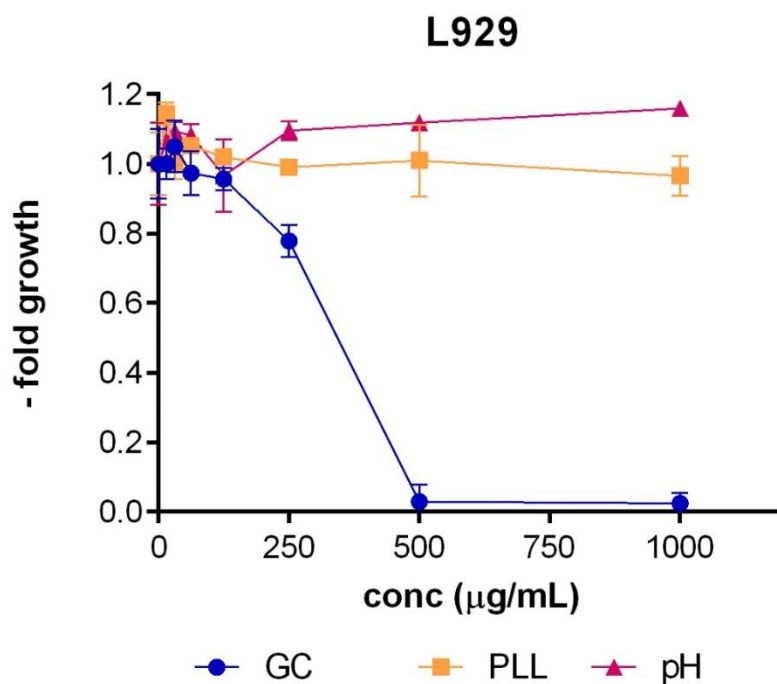


Figure S7. Cell viability of DMEM cell culture medium altered with changes in pH, glucolipid GC18:1 (GC) and poly-L-lysine (PLL) on L929 fibroblasts mouse cell line.

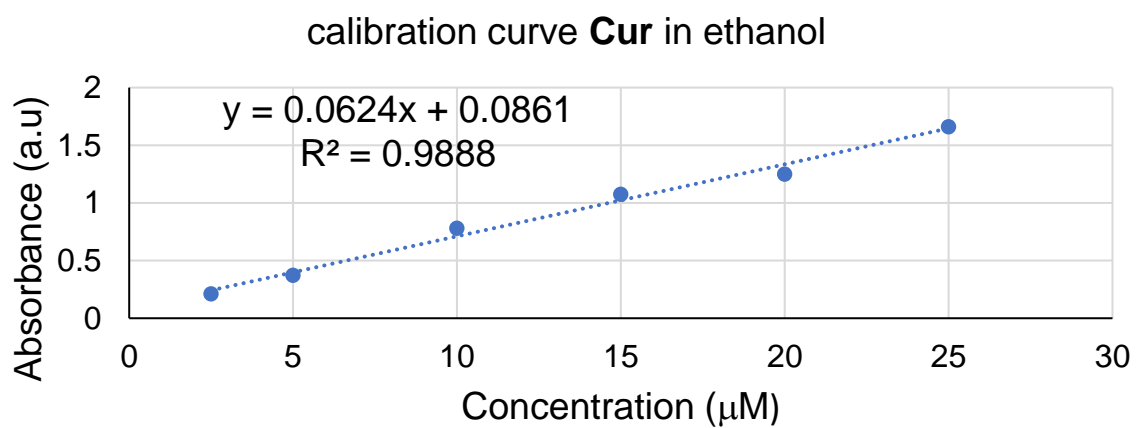
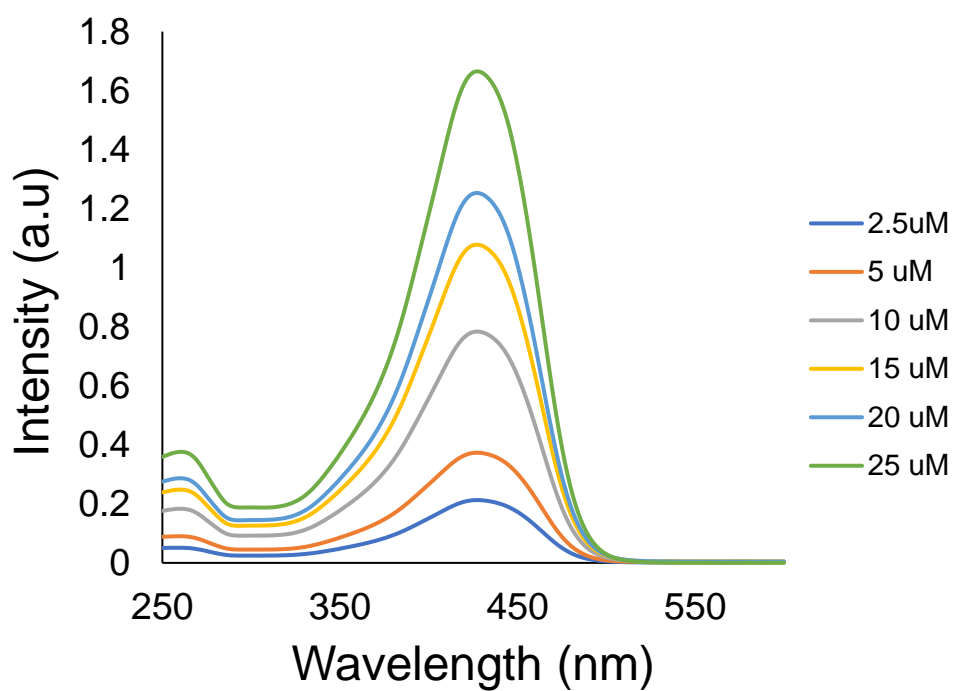


Figure S8. UV-Vis spectra of the calibration curve of Curcumin in ethanol.

*Fluorescence Microscopy*

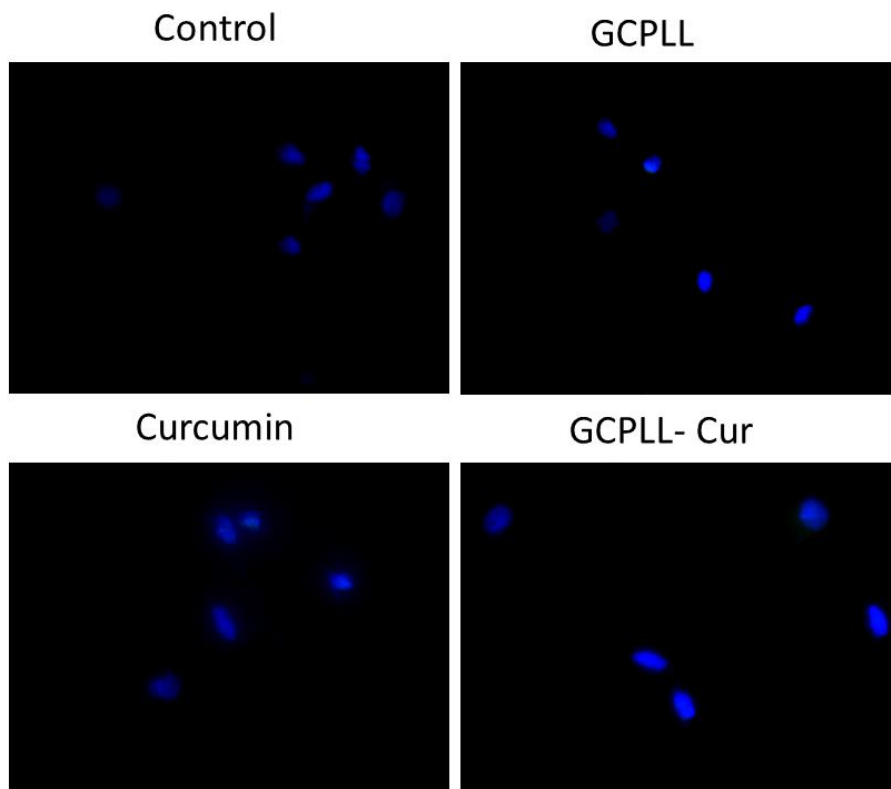


Figure S9. Fluorescence microscopy of **NHDF** cells stained with DAPI dye for nucleus.

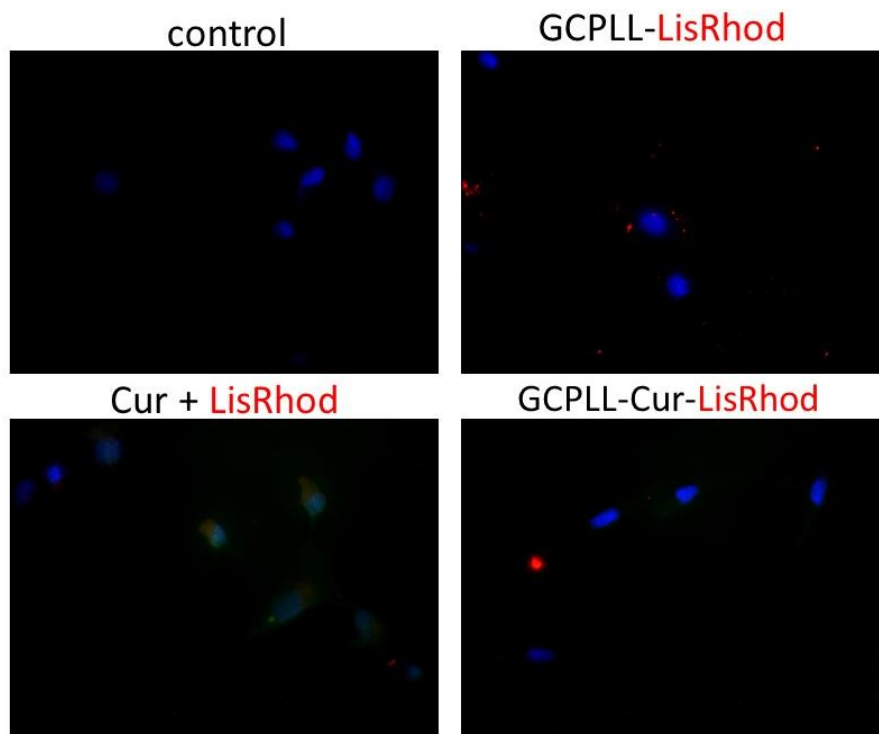


Figure S10. Fluorescence microscopy of **NHDF** cells stained with DAPI dye for nucleus and GCPLL labelled with Rhodamine-lipid (Liss-Rhod).

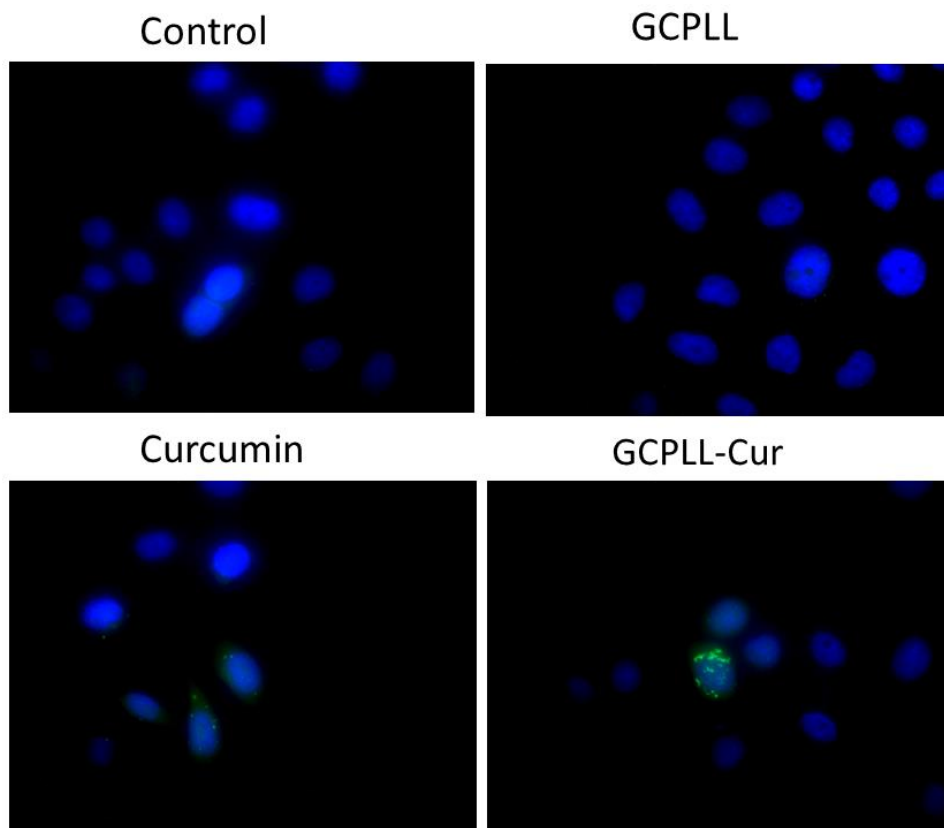


Figure S11. Fluorescence microscopy of **HeLa** cells stained with DAPI dye for nucleus.

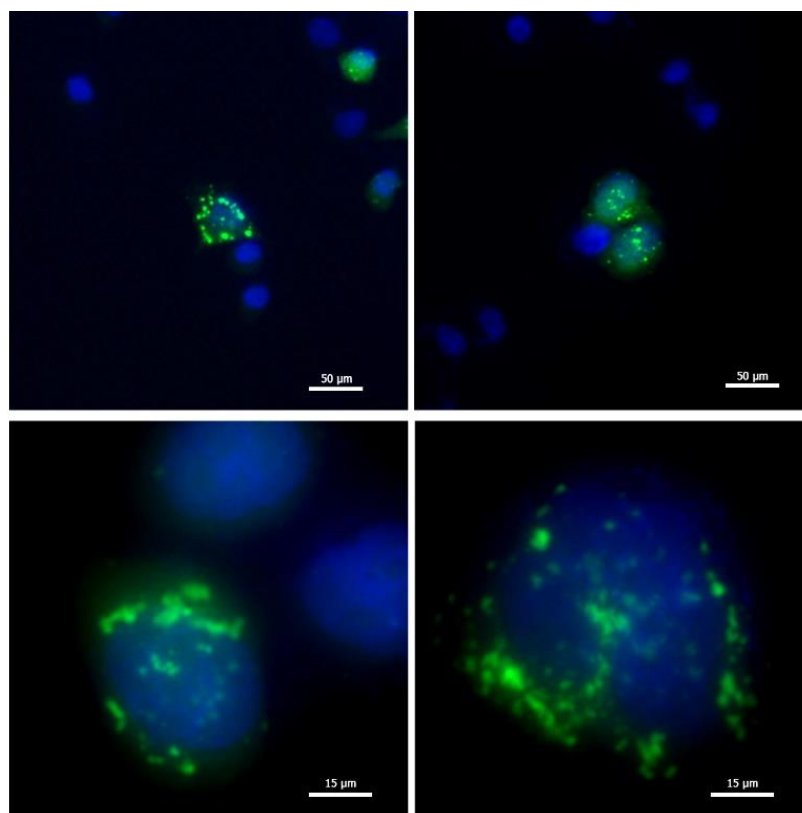


Figure S12. Fluorescence microscopy zoom images of **HeLa** cells stained with DAPI dye for nucleus after administration with GCPLL-Cur.

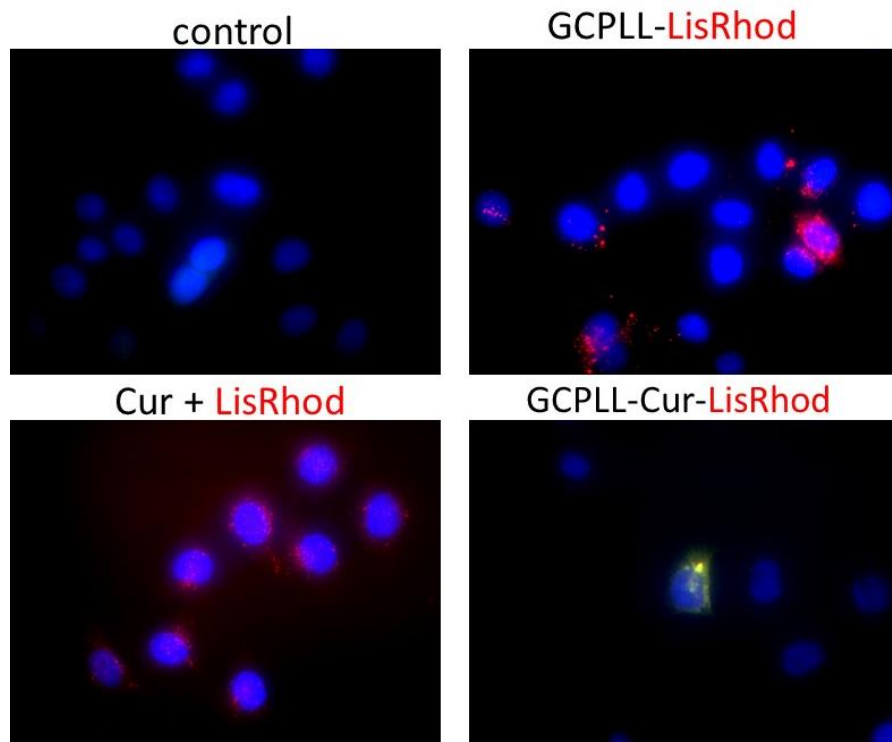


Figure S13. Fluorescence microscopy of **HeLa** cells stained with DAPI dye for nucleus and GCPLL labelled with Rhodamine-lipid (Liss-Rhod).

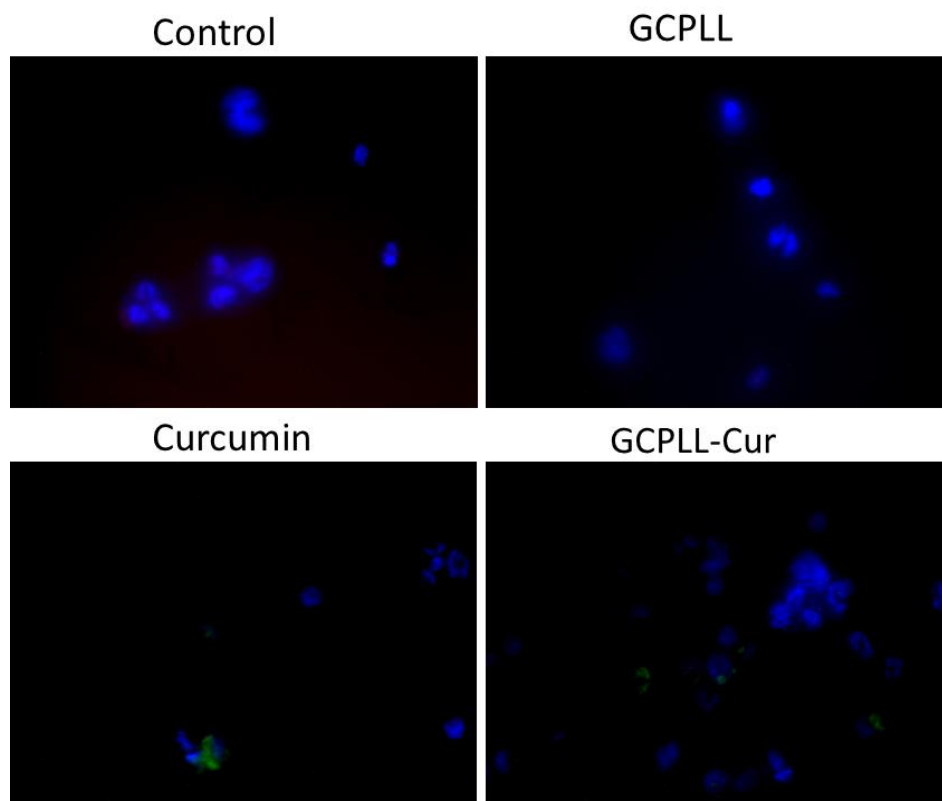


Figure S14. Fluorescence microscopy of **THP-1** cells stained with DAPI dye for nucleus.



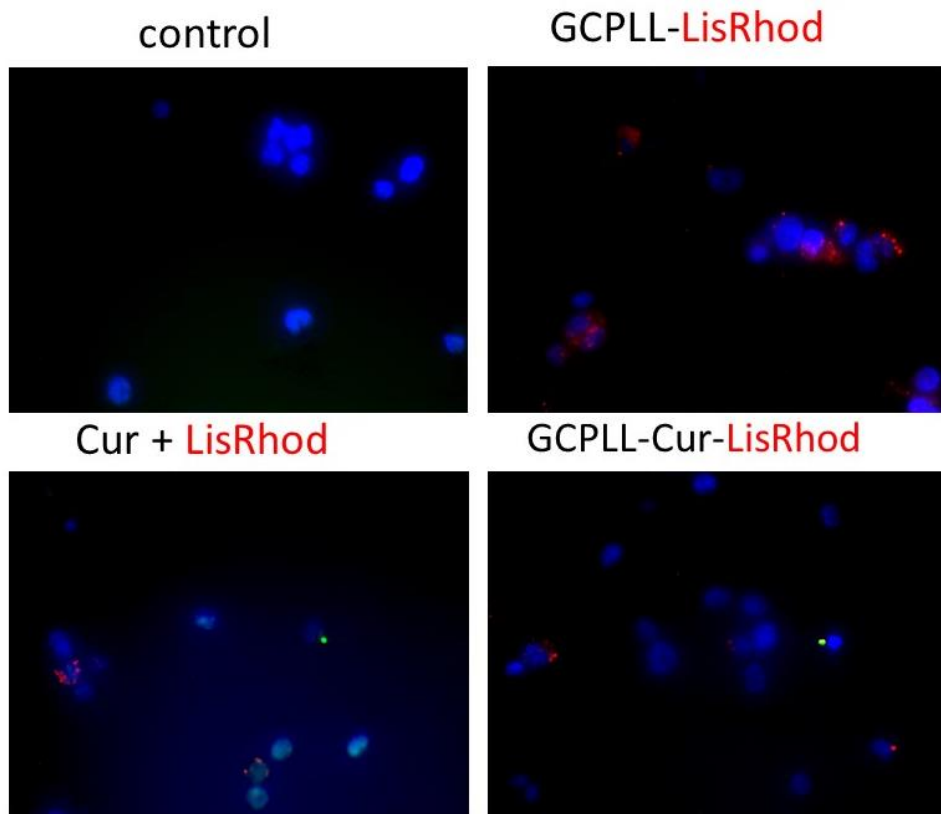
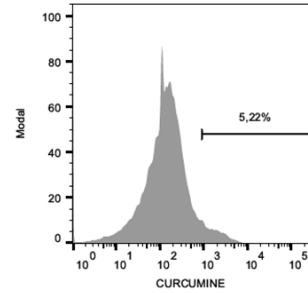
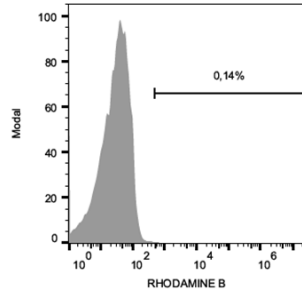
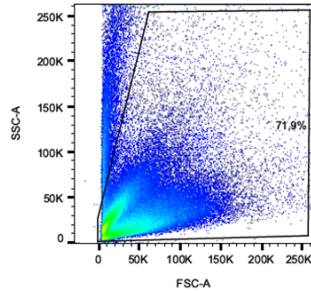


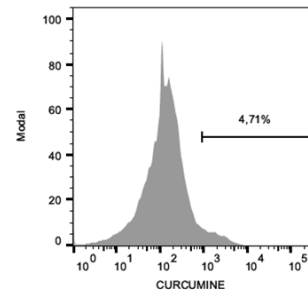
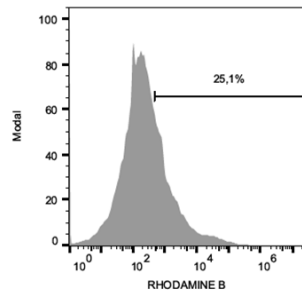
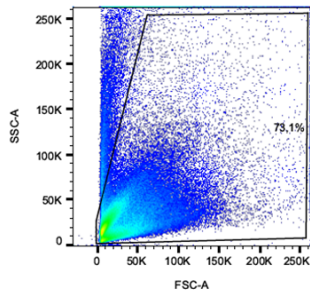
Figure S15. Fluorescence microscopy of **THP-1** cells stained with DAPI dye for nucleus and GCPLL labelled with Rhodamine-lipid (Liss-Rhod).

# Flow Cytometry FACS

HeLa  
blank



HeLa  
GCPLL-Rhod



HeLa  
GCPLL-Rhod-Cur

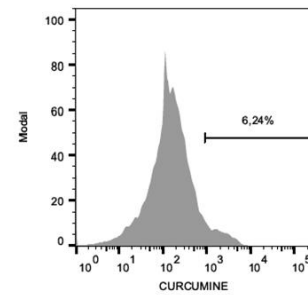
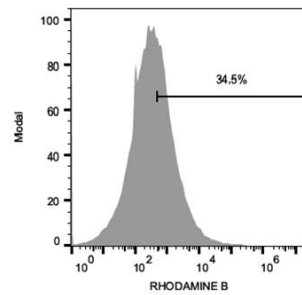
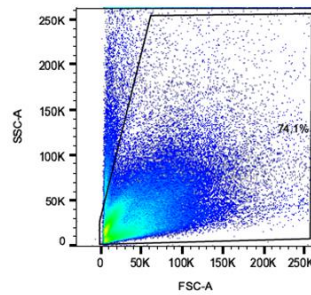


Figure S16. Flow cytometry raw data examples of the triplicate experiments with 3 replicates each one in HeLa cells, for: blank; GCPLL-Rhod and GCPLL-Rhod-Cur.

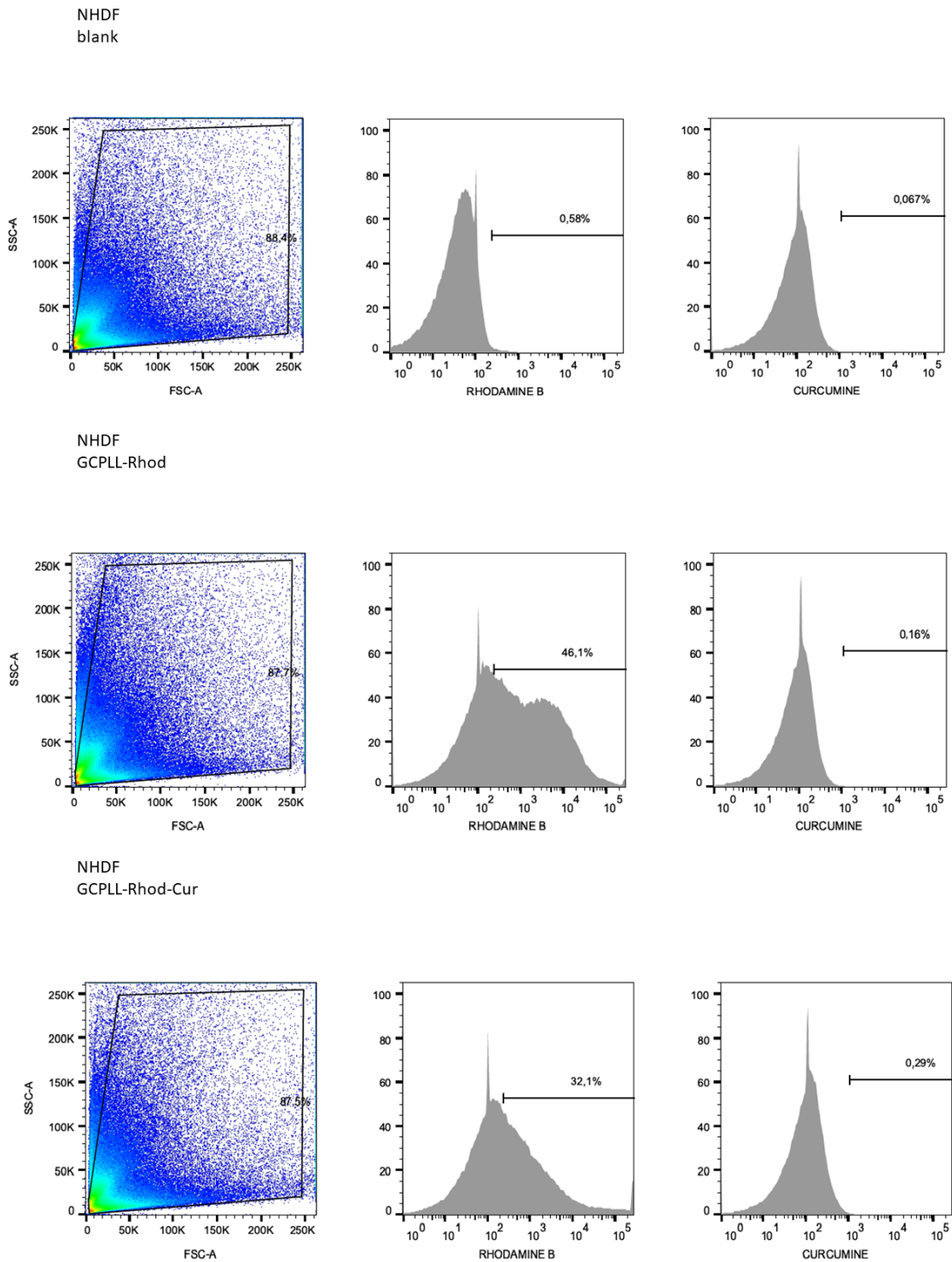
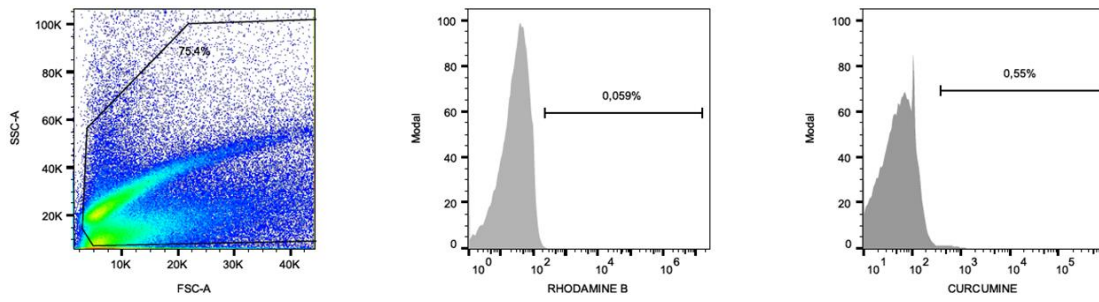
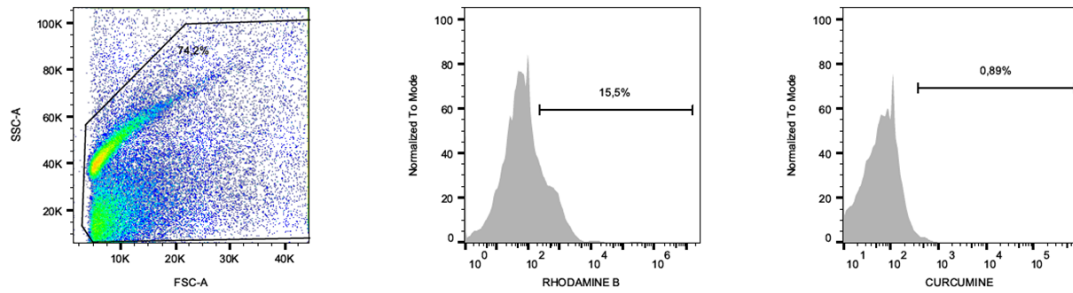


Figure S17. Flow cytometry raw data examples of the triplicate experiments with 3 replicates each one in NHDF cells, for: blank; GCPLL-Rhod and GCPLL-Rhod-Cur.

THP-1  
blank



THP-1  
GCPLL-Rhod



THP-1  
GCPLL-Rhod-Cur

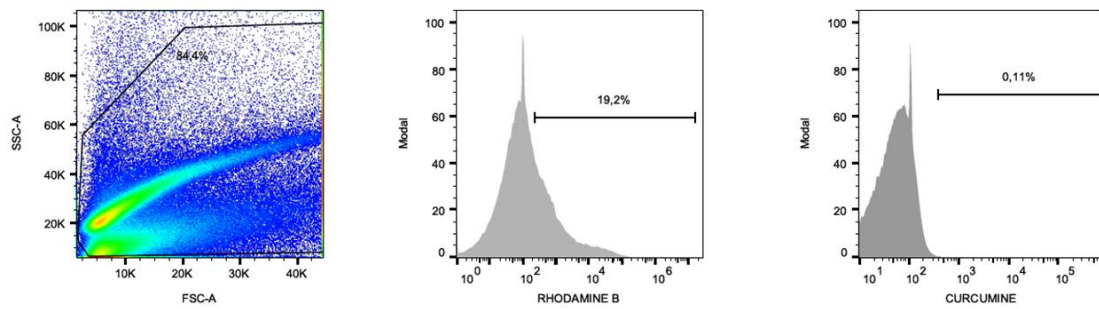


Figure S18. Flow cytometry raw data examples of the triplicate experiments with 3 replicates each one in THP-1 cells, for: blank; GCPLL-Rhod and GCPLL-Rhod-Cur.

*Confocal Microscopy*

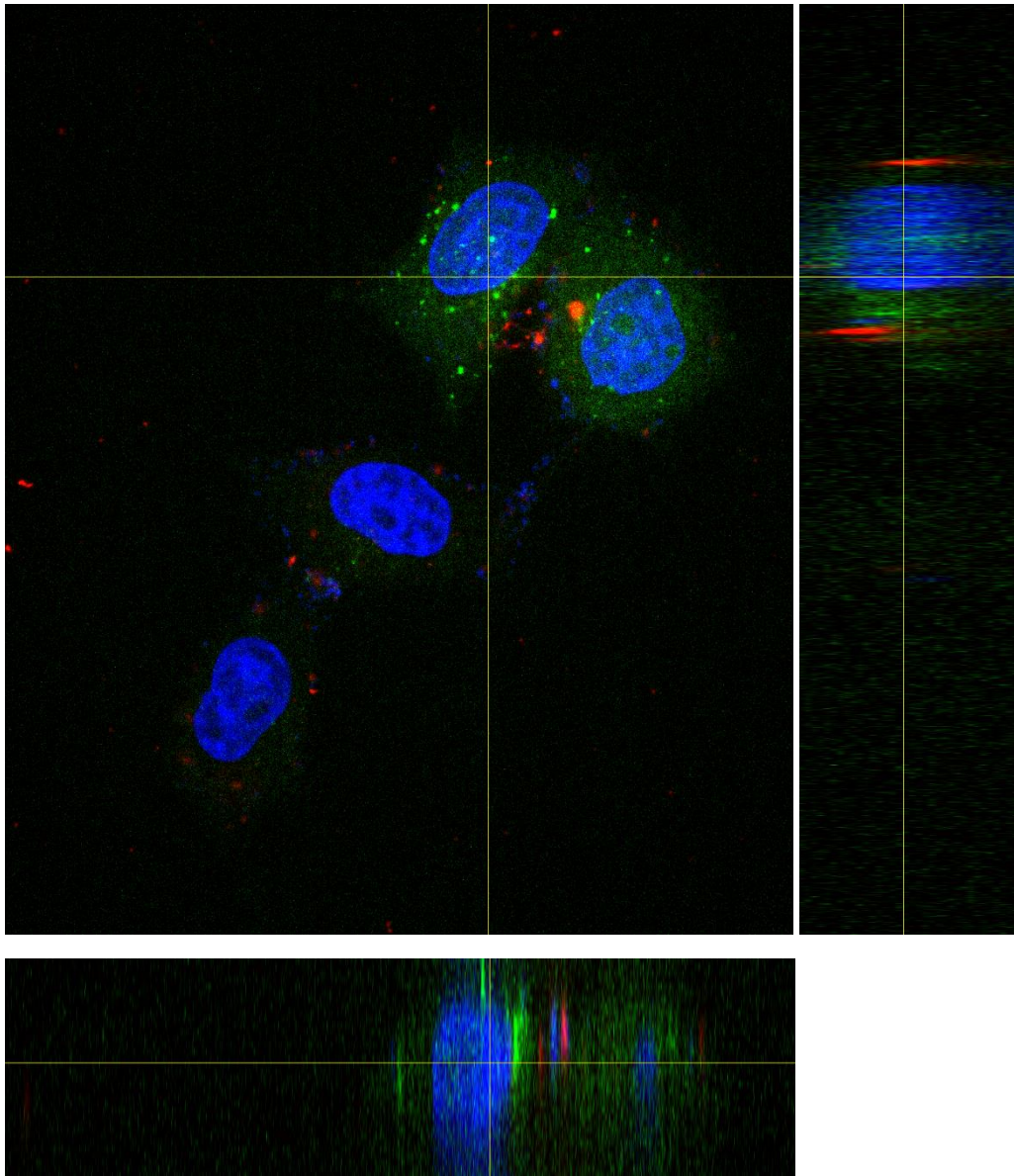


Figure S19. Orthogonal views of confocal microscopy images