



Formulation of liponanoparticles for dual chemo/photodynamic therapy of retinoblastoma

Marline N'Diaye

► To cite this version:

Marline N'Diaye. Formulation of liponanoparticles for dual chemo/photodynamic therapy of retinoblastoma. Galenic pharmacology. Université Paris Saclay (COMUE), 2018. English. NNT : 2018SACLS572 . tel-03491860

HAL Id: tel-03491860

<https://theses.hal.science/tel-03491860>

Submitted on 18 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Formulation de liponanoparticules pour le traitement du rétinoblastome par bithérapie chimio/ photodynamique

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'université Paris-Sud (Faculté de Pharmacie)

ED569 : Innovation thérapeutique : du fondamental à l'appliqué (ITFA)
Pôle : Pharmacotechnie et Physico-chimie Pharmaceutique

Thèse présentée et soutenue à Châtenay-Malabry, le 17 Décembre 2018, par

Marline N'DIAYE

Composition du Jury :

Amélie Bochot Professeur, Université de Paris-Sud	Présidente
Catherine Ladavière Directrice de recherche, Université Lyon 1	Rapporteur
Jean-Olivier Durand Directeur de recherche, Université de Montpellier	Rapporteur
Iván López-Montero Associé professeur, Universidad Complutense de Madrid	Examineur
Véronique Rosilio Professeur, Université de Paris-Sud	Directrice de thèse
Catherine Ladavière Directrice de recherche, Université Lyon 1	Rapporteur

Formulation of liponanoparticles for dual chemo/photodynamic therapy of retinoblastoma

PhD thesis of Paris-Saclay University
Research performed at University of Paris-Sud (Faculty of Pharmacy)

ED569: Therapeutic Innovation: from basics to applications (ITFA)
Pole: Pharmaceutical technology and Pharmaceutical Physico-chemistry

Defended at Châtenay-Malabry, on December 17th, 2018, by

Marline N'DIAYE

Thesis committee:

Amélie Bochot Professor, University of Paris-Sud	President
Catherine Ladavière Research director, University Lyon 1	Referee
Jean-Olivier Durand Research director, University of Montpellier	Referee
Iván López-Montero Associate professor, Universidad Complutense de Madrid	Member
Véronique Rosilio Professor, University of Paris-Sud	Thesis supervisor

Table of contents

List of figures	vi
List of tables	xii
Abbreviations	xiv
INTRODUCTION.....	2
Chapter I: Photodynamic therapy, retinoblastoma, and photosensitizer delivery systems	5
I-1. Photodynamic therapy (PDT): State-of-the-art	5
I-1.1. Principle and mechanism of action	5
I-1.2. Photosensitizer (PS)	10
I-1.2.1. Properties of a photosensitizer	10
I-1.2.2. First generation of photosensitizers.....	11
I-1.2.3. Second generation of photosensitizers	13
I-1.2.4. Third generation of photosensitizers	20
I-2. Retinoblastoma	21
I-2.1. Diagnosis and conventional treatments.....	21
I-2.2. Application of PDT to retinoblastoma.....	23
I-3. Photosensitizer delivery systems	24
I-3.1. Micelles.....	25
I-3.2. Liposomes	26
I-3.3. Nanoparticles	27
I-3.3.1. Biodegradable NPs	27
I-3.3.2. Non-biodegradable NPs	28

I-3.4. Liponanoparticles (LNP).....	29
I-3.4.1. Preparation methods for LNPs	30
I-3.4.2. Photosensitizer-loaded LNPs	39
I-4. Conclusion	39
Chapter II: Formulation and physicochemical characterization of liponanoparticles.....	42
II-1. Introduction	42
II-2. Materials and methods.....	42
II-2.1. Materials	42
II-2.2. Methods	43
II-2.2.1. Liposomes preparation.....	43
II-2.2.2. Preparation of PLA nanoparticles	44
II-2.2.3. Lipo-nanoparticles (LNPs) preparation	44
II-2.2.4. Particles characterization	45
II-2.2.5. Stability of LNPs.....	47
II-2.2.6. Interaction of LNPs with Y79 cells.....	47
II-3. Results and discussion.....	48
II-3.1. Nanoprecipitation conditions influencing NP characteristics.....	48
II-3.2. Liposomes.....	51
II-3.3. LNPs	53
II-3.3.1. Evaluation of various protocols for LNPs formation.....	53
II-3.3.2. Lipid assay	59
II-3.3.3. Assessment of LNP core-shell organization by confocal microscopy.....	66
II-3.3.4. Stability of the lipo-nanoparticles	67

II-3.3.5. Determination of the mechanism of interaction of PSs and LNPs with cells	71
II-4. Conclusion.....	75
Chapter III: Combined QCM-D and AFM experiments for deciphering the mechanism of fusion of lipid vesicles with polymer nanoparticles	77
III-1. Introduction.....	77
III-2. Materials and methods	78
III-2.1 Materials	78
III-2.2 Methods	78
III-2.2.1. Surface pressure-area (π -A) measurements	78
III-2.2.2. Surface potential measurements	79
III-2.2.3. Brewster Angle Microscopy (BAM)	80
III-2.2.4. Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) transfer of PLA monolayers	81
III-2.2.5. Quartz Crystal Microbalance with Dissipation (QCM-D) measurements.....	82
III-2.2.6. Atomic force microscopy experiments	84
III-3. Results and discussion	85
III-3.1. Interfacial behavior of PLA film at the air-water interface.....	85
III-3.2. Analysis by QCM-D and AFM of liposome interaction with a PLA layer adsorbed onto a HMDS-modified surface.....	87
III-3.2.1. Transfer of the PLA monolayer onto a HMDS-functionalized glass plate	88
III-3.2.2. Interaction of POPC and POPC-DOTAP liposomes with a SiO ₂ surface	92
III-3.2.3. Interaction of POPC and POPC-DOTAP liposomes with a PLA surface	94
III-3.2.4. Interaction with POPC-DOTAP liposomes with the PLA surface	96
III-4. Conclusion	107

Chapter IV: Co-encapsulation of a photosensitizer and beta-lapachone in liponanoparticles and <i>in vitro</i> evaluation of their cytotoxicity and phototoxicity for dual PDT/chemotherapy.	110
IV.1. Introduction	110
IV.2. Materials and methods	112
IV.2.1. Materials	112
IV.2.2. Methods	113
IV.2.2.1. Preparation of PS-loaded liposomes	113
IV.2.2.2. Preparation of β -Lap nanoparticles (β -Lap NPs).....	113
IV.2.2.3. Preparation of β -Lap and PSs-loaded LNPs	114
IV.2.2.4. Particles characterization	115
IV.2.2.5. Cell culture.....	116
IV.2.2.6. Cytotoxicity and phototoxicity experiments.....	116
IV.3. Results and discussion	119
IV.3.1. Physico-chemical characterization of drug loaded particles	120
IV.3.2. Encapsulation efficiency (EE) and drug/PS loading (DL) in LNPs	121
IV.3.3. Cytotoxicity of the studied formulations.....	122
IV.3.3.1. Cytotoxicity of the excipients	123
IV.3.3.2. Cytotoxicity of free drugs	125
IV.3.3.3. Cytotoxicity of beta-lapachone-loaded NPs and LNPs	127
IV.3.3.4. Cytotoxicity of photosensitizer-loaded LNPs.....	129
IV.3.3.5. Cytotoxicity of beta-lapachone and photosensitizers co-encapsulated in LNPs	130
IV.3.4 Phototoxicity of the studied formulations	131

IV.3.4.1. Phototoxicity of excipients	131
IV.3.4.2. Phototoxicity of free β -lapachone and β -Lap-loaded LNPs	133
IV.3.4.3. Phototoxicity of free PS and PS-loaded LNPs.....	134
IV.3.4.4. Phototoxicity of PS and β -Lap co-encapsulated in LNPs.....	135
IV.4. Conclusion	140
General discussion.....	142
Conclusion and perspectives	152
References	156

List of figures

Figure 1: The Jablonski diagram illustrating the mechanisms of production of reactive oxygen species upon illumination of a photosensitizer in PDT (from Dai et al., 2012).....	6
Figure 2: Illustration of the PDT optical therapeutic window (A) (from Plaetzer et al., 2009) and light penetration through human tissue (B) (from Sternberg et al., 1998 with slight modification).	9
Figure 3: Structures of the major components found in haematoporphyrin derivative (HpD) or Photofrin®. (A) Hematoporphyrin; (B) Hydroxyvinyldeuteroporphyrin; (C) Protoporphyrin IX; (D) Dihematoporphyrin with an ether linkage; (E) Dihematoporphyrin with an ester linkage (from Denis et al., 2013).	13
Figure 4: Basic structure of most second-generation tetrapyrrolic photosensitizers (A) and their typical absorbance spectrum showing the position of their Q-band I in the near-infrared region (from Moser et al., 2015).....	14
Figure 5: A) Structure of porphin, the tetrapyrrolic backbone of porphyrins; B) Characteristic absorption spectrum of porphyrins (from Josefsen and Boyle, 2008).	15
Figure 6: Chemical structure of A) 5-aminolevulinic acid and its derivatives; and B) Protoporphyrin IX (Pp IX) (from Gomes et al., 2018).....	17
Figure 7: Chemical structure of <i>m</i> -THPP.....	17
Figure 8: Structural comparison of porphyrin and chlorin (from Yoon et al., 2013).....	18
Figure 9: Clinical characteristics of retinoblastoma with a) leukocoria and b) bifocal tumors visible in the eye (from Dimaras and Corson, 2018).	23
Figure 10: Schematic illustration of the structure of a LNP (from Tan et al., 2013).	30
Figure 11: Particles size (A, B), polydispersity index (C) and zeta potential (D) for PLA NPs, sonicated liposomes and LNPs prepared under condition iii	50
Figure 12: Illustration of the cross-section of a LNP; R_{NP} is the radius of the PLA NP; T_w is the thickness of the water layer lying between the NP and lipid bilayer, and T_b is the thickness of the lipid bilayer. The theoretical diameter of LNP expected is calculated as $D_{LNP}=2R_{NP}+2$	

$T_w + 2T_b$, where T_b is ~ 4 nm and $T_w \sim 2$ (Bayerl and Bloom, 1990; Mornet et al., 2005; Savarala et al., 2011).....	55
Figure 13: Cryo-TEM image of PLA NPs.	56
Figure 14: Cryo-TEM image of LNPs 90/10(i) obtained after 1h vortexing. Black arrows show the presence of some unbroken vesicles or lipid membranes adsorbed on the NPs core.....	57
Figure 15: Cryo-TEM image of LNPs 75/25 (i) obtained after 1h vortexing. Black arrows show the presence of lipid membranes adsorbed on the NPs core which confer roughness to the surface. Some non-adsorbed lipid vesicles (blue arrows) are also visible in the suspensions.	57
Figure 16: Cryo-TEM images of LNPs 100/0(iii) (A), LNPs 75/25(iii) (B), LNPs 50/50(iii) (C) and LNPs 25/75(iii) (D) obtained after 6 min of sonication followed by 1h of incubation. The black arrows indicate the presence of adsorbed vesicles onto the NPs surface.	58
Figure 17: Cryo-TEM image of LNPs 75/25 (ii) obtained after 1h incubation. Black arrows show the presence of lipid membranes adsorbed on the NPs core which confer roughness to the surface. The yellow arrows indicate the presence of some lipid bridges.	59
Figure 18: Mechanism of analyte detection by an evaporative light scattering detector (ELSD). Following the effective separation of molecules in the HPLC column, the eluate is mixed with N_2 gas in the nebulizing chamber leading to a dispersion of droplets; then the solvents are evaporated, condensed and removed leading to solid analytes which cross an optical cell illuminated by a light laser source. Analytes absorb the laser beam and scatter the light which is detected and converted into a signal (from Rocés et al., 2016).....	60
Figure 19: HPLC-ELSD chromatogram for LNPs (90/10, 75/25 and 50/50 mol%) (iii) and PLA NPs.	63
Figure 20: Confocal microscopy images of LNPs 75/25(iii). The NP core was labelled with 0.01% NR (red dye) and the lipid shell with 1% NBD-PE (green dye). The green and red fluorescence signals of the LNPs are shown in A and B respectively. C corresponds to the overlay of both fluorescence and D shows the plotted fluorescence intensity profiles of a selected region of interest (ROI).	66
Figure 21: Confocal microscopy images of LNPs 100/0(iii). The NP core was labelled with 0.01% NR (red dye) and the lipid shell with 1% NBD-PE (green dye). The green and red fluorescence signals of the LNPs are shown in A and B respectively. C corresponds to the	

overlay of both fluorescence and D shows the fluorescence intensity profiles of a selected region of interest (ROI).	67
Figure 22: Evaluation of the stability of LNP 75/25(iii) in DMEM and DPBS by DLS.	68
Figure 23: Evaluation of the stability of LNPs 75/25(iii) by cryo-TEM in DPBS (A) and DMEM (B). Lipid bridges are visible between particles (black arrows). These bridges probably formed due to the screening of DOTAP charge at high ionic strength, and consecutive closer contact between liponanoparticles.....	69
Figure 24: Confocal microscopy images of fluorescent LNPs labelled with 0.001 mol% NR and 2 mol% NBD-PE suspended in DMEM (A) and DMEM supplemented with 10% FBS (B); the green fluorescence reveals the presence of lipids and the red fluorescence that of the NPs. Overlay of both fluorescence leads to a yellow colour which confirms the colocalization of lipid shells with polymer cores.	70
Figure 25: Confocal microscopy images of m-THPP LNPs dispersed in pure water (A), DMEM (B) and DMEM supplemented with 10% FBS (C). The red fluorescence confirms the presence of m-THPP in the lipid shell of the LNPs.	71
Figure 26: Confocal microscopy images depicting the interaction and internalization of fluorescent LNPs (iii) in Y79 cells. NPs were labelled with 0.001% NR (red dye) and lipid shells by 2 mol% NBD-PE (green dye). The autofluorescence of cells was assessed at 488 nm and 543 nm (A). Cells were incubated with fluorescent LNPs at 37°C at different times (B). Fluorescence overlays in the images and intensity profiles confirm the penetration of both NPs and lipid shells in cells.	73
Figure 27: Confocal microscopy images of Y79 cells incubated with m-THPP LNPs at different times (4h, 24h and 48h). For more clarity, the red fluorescence of m-THPP at 633 nm (left) is also shown on transmitted light images (right). No autofluorescence of Y79 cells was observed at 633 nm.	74
Figure 28: Confocal microscopy images of Y79 cells incubated with free m-THPP at different times (1h, 2h, 4h, 24h and 48h). For more clarity, the red fluorescence of m-THPP at 633 nm (left) is also shown on transmitted light images (right). No autofluorescence of Y79 cells was observed at 633 nm.....	74
Figure 29: (Left) Langmuir trough. (Right) Langmuir isotherm. LE: liquid-expanded; LC: liquid condensed; SP: solid phase.	79

Figure 30: Principle of the Brewster Angle Microscope.....	80
Figure 31: LB (A) and LS (B) transfers of a PLA the monolayer onto a solid substrate.....	82
Figure 32: Adsorption-rupture of phospholipid vesicles onto the PLA film.....	83
Figure 33: Surface pressure-area isotherm and Brewster angle (BAM) microscope images of a PLA film spread at the air-water interface (22°C): (a) 8 mN/m; (c) 15 mN/m; (f) 25 mN/m; (h) 50 mN/m; (j) expansion of the monolayer. The film was LB-transferred onto mica and imaged by atomic force microscopy (AFM) in the air: (b) 8 mN/m (height); (d,e) height and phase images at 15 mN/m; (g) 25 mN/m (height).....	86
Figure 34: (A) Surface pressure and surface potential-area isotherms; (B) Compressibility moduli deduced from the PLA π -A isotherm in (A).	87
Figure 35: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS. Height cross section performed along the black line is shown on the right side.	88
Figure 36: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS after LS transfer of PLA monolayers at 18 (A) and 35 mN/m (B). Height cross section performed along black line depicted on height picture at 35 mN/m is shown in (C).	90
Figure 37: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS following LS transfer of PLA at the surface pressure of 25 mN/m. Height cross sections performed along black line depicted on height pictures are shown on the right.	91
Figure 38: Force-distance measurement performed in water on the PLA film transferred at 25 mN/m. Approach and retract curves are displayed as vertical deflection (in nN) of the cantilever versus the AFM head height (in nm).....	91
Figure 39: ΔF and ΔD values for (A) POPC (0.4 mM), (B) and (C) POPC-DOTAP (75:25) liposomes (4 mM) on the SiO ₂ -coated quartz crystal QCM-D sensor. Whereas (B) is an enlargement of the plot for the first hour, (C) shows the whole QCM-D experiment. Arrows indicate the rinsing step.....	94
Figure 40: Interaction between 4 mM pure POPC liposomes and the PLA monolayer transferred at 25 mN/m: (A) ΔF -time and ΔD -time relationships monitored by QCM-D; (B) is	

the enlargement of the QCM plot in (A); (C) height (left) and phase (right) pictures of the PLA surface after 1h incubation with the liposomes and abundant water rinsing..... 95

Figure 41: Interaction between 0.4 mM POPC-DOTAP (75:25) liposomes and the PLA monolayer transferred at 25 mN/m: (A) ΔF -time and ΔD -time relationships monitored by QCM-D. (B) close-up on the 60 first minutes of ΔF -time and ΔD -time relationships. (C) height and (D) phase pictures of the PLA surface before and after 1h incubation with the liposomes and abundant water rinsing (same area scanned). Height cross sections performed along black and white lines are shown below C. (E) Enlargement of height and phase pictures showing evidence of large holes in the lipid bilayer covering the PLA monolayer. 98

Figure 42: The four steps of phospholipid bilayer formation (adapted from Sundh et al., 2010). 99

Figure 43: ΔF -time and ΔD -time relationships monitored by QCM-D for the interaction of 4 mM POPC-DOTAP (75:25) liposomes with a PLA monolayer transferred at 25 mN/m : (A) Full experiment with rinsing at $t = 80$ min; (B) Close-up on the initial times of the experiment. 100

Figure 44: The receding-top and parachute models (from Fuhrmans and Müller, 2013). 102

Figure 45: ΔD - ΔF relationships for (A) POPC/SiO₂; (B) POPC-DOTAP/SiO₂ (4 mM); (C) POPC/PLA (4 mM); (D) POPC-DOTAP/PLA (4 mM)..... 105

Figure 46: 7th overtone of the ΔD - ΔF relationships for POPC-DOTAP/SiO₂ (4 mM). 106

Figure 47: Removal of β -Lap aggregates following nanoprecipitation. The organic solvent was evaporated in a round bottom flask (A) followed by the filtration of the suspension (B and C). 114

Figure 48: UV-visible spectrum of free β -Lap (blue line), β -Lap NPs (red line) and β -Lap LNPs (green line). Both lipids and PLA did not interfere with the absorbance spectrum of β -Lap. 116

Figure 49: Summary of the procedure used for in vitro cytotoxicity and phototoxicity experiments. 117

Figure 50: Viability of Y79 cells in contact with POPC/DOTAP 100/0, 75/25 and 0/100 vesicles for 72h..... 123

Figure 51: Viability of Y79 cells in contact with PLA NPs and LNPs 75/25 for 72h. 124

Figure 52: Viability of Y79 cells in contact with free <i>m</i> -THPP (A) and <i>m</i> -THPC (B) after 72h and 96h of treatment.....	126
Figure 53: Viability of Y79 cells following contact with β -Lapachone for 96h.....	127
Figure 54: Viability of Y79 cells in contact with free β -Lap, β -Lap loaded NPs and β -Lap loaded LNPs for 96h.....	128
Figure 55: Cytotoxic effect of <i>m</i> -THPP LNPs (A) and <i>m</i> -THPC LNPs (B) on Y79 cells, after incubation for 72h and 96h, respectively.	129
Figure 56: Cytotoxic effect of β -Lap co-encapsulated with <i>m</i> -THPP (A) or <i>m</i> -THPC (B) in LNPs after 96h incubation with Y79 cells. PS concentrations are indicated in black, and β -Lapachone ones in red.....	130
Figure 57: Phototoxic effect of A) POPC/DOTAP 100/0, 75/25 and 0/100 liposomes, and, B) PLA NPs and LNP 75/25 on Y79 cells after 96h of treatment.	132
Figure 58: A) Cytotoxicity/phototoxicity of free β -Lap, and, B) phototoxicity of β -Lap loaded encapsulated in LNPs on Y79 cells after 96h of treatment of Y79 cells.....	134
Figure 59: Phototoxicity of <i>m</i> -THPP and <i>m</i> -THPP LNPs (A), and, <i>m</i> -THPC and <i>m</i> -THPC LNPs (B) on Y79 cells after 96h of treatment.....	135
Figure 60: A) Cyto/phototoxicity of β -lap/ <i>m</i> -THPP LNPs and B) Comparison of the phototoxicity of β -lap/ <i>m</i> -THPP LNPs with that of <i>m</i> -THPP LNPs; C) Cyto/phototoxicity of β -lap/ <i>m</i> -THPC LNPs and D) Comparison of the phototoxicity of β -lap/ <i>m</i> -THPC LNPs with that of <i>m</i> -THPC LNPs; All these plots were obtained after 96h of incubation with Y79 cells. ...	136
Figure 61: FA plots for (A) β -Lap/ <i>m</i> -THPC and β -Lap/ <i>m</i> -THPP LNPs, and (B) the pure drugs.	138
Figure 62: Combination index-fraction affected relationships for β -lap/ <i>m</i> -THPC and β -lap/ <i>m</i> -THPP LNPs, calculated using the CompuSyn software.....	139
Figure 63: Futile oxidoreduction cycle of beta-lapachone catalyzed by NQO1 (from Kung, 2014).....	145
Figure 64: Western blot demonstrating the expression of NQO1 by Y79 cells.....	145

List of tables

Table 1: Summary of the reactions taking place after PS activation by light and conditions under which it may happen (from Kriska et al., 1998).....	7
Table 2: Effect of nanoprecipitation conditions on the characteristics of PLA NPs. m_{PLA} is the mass of PLA initially used; C_{orgPLA} is the polymer concentration in the organic solvent; OP/AP ratio is the organic-to-aqueous phase ratio; PDI is the polydispersity index and ζ the zeta potential. Nd: not determined.	49
Table 3: Mean size of extruded and tip-sonicated POPC/DOTAP liposomes.	52
Table 4: LNP physicochemical characterization by DLS and zeta potential depending on the mixing condition. ΔHD is the difference in the hydrodynamic diameter (HD) between LNP and bare PLA NPs ($\Delta HD = HD_{LNPs} - HD_{NPs}$).	54
Table 5: Quantification of POPC and DOTAP in two liposome and LNP samples by HPLC-ELSD; expression of the experimental ratio of POPC/DOTAP, lipid recovery as function of the initial amount of lipids used, proportion of lipid and number of bilayers covering NPs surface. * Calculated using equation (2 to 7).....	62
Table 6: Characteristics of the various studied surfaces in contact with liposomes, as deduced from AFM measurements in water. RMS: roughness. *The thickness of the PLA monolayer was measured 1.5h after spreading at the air-water interface and transfer at 25 mN/m. No change in thickness was observed afterwards. The bilayer surface coverage was deduced from the surface coverage of PLA (87%), and the final ΔF value obtained in the QCM-D experiment.	92
Table 7: QCM-D characteristics values for various studied samples. The time refers to the duration of the frequency and dissipation changes for the adsorption and following steps.....	97
Table 8: Summary of the tested concentration ranges for in vitro cyto/phototoxicity experiments.	119
Table 9: Size, PDI and zeta potential of unloaded and loaded NPs and LNPs.....	121
Table 10: Summary of the encapsulation efficiency (EE) and drug loading (DL) for β -Lapachone-loaded PLA NPs and PS (m-THPP or m-THPC)-loaded LNPs.	121

Table 11: Cytotoxicity of excipients, m-THPP and m-THPP LNPs against Y79 cells after treatment for 72h. In the first two columns are presented the IC ₅₀ values for drugs or excipients, while the mass concentration of LNPs or NPs containing encapsulated drugs is indicated in the last column (right).	125
Table 12: Cytotoxicity of free drug and PSs, and drug and PS encapsulated in PLA NPs or LNPs after 96h incubation with Y79 cells. IC ₅₀ values of drugs are presented in the first two columns and the last column (right) indicates the mass concentration of the nanocarriers in which the drugs were entrapped.	129
Table 13: Phototoxicity of excipients, free drug and PSs, and drug/PSs encapsulated in PLA NPs or LNPs on Y79 cells after 96h of incubation with Y79 cells. The first two columns show the IC ₅₀ values of drugs or excipients. The last column (right) indicates the mass concentration of the nanocarriers containing the drugs.	133

Abbreviations

AFM: Atomic Force Microscopy
ALA: 5-aminolevulinic acid
ALAS: Aminolevulinic acid synthase
ATB: Antibiotic
BAM: Brewster Angle microscopy
BCCS: Basal Cell Carcinoma of Skin
 β -Lap: Beta-Lapachone
BPD-MA: Benzoporphyrin derivative monoacid
BPD-NP: Benzoporphyrin derivative monoacid loaded nanoparticles
BSA: Bovine Serum Albumin
CI: Combination Index
CMC: Critical Micelle Concentration
Cryo-TEM: Cryogenic Transmission Electron Microscopy
CT: Chemotherapy
DCM: Dichloromethane
DESE: Double Emulsion-Solvent Evaporation
DHE: Dihematoporphyrin Esters
DL: Drug Loading
DLS: Dynamic Light Scattering
DMEM: Dulbecco's Modified Eagle's Medium
DMPC: 1,2-Dimyristoyl Phosphatidylcholine
DMSO: Dimethyl sulfoxide
DNA: DeoxyriboNucleic Acid
DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPS: 1,2-dioleoyl-sn-glycero-3-phospho-L-serine

DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane
DPBS: Dulbecco Phosphate Buffer Saline
DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPTAP: 1,2-dipalmitoyl-3-trimethylammonium-propane
EBR: External Beam Radiation
EE: Encapsulation Efficiency
EGFR: Epidermal Growth Factor Receptor
ELSD: Evaporative light scattering detector
EPR: Enhanced Permeability and Retention
ESE: Single Emulsion-Solvent Evaporation
FA: Fraction Affected
FAc: Formic Acid
FBS: Fetal Bovine Serum
H₂O₂: Hydrogen peroxide
H₂SO₄: Sulfuric acid
HD: Hydrodynamic Diameter
HEK 293T: Human embryonic kidney 293 cells
HMDS: 1,1,1,3,3,3- Hexamethyldisilazane
Hp: Haematoporphyrin
HpD: Hp Derivative
HPLC: High-performance liquid chromatography
HPPH: 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide
IV: Intravenous
KTP: Potassium–titanium–phosphate
LB: Langmuir-Blodgett
LDL: low density lipoproteins
LED: Light-Emitting Diode
LNP: Liponanoparticle
LS: Langmuir-Schaefer
LUV: Large Unilamellar Vesicles

MAbs: Monoclonal antibodies

MLV: MultiLamellar Vesicles

mPEG₂₀₀₀-DSPE: 1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000

MRI: Magnetic Resonance Imaging

MSN: Mesoporous Silica Nanoparticles

m-THPC: 5,10,15,20-tetrakis(m-hydroxyphenyl)chlorin

m-THPP: 5,10,15,20-tetrakis-(m-hydroxyphenyl) porphyrin

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NaCl: Sodium chloride

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NBD-PE: 1-oleoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphoethanolamine

Nd:YAG: Doubled neodymium:yttrium-aluminum-garnet

NP: Nanoparticle

NPe6: N-aspartyl chlorin e6

NQO1: NAD(P)H:Quinone Oxidoreductase

NR: Nile Red

¹O₂: Singlet oxygen

O₂^{•-}: Superoxide anion

OEP: Octaethylporphyrin

OH: Hydroxyl

PARP: Poly ADP Ribose Polymerase

PBS: Phosphate Buffer Saline

PEG-b-PCL: Poly(ethylene oxide)-b-poly(ε-caprolactone)

PDI: Polydispersity Index

PDT: Photodynamic Therapy

PEG-PLA: poly(ethylene glycol)-co-poly(D,L-lactic acid)

PLA: poly(D,L-lactic acid)

PLGA: poly(D,L-lactide-co-glycolide)

POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

Pp IX: Protoporphyrin IX
PS: Photosensitizer
PVA: Polyvinyl Alcohol
QCM-D: Quartz Crystal Microbalance with Dissipation
RB: Retinoblastoma
RBM: Retinoblastoma cell line
RBC: Red Blood Cells
RMS: Root Mean Square
ROS: Reactive Oxygen Species
SnET2: Tin ethyl etiopurpurin
SUV: Small Unilamellar Vesicles
TGA: Thermogravimetric Analysis
THF: Tetrahydrofuran
THPTS: Tetrahydroporphyrin tetratosylat
TPGS: Tocopherol Polyethylene Glycol Succinate
TPP: Tetraphenylporphyrin
UV: Ultraviolet
VEGF: Vascular Endothelial Growth Factor
WERI-RB1: Retinoblastoma cell line
Y79: Retinoblastoma cell line

INTRODUCTION

INTRODUCTION

Photodynamic therapy (PDT) is a treatment modality which requires the combination of a photosensitizer (PS), light and oxygen to cause a cytotoxic effect. PDT is a non-invasive method which allows the destruction of pathological cells in several diseases including age-related macular degeneration, a large variety of small solid tumors (skin, head, neck, lung, esophageal, brain, bladder etc.) and microbial infections. Once systemically administered, the PS that is not cytotoxic in the dark, accumulates in the diseased tissue. Subsequent illumination of this tissue with a laser at PS-specific wavelength generates the production of reactive oxygen species (ROS) leading to cells death by apoptosis or necrosis. Because conventional anti-cancer therapies are associated to severe side effects and risk of developing secondary tumors, PDT appears as a promising alternative treatment thanks to its high selectivity, its minimal side effects and relatively low cost. Some PSs like porphyrins do not penetrate in the nucleus and therefore, there is no risk of inducing DNA damage or gene mutations (O'Connor *et al.*, 2009). PDT presents, however, some limitations. It is mainly effective against small solid tumors located at regions that can be easily reached by light. One other important parameter is the level of oxygen in the tumors. PDT is ineffective against large and deeply localized tumors, and against metastases (Calixto *et al.*, 2016).

Considering the above-mentioned facts, PDT is an attractive treatment modality for retinoblastoma (RB), a rare malignancy of the retina in infants. Current treatments consist in enucleation and/or conservative treatments including chemotherapy, cryotherapy, or radiotherapy (Aerts *et al.*, 2006). For many years, a research devoted to PDT application to RB has been developed at the Institut Curie. Several glycoconjugated derivatives of the tetraphenylporphyrin m-THPP have been synthesized by Dr Philippe Maillard's team. However, these compounds are difficult to deliver to the body because of their high hydrophobicity.

We have envisaged a system in which both a PS and a cytotoxic agent could be co-encapsulated for combined photodynamic and chemo therapy. This system is a hybrid core-shell nanoparticle (referred to as liponanoparticle or LNP) which would contain the cytotoxic agent in the

polymeric core and the PS in the lipid bilayer shell. Liponanoparticles could be injected intravitreally for PDT treatment of the RB tumor and chemotherapy against forming metastases.

The objectives of this PhD thesis were three-fold: i) to design and characterize the LNPs; ii) to co-encapsulate a photosensitizer, e.g., *m*-THPC or *m*-THPP, and beta-lapachone (β -Lap), an anticancer agent which has demonstrated in vitro efficacy against RB, and which is believed to possess a certain specificity; and iii) to evaluate LNPs phototoxic efficacy on RB cells.

**Chapter I: Photodynamic therapy, retinoblastoma, and photosensitizer
delivery systems**

Chapter I: Photodynamic therapy, retinoblastoma, and photosensitizer delivery systems

I-1. Photodynamic therapy (PDT): State-of-the-art

I-1.1. Principle and mechanism of action

The therapeutic effect of light is known and has been exploited since the antiquity for the treatment of many diseases such as vitiligo, psoriasis, rickets and even skin tumors (Spikes, 1985). At this time, there were already two modalities of treatment using light as therapeutic agent, namely phototherapy and photochemotherapy, the latter being now known as photodynamic therapy (PDT). The concept of phototherapy was introduced by the Danish physician Niels Finsen in 1903 (Dolmans *et al.*, 2003). He found that patients suffering from smallpox pustules could be cured by red light, and those suffering from cutaneous tuberculosis by ultraviolet (UV) light from the sun.

Although the photodynamic effect had been previously observed, it was only in the early 1900s that this new method of treatment was highlighted, thanks to a student in Von Tappeiner's group, who accidentally discovered the lethal effect of both a dye (acridine orange) and light on microorganisms (Daniell and Hill, 1991). PDT was approved for the first time in 1994 by the Canadian FDA for the treatment of skin cancer. Today, it is considered as a promising treatment substitute to current treatments in oncology, dermatology but also in ophthalmology, rheumatology, cardiovascular and infectious diseases (Benov, 2015; Dolmans *et al.*, 2003). It is based on the interaction between a photosensitizer (PS), light and molecular oxygen (Figure 1), which are non-toxic by themselves. Depending on the part of the body that is treated, the PS is administered topically or systemically. After PS distribution all over the body, the diseased tissue or organ is illuminated by a light of appropriate wavelength that transfers its energy to the PS. Following absorption of light energy, the PS which is pharmacologically inactive and stable at its ground state (S_0), goes to a higher energetic level, short-lived singlet state (S_1), that is extremely unstable. At this stage, two options are possible. In the first one, S_1 -PS goes

back to S_0 , emitting fluorescence at a nanosecond scale. This phenomenon can be used for photodiagnosis purposes (Ackroyd *et al.*, 2001; Nokes *et al.*, 2013). In the second one, S_1 -PS converts into a relatively long-lived excited triplet state (T_1) by intercrossing system, its lifetime ranging from micro to milliseconds. PS in its T_1 state can return to the ground state through a phosphorescence process or may transfer its energy to other molecules via two kinds of photochemical reactions (type I and type II reactions) that both induce a cytotoxic effect.

In type I reactions, the PS in its triplet state transfers a hydrogen atom or an electron directly to a biological substrate such as a cell membrane (Dolmans *et al.*, 2003), amino acids in their free form or included in peptides and proteins, etc. (Silva *et al.*, 2008), leading to the formation of radicals which react with oxygen to produce reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl ($\cdot OH$) radicals, and hydrogen peroxide (H_2O_2). In type II reactions, PS in its T_1 state transfers its energy directly to ground-state molecular oxygen present in the environment to generate highly reactive and cytotoxic singlet oxygen (1O_2) that has a very short half-life (less than $0.04\ \mu s$) and a diffusion distance shorter than $0.02\ \mu m$ (Moan and Berg, 1991). Although both type I and type II reactions occur simultaneously, type II reaction is usually considered as predominant (Allison and Moghissi, 2013; Bozzini *et al.*, 2012; Michels and Schmidt-erfurth, 2001). However, type I reactions may prevail under hypoxic conditions (Ochsner, 1997).

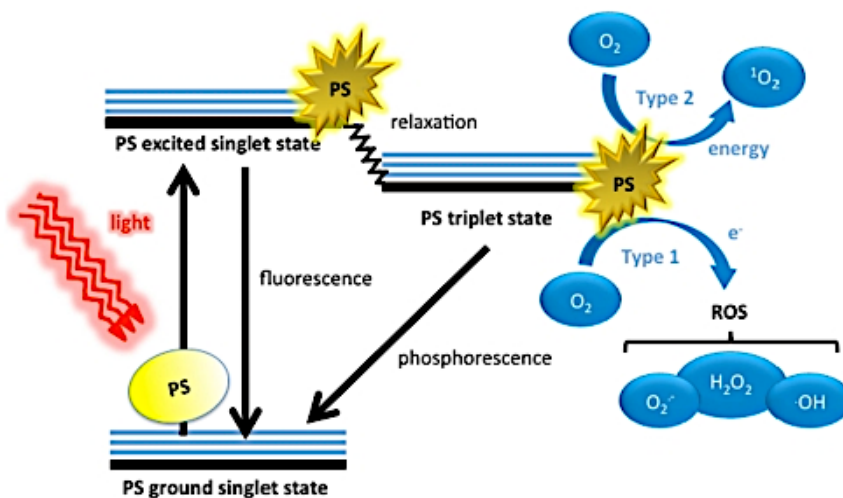


Figure 1: The Jablonski diagram illustrating the mechanisms of production of reactive oxygen species upon illumination of a photosensitizer in PDT (from Dai *et al.*, 2012).

A third reaction has been suggested to take place competitively with the two previously described mechanisms depending on the conditions in which the excited PS is generated (Table 1). In this type III reaction also called triplet-doublet interaction, the T1-PS may directly interact with free radicals present in a biological tissue independently of the presence of oxygen, and produce a photodynamic effect (Gál, 1992; Kriska *et al.*, 1998; Roblero-Bartolón and Ramón-Gallegos, 2015; Vidóczy *et al.*, 1992).

Type	main primary step	condition for main contribution
I	$^3\text{PS} \rightarrow \text{PS}$	<i>de-aerated system with low radical content (strong hypoxia and lack of radicals)</i>
II	$^3\text{PS} + ^3\text{O}_2 \rightarrow ^1\text{PS} + ^1\text{O}_2$	<i>normal cells</i>
III	$^3\text{PS} + \text{Rad}^\bullet \rightarrow \text{e}^- \text{ transfer}$	<i>hypoxia; high concentrations of (native) free radicals</i>

Table 1: Summary of the reactions taking place after PS activation by light and conditions under which it may happen (from Kriska *et al.*, 1998).

The overall effect of these reactions is the occurrence of irreversible photo-oxidative damage to biological structures. As a result, cell death may directly occur (i) by apoptosis or necrosis depending on the subcellular localization of the photosensitizer (Godar, 1999; Moor, 2000), (ii) by autophagy (Yoo and Ha, 2012), and/or indirectly in cancer (iii) by destruction of tumor blood vessels (Buzzá *et al.*, 2014; Fingar, 1996; Rodriguez *et al.*, 2004) and (iv) stimulation of the immune system of the host against cancer cells (Gollnick *et al.*, 2002; Maira *et al.*, 2007). Mitochondrial localization of PSs is often associated to apoptosis while PSs located in plasma membranes are more likely to induce necrosis (Lupu *et al.*, 2016; O'Connor *et al.*, 2009; Oleinick and Evans, 1998).

PDT presents many advantages compared to conventional anticancer treatments. These advantages include: (i) minimal invasive character regarding both the route of administration and light used, (ii) high selectivity because the phototoxic effect is only limited to the illuminated area, (iii) minimal systemic toxicity due to the low toxicity of the PSs in darkness

and its selective accumulation in tumors, preserving healthy tissues from the toxic effect, (iv) absence of long-term adverse effects, (v) possible combination with another therapy (e.g., chemotherapy, radiotherapy, surgery), (vi) repeatability without harmful effect, (vii) low cost-effectiveness compared to other cancer treatments, and (viii) possible application in outpatient setting (Calixto *et al.*, 2016; Dai *et al.*, 2012; Li, 2013; Sharman *et al.*, 1999). However, it has also some limitations. As the photodynamic effect occurs only in the illuminated area, PDT is useless for disseminated metastases. Hypoxic conditions that are observed in most solid tumors, also lead to PDT failure since oxygen plays a key role in the photodynamic effect. Tumors localized deep in the body cannot be treated by PDT because of the low penetration of visible light through biological tissues (Calixto *et al.*, 2016; Dolmans *et al.*, 2003).

Tumor accessibility, photophysical properties of the PS and light dose are important parameters guiding the choice of the light source. Different types of light sources have been proposed for PDT application including non-coherent broad-spectrum lamps and lasers. Examples of non-coherent broad-spectrum lamps are xenon arc, tungsten filament quartz halogen, metal halide and fluorescent lamps, emitting diodes lamps (LEDs) and intense pulsed lights (Breskey *et al.*, 2013; Juzeniene *et al.*, 2007). A variety of lasers are also available, including argon or argon-pumped dye lasers, metal vapor lasers (gold or copper), potassium–titanium–phosphate (KTP) dye laser, doubled neodymium:yttrium-aluminum-garnet (Nd:YAG) solid state dye lasers and diode lasers (Brancaleon and Moseley, 2002; Kostron and Hasan, 2016; Lapes *et al.*, 1996; Zhu and Finlay, 2008). While both types of light sources are described for easily accessible diseases especially in dermatology, lasers are more frequently used, and they are preferred for the treatment of tumors located elsewhere (e.g., urinary bladder, lung and digestive tract) because they can be easily coupled to optical fibers which allow reaching the desired location (Brancaleon and Moseley, 2002; Juzeniene *et al.*, 2007; Teixeira *et al.*, 2015). Non-coherent lamps are considered superior to lasers in dermatology because they are cheaper, and because they offer a large range of wavelengths, thus allowing use of various PSs. LEDs and diode lasers are the most universal light sources used (Kostron and Hasan, 2016).

It has been shown that the fluence rates of light delivered in PDT is dependent on the PS and varied from 5 to almost 300 J/cm² (Kharkwal *et al.*, 2011; Kostron and Hasan, 2016; Lapes *et al.*, 1996; Schmidt-Erfurth *et al.*, 1994; Silva *et al.*, 2008). For example, the fluence rate necessary to treat head and neck cancers using *m*-THPC was evaluated to ~10-20 J/cm² (Triesscheijn *et al.*, 2006). Meso-tetraphenylporphinesulfonate tetrasodium salt (TPPS) used

for the treatment of superficial basal cell carcinoma of skin was illuminated at 120-150 J/cm², while for hypericin, the illumination could be performed at 75 J/cm² only (Kostron and Hasan, 2016).

Several endogenous tissue chromophores such as hemoglobin, melanin, collagen, water etc., strongly absorb light at wavelengths lower than 650 nm and higher than 850. When tissues are illuminated at these wavelengths (< 650 nm and > 850 nm), important light scattering is observed, which hampers the penetration of light deeper in the tissues. Tissues are considered as a non-optically transparent medium. They become transparent at wavelengths between 650-850 nm because of the low absorption of the chromophores and consequent limitation of light scattering (Lee and Kopelman, 2011; Yoon *et al.*, 2013). For this reason, wavelengths in the 650-850 nm range are defined as PDT “optical therapeutic window” (Figure 2A). Maximum light penetration in human tissues is observed with red light. Blue light penetrates less deeply than green and yellow light (Figure 2B) (Agostinis *et al.*, 2011; Avci *et al.*, 2013). Depending on the location of the tumor, one photosensitizer will be preferred to the others.

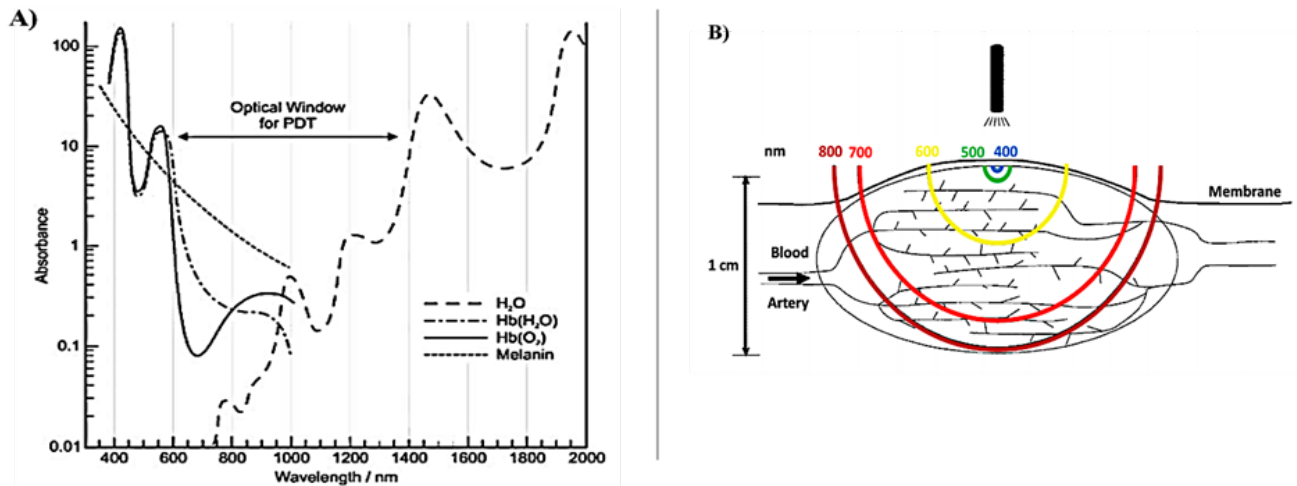


Figure 2: Illustration of the PDT optical therapeutic window (A) (from Plaetzer *et al.*, 2009) and light penetration through human tissue (B) (from Sternberg *et al.*, 1998 with slight modification).

I-1.2. Photosensitizer (PS)

PSs are natural or synthetic photo-sensitive agents able to absorb light energy and to transfer it to other molecules. According to their chemical structure, PSs are categorized as porphyrin- and non-porphyrin derivatives. Porphyrin-derived compounds are macrocycles which chemical structure looking like that of porphyrins.

In PDT, PSs are also classified as first-, second- and third-generation compounds. First-generation PSs refer to the first PSs that have been developed and clinically used between the sixties and early eighties. Second-generation PSs were synthesized to overcome the limitations of first-generation PSs. The third generation of PSs gathers chemically modified compounds for specific targeting of tumor tissues.

I-1.2.1. Properties of a photosensitizer

Extensive studies of first-generation PSs have led researchers to define the following prerequisite characteristics of an ideal photosensitizer. A good PS candidate should be pharmacologically stable, non-mutagenic and/or carcinogenic, non-toxic (as well as its metabolites) until activation by a light of appropriate wavelength (Allison *et al.*, 2004; Allison and Moghissi, 2013; Huang, 2005; Zane *et al.*, 2001). It should be commercially available, chemically pure to avoid unwanted side effects (Teixo *et al.*, 2015), and should not provoke serious allergic effects upon administration (Allison *et al.*, 2004; Plaetzer *et al.*, 2009). Pain should be avoided during the PDT process. The probability for a PS to undergo a transition from its S1 to T1 state should be high (triplet quantum yield $\Phi_T \geq 0.5$) (Huang, 2005; Josefsen and Boyle, 2008; Zhu and Finlay, 2008) and the PS should have a high quantum yield for substantial $^1\text{O}_2$ and ROS production (oxygen quantum yield $\Phi_\Delta \geq 0.5$) to guarantee an effective photodynamic effect (Josefsen and Boyle, 2008; Zane *et al.*, 2001). The T1 state should have a relatively long lifetime of the order of microseconds (triplet state lifetime τ_T in the microsecond to millisecond range), and a high energy ($\geq 94 \text{ kJ mol}^{-1}$) (Da and Vicente, 1996; Josefsen and Boyle, 2008). Also, the PS should absorb light at wavelengths far from those at which biological molecules such as hemoglobin do (Bozzini *et al.*, 2012). Its absorbance should be strong enough

in the near to infrared spectral region (650-800 nm), for deeper tissue penetration, and because single photon absorption at wavelengths higher than 800 nm does not provide sufficient energy to transform oxygen into its singlet state (Abrahamse and Hamblin, 2016; Allison *et al.*, 2004; Huang, 2005). The PS should also have a high extinction coefficient ($\epsilon > 30000 \text{ L.mol}^{-1}.\text{cm}^{-1}$) (Da and Vicente, 1996). It ought to be amphiphilic in order to be easily administered through various routes and able to cross a cell membrane. Ideally, it should rapidly and selectively accumulate in the diseased tissue, as already reported for tumor cells, but should also have short lifetime and be rapidly cleared from the patient normal tissues to minimize healthy skin sensitization (Ackroyd *et al.*, 2001; Allison and Moghissi, 2013; Bozzini *et al.*, 2012; Huang, 2005; Plaetzer *et al.*, 2009; Zane *et al.*, 2001). Selective localization of PS at the targeted site is an important issue as $^1\text{O}_2$ is only active in the immediate vicinity of the PS (Abrahamse and Hamblin, 2016; Nyokong and Ahsen, 2012). Finally, it should be less expensive than conventional therapies (Abrahamse and Hamblin, 2016).

Of course, the perfect PS that possesses all these qualities at once does not exist. However, various strategies have been adopted to obtain the ideal candidate (s). A few photosensitizers that generally meet the above-mentioned properties have been described, though (Calixto *et al.*, 2016).

I-1.2.2. First generation of photosensitizers

The first generation of PSs includes hematoporphyrin (Hp) and its derivatives (HpD and porfimer sodium, Photofrin[®]) which are naturally occurring porphyrins. Hp is the first PS isolated from dried blood by Scherer in 1841. Studies conducted on Hp have shown its fluorescence property, specific uptake and retention in tumors as well as its photodynamic action. However, high dosage of Hp was required for significant accumulation in the diseased tissue and, therefore, Hp was associated to long lasting skin photosensitivity (Daniell and Hill, 1991; Lipson *et al.*, 1961; O'Connor *et al.*, 2009). Chromatographic analysis of Hp showed that it was composed of a complex mixture of porphyrin-derived compounds. Two fractions have been identified, the first one being pure Hp with poor affinity to tumors, whilst the second one corresponded to impurity components and showed the greatest affinity to tumors.

Attempts to purify Hp by treatment with a mixture of sulfuric and acetic acids, then neutralization with a sodium acetate solution, led to the formation of a Hp derivative (HpD) which exhibited improved affinity to tumors compared to Hp (Dougherty, 1983; Juzeniene *et al.*, 2007; Lipson *et al.*, 1961). Like Hp, HpD is not a pure compound. It is composed of a mixture of porphyrin monomers, dimers and oligomers, linked by ether or ester bond or a combination of both (Figure 3). The monomers including hematoporphyrin stereoisomers and hematoporphyrin vinyl deuteroporphyrin isomers showed the lowest photodynamic activity because of their poorest selectivity to tumors (Moan and Berg, 1991). Photodynamic activity was attributed to ether-linked and ester-linked porphyrin aggregates also called dihematoporphyrin esters which are hydrophobic (Dougherty, 1987). Partial purification of HpD obtained by reducing most of the monomer components led to the most widely used PS in clinical PDT, marketed as Photofrin[®] (Agostinis *et al.*, 2011; Da and Vicente, 1996; Sharman *et al.*, 1999; Zane *et al.*, 2001). Although Photofrin[®] showed superior efficacy compared to HpD and Hp, the clinical use of all these first generation PSs was limited because: i) their complex mixture is difficult to reproduce (Da and Vicente, 1996; Denis *et al.*, 2013), ii) they have a weak Q-band absorption at 630 nm and this wavelength does not allow deep penetration of light in tissues, iii) their selectivity for tumor cells is poor, and, iv) they tend to accumulate and stay in healthy skin leading to serious cutaneous phototoxic side effects that last for about 2 months or more (Da and Vicente, 1996; Denis *et al.*, 2013; Ochsner, 1997). In addition, Photofrin[®] is not stable beyond 6 months (Dougherty, 1987).

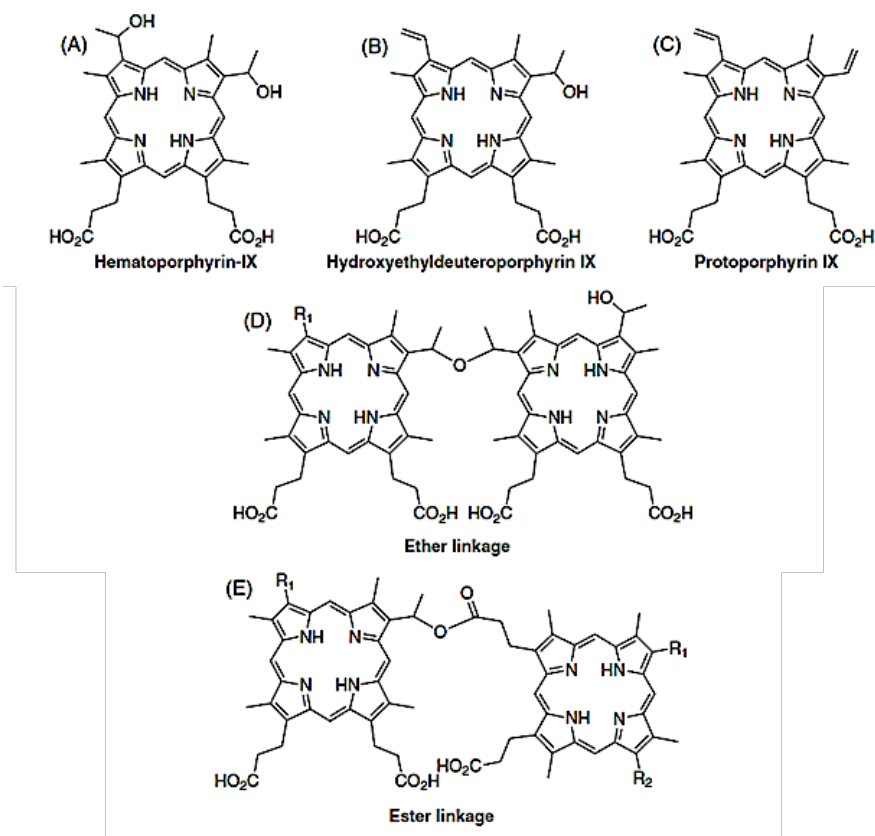


Figure 3: Structures of the major components found in haematoporphyrin derivative (HpD) or Photofrin®. (A) Hematoporphyrin; (B) Hydroxyvinyldeuteroporphyrin; (C) Protoporphyrin IX; (D) Dihematoporphyrin with an ether linkage; (E) Dihematoporphyrin with an ester linkage (from Denis et al., 2013).

I-1.2.3. Second generation of photosensitizers

Efforts have been made to synthesize chemically pure PSs with improved photophysical properties such as a strong absorption in the red spectrum at wavelengths longer than 630 nm, reduced skin photosensitivity, and higher specificity to tumors (Figure 4B). Second-generation PSs mostly encompass tetrapyrrolic compounds such as porphyrin derivatives, chlorins (Foscan®), bacteriochlorins (Tookad®), phthalocyanines (aluminum phthalocyanine tetrasulfonate or AlPcS4), porphycenes, purpurins (Purlytin®) and texaphyrins (Lu-tex®) (Figure 4A) but also non-porphyrin dyes, including phenothiazines (blue methylene), cyanines (merocyanine 540), dipyrromethenes, hypericin, xanthenes (rose bengal), curcumin and

chalcogenopyrylium dyes (DeRosa and Crutchley, 2002; Kostron and Hasan, 2016; O'Connor *et al.*, 2009; Zhang *et al.*, 2018).

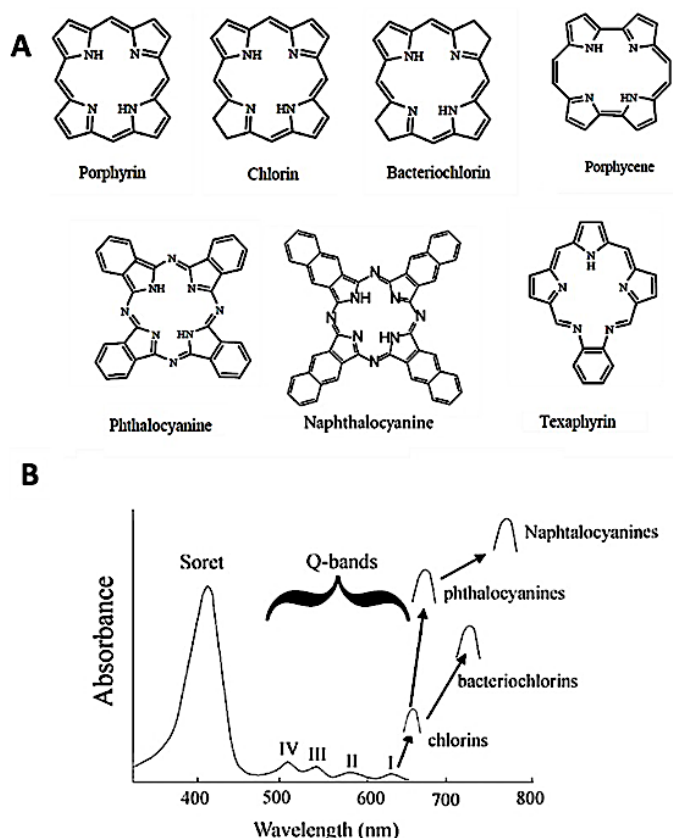


Figure 4: Basic structure of most second-generation tetrapyrrolic photosensitizers (A) and their typical absorbance spectrum showing the position of their Q-band I in the near-infrared region (from Moser *et al.*, 2015).

Tetrapyrrolic derivatives

Tetrapyrrolic PSs include porphyrins, chlorins, bacteriochlorins, phthalocyanines, porphycene, purpurins and texaphyrins. They are the PSs that have been employed the most in PDT applications. They are macrocycles with a tetrapyrrolic backbone similar to that of chlorophyll or hemoglobin.

Porphyrins represent a class of intense purple dyes which possess a tetrapyrrole backbone (porphine) consisting of four pyrrolic moieties generally connected to each other via methine

bridges (Figure 5A) (Josefsen and Boyle, 2008). They play essential roles in the biological activity of all living organisms (Lesage *et al.*, 1993) and their efficacy as PS is based on their ability to absorb light in the red to near infrared region of the optical spectrum. Actually, their absorption spectrum includes a strong band called the Soret band near 400 nm, followed by four weaker bands between 600 and 800 nm also referred to as the satellite bands or Q-bands (Figure 5B). Porphyrins are present in large quantities in natural sources (mammalian blood) or can be entirely synthesized (Sternberg *et al.*, 1998). Several approaches have been used to develop second-generation porphyrins. Among them, we can mention the substitution of the porphine macrocycle at its β and/or meso positions and the modification of the side chains in naturally available porphyrins such as Hp and protoporphyrin, which result in a slight modification of PS absorption spectra and solubility (Denis *et al.*, 2013; Sternberg *et al.*, 1998). Metal insertion at the center of porphyrins induces a drastic red shift of a porphyrin absorption spectrum as well as its photostability and photophysical properties. These porphyrins are referred to as metalloporphyrins. Depending on their nature, the central ion may prolong the T1 lifetime, thus improving the PDT effect, or quickly deactivate the excited state, thereby inhibiting ROS formation (Denis *et al.*, 2013).

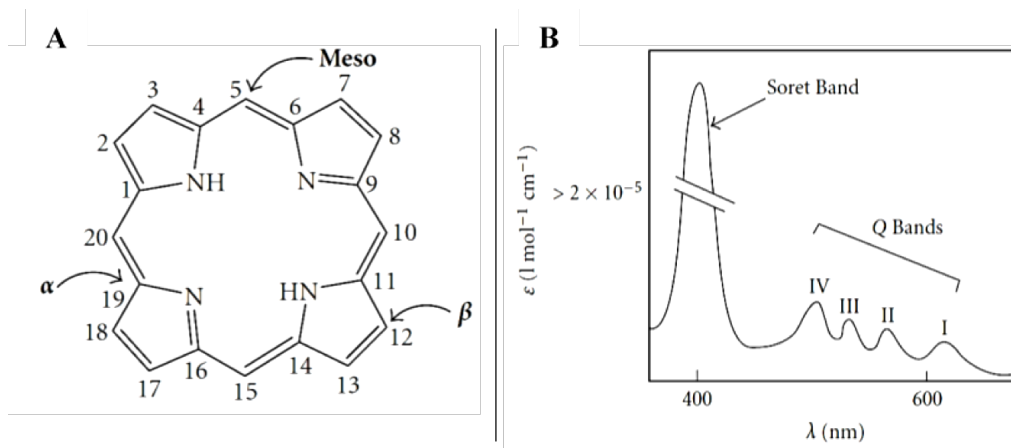


Figure 5: A) Structure of porphine, the tetrapyrrolic backbone of porphyrins; B) Characteristic absorption spectrum of porphyrins (from Josefsen and Boyle, 2008).

Among the second-generation porphyrin derivatives, the most attractive are 5-aminolevulinic acid (ALA) and the 5,10,15,20-tetrakis-(m-hydroxyphenyl) porphyrin (m-THPP).

5-aminolevulinic acid (ALA)

ALA has been introduced in 1990 as a prodrug PS (Kennedy *et al.*, 1990). It is administered topically or systemically (oral, intravenous and intradermal routes) and is marketed in its pure form as Levulan[®] kerastic (DUSA Pharmaceuticals, Valhalla, NY, USA), and in the form of alkyl ester derivatives (methyl-ALA, Metvix[®] and benzyl-ALA, Benxvix[®]) (Figure 6A). The higher lipophilicity of these derivatives compared to ALA allows their deeper penetration in the tissues (Gomes *et al.*, 2018).

ALA is naturally formed within the body by the condensation of glycine and coenzyme-A through ALA Synthase (ALAS), and undergoes a series of reactions leading to the formation of protoporphyrin IX (Pp IX) (Figure 6B), the immediate precursor of heme. Pp IX is converted into heme by insertion of ferrous ion in its center. This reaction is catalyzed by a ferrochelatase. Under normal conditions, the rate at which all these reactions occur is tightly regulated by the needs for heme, and a feedback inhibition mechanism maintains low level of porphyrin intermediates potentially responsible for oxidative stress and cellular damage. Administration of exogenous ALA in excess is believed to bypass the regulatory process thereby leading to an accumulation of Pp IX in cells. Pp IX is an effective PS which absorbs light at 635 nm. The photodynamic effect of ALA-induced Pp IX has been demonstrated in vitro and in vivo on a variety of tumors (Nokes *et al.*, 2013). ALA-induced Pp IX tends to accumulate selectively in tumors that arise from the epidermis (basal and squamous cell carcinoma, adenocarcinoma of the sebaceous gland), the bronchi (lung squamous cell carcinoma), the mammary tissue (breast adenocarcinoma) and the salivary gland (parotid squamous cell carcinoma) (Kennedy and Pottier, 1992). This selectivity towards tumor tissue might be explained by the significant variation of the level of heme-synthesis-rate limiting enzymes (ALAS, ferrochelatase, porphobilinogen deaminase), the presence of damaged epithelium, and tumor tissue vascular permeability (Krammer and Plaetzer, 2008; O'Connor *et al.*, 2009; Yang *et al.*, 2015). Pp IX is rapidly cleared from the body within 24h whatever the route of administration used (Kelty *et al.*, 2002; Krammer and Plaetzer, 2008), which limits skin photosensitization.

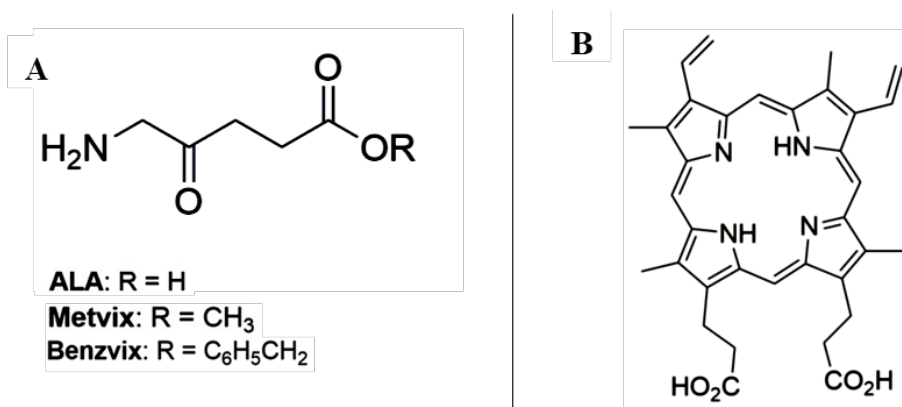


Figure 6: Chemical structure of A) 5-aminolevulinic acid and its derivatives; and B) Protoporphyrin IX (Pp IX) (from Gomes *et al.*, 2018).

5,10,15,20-tetrakis-(*m*-hydroxyphenyl) porphyrin (*m*-THPP)

m-THPP is one of the first synthetic porphyrin derivatives that have been easily synthesized by single-step reaction from pyrrole and 3-methoxybenzaldehyde (Figure 7) (Berenbaum *et al.*, 1986). It is a potent hydrophobic PS with good photophysical properties (λ_{max} : 644 nm, Φ_{T} : 0.69, τ_{T} : 120 μs , Φ_{Δ} : 0.46) (Bonnett *et al.*, 1999) and better selectivity for tumors than first-generation PSs. It was found to be at least 25 times more effective than HpD, with reduced skin sensitization (Berenbaum *et al.*, 1986).

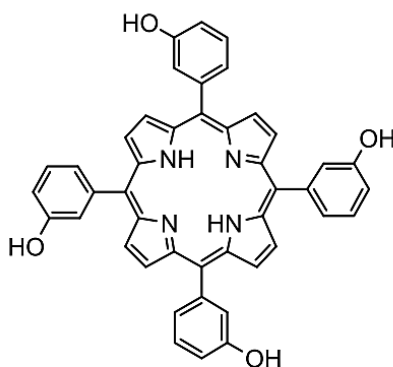


Figure 7: Chemical structure of *m*-THPP.

Chlorins differ from porphyrins by the lack of a double bond on the tetrapyrrolic ring (Figure 8). They are synthesized starting with natural materials such as chlorophyll-a (Chl a) (Ali and Van Lier, 1999; Denis *et al.*, 2013; Montforts, 1995), and Pp IX (Denis *et al.*, 2013). They are also prepared by diimide reduction of synthetic tetraphenylporphyrin (TPP) or octaethylporphyrin (OEP) derivatives (Bonnett *et al.*, 1989; Da and Vicente, 1996). This chemical reduction leads to a shift of the porphyrin absorption Q band towards longer wavelengths, between 650-690 nm. Thus, chlorins are good PS candidates because of this absorption band in the red region which allows deeper penetration of light into biological tissues, and their high molar extinction coefficient. Most of the promising PSs already marketed or under clinical trials are chlorin analogs which include 5,10,15,20-tetrakis(*m*-hydroxyphenyl)chlorin (*m*-THPC, Foscan®), benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin®), tin ethyl etiopurpurin (SnET2, Rostaporfin, Purlytin®), N-aspartyl chlorin e6 (NPe6, Talaporfin, Ls11), and 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH, Photochlor®) (Ormond and Freeman, 2013).

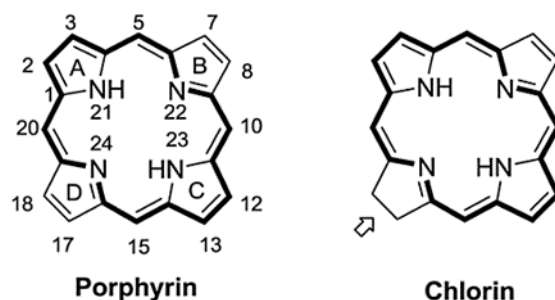


Figure 8: Structural comparison of porphyrin and chlorin (from Yoon *et al.*, 2013).

5,10,15,20-tetrakis(*m*-hydroxyphenyl) chlorin (*m*-THPC), Temoporfin (Foscan®), is prepared by diimide reduction of its porphyrin parent *m*-THPP, and is one of the most effective chlorin derivative, fulfilling almost all prerequisites of the ideal PS. Indeed, its photophysical properties (λ_{max} : 650 nm, Φ_{T} : 0.89, τ_{T} : 50 μs , Φ_{A} : 0.43 and ϵ : 29,600 $\text{dm}^3 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) allow inducing an effective PDT response. *m*-THPC exhibits greater selectivity to various tumors and is much more effective at very low concentration (~ 0.1 -0.15 mg/kg) and low light dose (~ 10 -

20 J/cm²) than HpD, Photofrin® (Bonnett, 1995; Bonnett *et al.*, 1999; Dougherty *et al.*, 1993; Mitra and Foster, 2005), ALA (Bourré *et al.*, 2002) and *m*-THPP (Berenbaum *et al.*, 1993). Unfortunately, because of its high effectiveness, patient exposure to room light may cause serious burns (Friedberg *et al.*, 2003). Nevertheless, it shows reduced skin sensitization compared to Photofrin® (2 vs 4-8 weeks) (Da and Vicente, 1996; O'Connor *et al.*, 2009; Rodriguez *et al.*, 2004; Triesscheijn *et al.*, 2006) and is painful (Allison *et al.*, 2004).

Benzoporphyrin Derivative Monoacid Ring A (BPD-MA) or verteporfin (Visudyne®), a semi-synthetic chlorin-type compound rather than a porphyrin one, is formed by Diels–Alder reaction of Pp IX and dimethyl acetylene dicarboxylate (Denis *et al.*, 2013). This PS has a significant absorption at 690 nm. It accumulates in tumors within 30-150 min and is quickly removed from the body 24 hours following administration (Andrejevic Blant *et al.*, 2000; Richter *et al.*, 1990). It exhibits superior photodynamic effect and lower skin sensitization than *m*-THPC (Opitz *et al.*, 2006). Its photodynamic action is reported to be mediated by both type I and type II mechanisms (Hadjur *et al.*, 1997). BPD-MA induces significant destruction of tumor blood vessels. Because it is lipophilic, it has been incorporated into liposomes (Fingar *et al.*, 1999).

Tin ethyl etiopurpurin (SnET2, Rostaporfin) or Purlytin® is a metallochlorin based on a complex of tin with purpurin, an analogue of the degradation product of chlorophyll (Garbo, 1996). SnET2 has been tested in a cremophor emulsion in clinical trials because of its hydrophobicity (Pogue *et al.*, 1998). It has a strong absorption Q band at 660 nm and a high molar extinction coefficient. It has been reported to induce a strong tumoricidal effect. Although it also causes skin sensitization, this side effect rapidly disappears a week after treatment (Morgan *et al.*, 1990).

N-aspartyl chlorin e6 (NPe6, Talaporfin, Ls11) is an analogue of chlorin e6 (Ce6) which is a derivative of chlorophyll-a obtained by condensation of Ce6 with L-aspartic acid. Thanks to its 4 carboxylic acid substituent groups, NPe6 is amphiphilic. Its absorption peak shows maximum absorption at 664 nm with high molar extinction coefficient, and is a good generator of singlet oxygen (Spikes and Bommer, 1993). NPe6 is an excellent tumor PS since it accumulates quickly in tumors, about 4 to 6 h following administration.

I-1.2.4. Third generation of photosensitizers

Although second-generation PSs have unmistakably proven their superior efficacy over first-generation PSs, they exhibit, however, a modest specificity toward tumor cells (Calixto *et al.*, 2016; Knop *et al.*, 2009; Paszko *et al.*, 2011). Moreover, most of them undergo aggregation in aqueous medium because of their lipophilic nature. Therefore, further investigations were devoted to the design of amphiphilic PSs with higher selectivity and affinity to tumor tissues (Allison *et al.*, 2004; Gomes *et al.*, 2018; Lee and Kopelman, 2011). These new PSs also called targeted PSs are referred to as third-generation PSs and most of them derive from second-generation PSs.

Two main strategies have been adopted to develop these PSs (Lupu *et al.*, 2016). The first one consisted in grafting specific ligands to existing second-generation PSs (DeRosa and Crutchley, 2002). Examples of ligands that have been used are monoclonal antibodies (MAbs) (e.g., human epidermal growth factor receptor 2 or interleukin-2 receptor) (Moore *et al.*, 2009; Van Dongen *et al.*, 2004), proteins and peptides (e.g., galectin-1, folic acid receptor, estrogen receptor, low density lipoproteins (LDL) receptor, bile acids, transferrin, shiga toxin or polyamines) (Diddens *et al.*, 1997; Ferreira *et al.*, 2013; Gomes *et al.*, 2018; Jiang *et al.*, 2010; Rogers *et al.*, 2013; Stallivieri *et al.*, 2017; Swamy *et al.*, 2006), or carbohydrates (e.g., hyaluronic acid, galactose, lactose, mannose or glucopyranose) (Bae and Na, 2012; Han *et al.*, 2016; Laville *et al.*, 2006; Lupu *et al.*, 2018; Maillard *et al.*, 2007; Soares *et al.*, 2010; Zheng *et al.*, 2001).

The second strategy consisted in encapsulating the PSs in nanocarriers. This vectorisation is described in section I.3.

I-2. Retinoblastoma

The first case of retinoblastoma (RB) mentioned in the literature dates back to 1597 when Petrus Pawius reported an autopsy he had performed on a young child (Dunphy, 1964; Grossniklaus, 2014). Retinoblastoma is a rare malignant tumor of the retina which affects children of less than 5 years old (Hilkert *et al.*, 2017; Rodriguez-Galindo *et al.*, 2015), and represents today 2-4% of all pediatric cancer diagnosed (Delhiwala *et al.*, 2016). It has an annual worldwide incidence of 1 case per 15,000 to 20,000 births (Delhiwala *et al.*, 2016; Lankowsky *et al.*, 2016) with the highest number of cases observed in developing countries (Hilkert *et al.*, 2017) or in countries with high birth rates (Grossniklaus, 2014).

I-2.1. Diagnosis and conventional treatments

Retinoblastoma is a visible tumor that can be detected easily without using sophisticated diagnostic imaging tools. The most characteristic sign of RB is an abnormal white reflection called leukocoria (Figure 9a) that is visible through the pupil (Dimaras and Corson, 2018; Dyer, 2016). Other signs are misaligned eyes also called strabismus, and proptosis (Dimaras and Corson, 2018) an abnormal bulging of the eye out of the orbit. This latter sign, often observed in developing countries, indicates an advanced stage of the tumor with a higher mortality rate (Hilkert *et al.*, 2017). Imaging techniques like ultrasonography, fluorescein angiography, magnetic resonance imaging (MRI), retinal camera (RetCam) imaging or computed tomography are also used for RB diagnosis (Aerts *et al.*, 2006; Lankowsky *et al.*, 2016).

Retinoblastoma is a genetic disease. The tumor suppressor gene RB1 has been implicated in disease occurrence, and it has been established that almost all RB patients present one mutation of this gene in both alleles of their chromosome 13q14 (Cowell, 1991). There are 2 forms of RB: (i) the hereditary form where all body cells already carry one inactive RB1 allele, and the inactivation of the second RB1 allele occurring in the retina initiates the development of the tumor, and (ii) the sporadic form where the inactivation of both RB1 alleles occurs spontaneously in a retinal cell (Aerts *et al.*, 2006; Dunphy, 1964). RB is also qualified as unilateral or bilateral whether it affects one eye or both of them, and as unifocal or multifocal when one or more tumors are found in each eye. The sporadic form, which represents ~60-70%

(Cerman and Çekiç, 2015) and is commonly diagnosed when the child is ~2 years old (Maillard *et al.*, 2007), is usually unilateral and unifocal. In general, bilateral and multifocal tumors (Figure 9b) are observed in the hereditary form of RB (Lanzkowsky *et al.*, 2016). They account for 30-40% cases. However, 15% of patients presenting unilateral RB were reported to have an inherited mutation.

Different treatment modalities are available depending on the type, the location and the stage of the tumor(s). For early diagnosed RB, conservative therapies are performed aiming to save the life and preserve the vision of the patient, while in case of late diagnosis, the priority is to save life and avoid the development of the cancer as the vision is already affected (Aerts *et al.*, 2006; Dimaras and Corson, 2018; Ramírez-Ortiz *et al.*, 2017). Focal therapies are applied to small tumors. They include external beam radiation (EBR), photocoagulation, cryotherapy, hyperthermia, or brachytherapy, sometimes associated. Patients are often treated first by chemotherapy (CT) to achieve chemoreduction of the tumor(s) size prior to the application of the focal therapies (Lanzkowsky *et al.*, 2016; Rodriguez-Galindo *et al.*, 2015; Teixeira *et al.*, 2015). For patients presenting medium-to-large size intraocular tumors as well as extraocular tumors, CT and/or enucleation are systematically indicated. Carboplatin, sarcolysin (Melphalan[®], Shields *et al.*, 2014), vincristine, cyclophosphamide, doxorubicin, ifosfamide and etoposide are common chemotherapeutic drugs, often administered as a cocktail (VDC for vincristine, cyclophosphamide and doxorubicin or VCE for vincristine, carboplatin and etoposide) (Grossniklaus, 2014; Lanzkowsky *et al.*, 2016). In addition to intravenous (IV) delivery of these chemotherapeutics agents, various routes of administration have been explored such as intra-arterial, periocular, and intravitreal route. These approaches also favor the destruction of RB seeds which disseminate in the fluid as dust, clouds or spheres and are responsible for disease relapse and/or metastasis (Francis *et al.*, 2017, 2014; Kalita *et al.*, 2014; Shields *et al.*, 2013). Intravitreal CT using methotrexate or sarcolysin (Shields *et al.*, 2013) have shown promising response for recurrent RB (Munier *et al.*, 2013).

Despite the real benefits brought in terms of prognosis, most of the above mentioned therapies are associated to severe adverse effects (Cerman and Çekiç, 2015; Francis *et al.*, 2017, 2014; Manjandavida and Shields, 2015; Munier *et al.*, 2013; Teixeira *et al.*, 2015). Some of them, mainly radiation therapies (EBR, radiotherapy) and CT favor the development of secondary tumors (Aerts *et al.*, 2006; Teixeira *et al.*, 2015). Multi-drug resistance is another issue encountered with CT (Das and Sahoo, 2012).

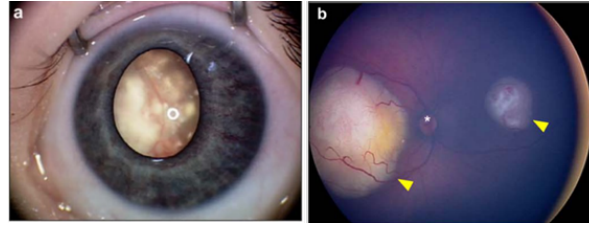


Figure 9: Clinical characteristics of retinoblastoma with a) leukocoria and b) bifocal tumors visible in the eye (from Dimaras and Corson, 2018).

I-2.2. Application of PDT to retinoblastoma

PDT has emerged as an interesting alternative to retinoblastoma conventional therapies as it is non-mutagenic, can activate the immune system to attack tumor cells, and generates less side effects than conventional therapies (Cerman and Çekiç, 2015; Teixeira *et al.*, 2015; Walther *et al.*, 2014). First clinical applications of PDT to retinoblastoma date from the eighties (Murphree *et al.*, 1987; Ohnishi *et al.*, 1986). Hematoporphyrin derivative (HpD) was effective against small RB when used alone, but an additional therapy was necessary in the case of large tumors (Teixeira *et al.*, 2015). Several preclinical studies were conducted in vitro and in vivo which demonstrated the potential efficacy of PDT for curing RB. However, no clinical application to RB has been reported (Lupu *et al.*, 2018). Tetrahydroporphyrin tetratosylat (THPTS) (Walther *et al.*, 2014), verteporfin (Stephan *et al.*, 2008), m-THPC (Aerts *et al.*, 2010) and ALA (Ruiz-Galindo *et al.*, 2007) are examples of PSs evaluated for RB treatment. In most studies, the intracellular localization of the photosensitizers was not determined, and it was not yet clear at which level of the cancer tissue PDT effects occurred. It has been recently suggested, however, that PDT acts preferentially by destroying RB vasculature (Teixeira *et al.*, 2015).

Different strategies have been proposed to favor specific accumulation of PSs in RB tissues. Chlorin e6-LDL conjugates were synthesized and showed significant uptake by RB cells (Diddens *et al.*, 1997). Maillard and coworkers developed porphyrin glycoconjugates (Laville *et al.*, 2006; Lupu *et al.*, 2018; Maillard *et al.*, 2007) to improve the amphiphilicity of the porphyrins and their affinity to RB cells, as it has been showed that RB cells overexpress sugar receptors (Gallud *et al.*, 2015; Griegel *et al.*, 1989). Specific interaction of the glycoconjugated

PSs with the sugar receptors of RB cells was demonstrated in our laboratory (Makky *et al.*, 2012, 2011).

All these investigations proved the rationale of using PDT as a conservative treatment of RB.

I-3. Photosensitizer delivery systems

The major concern about most PSs is their poor water-solubility leading to aggregation in aqueous environment (Da and Vicente, 1996; Dąbrowski *et al.*, 2016; Monge-Fuentes *et al.*, 2014), therefore inactivating their photodynamic activity. Moreover, because of their lipophilic nature and lack of specificity toward tumor cells, they accumulate into the skin, which induces the skin photosensitivity reported as the main PDT side effect (Friedberg *et al.*, 2003; Gijssens *et al.*, 2002; Paszko *et al.*, 2011). These PSs require to be solubilized in order to accumulate at sufficient high dose in the targeted tissue and lead to the desired therapeutic effect.

Several approaches have been tested to achieve this goal. Among them is the use of non-aqueous solvents such as DMSO (Richter *et al.*, 1990), ethanol, polyethylene glycol 400 (PEG 400) (Jones *et al.*, 2003) and propylene glycol (Saavedra *et al.*, 2014). However, these solvents are toxic (Strickley, 2004). Natural eutectic solvents were also proposed to solubilize PSs (Wikene *et al.*, 2015). Another strategy was their complexation with cyclodextrins (Mazzaglia *et al.*, 2010; Samaroo *et al.*, 2014). Beside the conventional solubilization approaches, incorporation of the PSs in nanomedicines was also envisioned. Nanomedicines have earned growing attention in many fields since their emergence few decades ago, because of their tremendous benefits. Indeed, different types of nanomedicines exist, which allow to encapsulate hydrophobic and hydrophilic drugs for solubilization or protection against degradation (Ge *et al.*, 2014). More than one drug can be loaded into these systems. They can cross biological barriers (Lee and Kopelman, 2011; Paszko *et al.*, 2011) and be specifically addressed to their target by decoration of their surface with specific ligands (Nichols and Bae, 2013). Their surface can also be functionalized with long PEG chains to prolong their circulation time. This increases their bioavailability in the body by avoiding opsonization that is the first step before rapid clearance from the blood (Cho *et al.*, 2008; Sobot *et al.*, 2016). Another important benefit of nanomedicines is that they can help reducing considerably drug side effects when the therapeutic index of the encapsulated drug is increased (De Jong and Borm, 2008; Liu *et al.*,

2015). Moreover, they have been reported to preferentially accumulate into tumors, thanks to their nanoscale size. This was attributed to the enhanced permeability and retention (EPR) effect (Fattal and Tsapis, 2014; Maeda, 2017; Maeda *et al.*, 2016). Finally, drug release can be controlled (Bobo *et al.*, 2016).

Various nanocarriers have been developed to solubilize PSs and protect them from the aqueous environment, but also to enhance their specific accumulation into tumors. The most frequent nanocarriers used were micelles (Van Nostrum, 2004), liposomes (Gaio *et al.*, 2016) and nanoparticles (Master *et al.*, 2013). PSs were also loaded in micro and nano-emulsions (Junping *et al.*, 2003; Maisch *et al.*, 2009; Primo *et al.*, 2008). Many of these nanocarriers were functionalized with grafted ligands in order to target specific receptors. The literature reports on micelles, carbon nanoparticles, liposomes, porous hollow nanospheres, gold NPs, in which PSs have been entrapped either physically or covalently (Gijssens *et al.*, 2002; Liang *et al.*, 2014; Lupu *et al.*, 2016; Ma *et al.*, 2014; Master *et al.*, 2013, 2012b; Simon *et al.*, 2010). In the latter case, they were generally bound to the nanocarrier's core (Brevet *et al.*, 2009; Gary-Bobo *et al.*, 2012) or to its surface (Mackowiak *et al.*, 2013). The covalent linkage of PSs to nanocarriers allows to bypass the premature leakage observed with physically loaded PSs.

I-3.1. Micelles

Amphiphilic surfactants and copolymers are used above their critical micelle concentration (CMC) to obtain micelles. Cremophor EL (polyoxyethylene glycerol triricinoleate) is a surfactant often used to solubilize PSs (Dąbrowski *et al.*, 2016; Huang *et al.*, 2012b). However, because it is toxic, Cremophor EL has been replaced by other copolymers including Pluronic P123, PEG-lipid conjugates (mPEG₂₀₀₀-DSPE) (Van Nostrum, 2004), poly (ϵ -caprolactone)-b-poly (ethylene oxide), poly (ethylene oxide)-b-o-poly (ϵ -caprolactone) (Hofman *et al.*, 2008), poly (ethyleneoxide-b- ϵ -caprolactone), poly (ethyleneoxide-b-D,L-lactide), poly (ethyleneoxide-b-styrene) (Gibot *et al.*, 2014), poly (ethylene glycol)-co-poly (D,L-lactic acid) (Ding *et al.*, 2011) and poly (2-ethyl-2-oxazoline)-b-poly (D,L-lactide) (Senge and Brandt, 2011). The hydrophilic segments of these surfactants are exposed to the aqueous environment while the hydrophobic ones form the core of the micelles in which PS molecules are entrapped. These micelles have a size ranging from 10 to 100 nm (Dąbrowski *et al.*, 2016; Van Nostrum, 2004). Micelles decorated with a peptide ligand (e.g., the epidermal growth factor receptor,

EGFR) showed rapid cellular uptake and improved photodynamic activity compared to non-specific micelles (Master *et al.*, 2012b).

I-3.2. Liposomes

Liposomes have been largely used as delivery systems for photosensitizers. They are unilamellar or multilamellar vesicles composed of phospholipid bilayer(s) enclosing an inner aqueous compartment. Liposomes are biocompatible and biodegradable thanks to their resemblance with biological membranes (Puri, 2014). Both hydrophilic and hydrophobic PSs have already been encapsulated into liposomes (Jin and Zheng, 2011). Hydrophobic PSs were incorporated in the lipid bilayer so that their aggregation was prevented, and they remained in their active form, while the hydrophilic PSs were solubilized in the aqueous core of the vesicles (Huang *et al.*, 2012a; Jin and Zheng, 2011).

Co-encapsulation of a PS with another hydrophobic active ingredient in the lipid bilayer is also possible (Song *et al.*, 2017). Various phospholipid compositions have been used in PSs liposomal formulations, such as DPPC (Jiang *et al.*, 1998), POPC/DOPS 90/10 (Isele *et al.*, 1994), egg phosphatidylcholine (Egg PC) (Wang *et al.*, 1999), DMPC (Senge and Brandt, 2011; Szurko *et al.*, 2009), DMPC/cationic surfactant mixture (Bombelli *et al.*, 2008), cetyl-PEI (cetylated polyethylenimine)/DPPC/cholesterol/DPPG mixture (Takeuchi *et al.*, 2004), and a mixture of ceramide/cholesterol/palmitic acid/cholesteryl sulfate specifically designed to mimic the mammalian stratum corneum lipid composition (Pierre *et al.*, 2001). Marketed liposome formulations include Visudyne[®], a formulation of verteporfin prepared with egg phosphatidylglycerol (Egg PG) and dimyristoyl phosphatidylcholine (DMPC) (Bulbake *et al.*, 2017) and Foslip[®], a liposomal formulation of m-THPC (temoporfin) prepared with a DPPC/DPPG lipid mixture (Lassalle *et al.*, 2009). Functionalized PS-loaded liposomes were also designed using PEG-lipid conjugates such as DSPE-mPEG₅₀₀₀ (Song *et al.*, 2017) terminated by antibodies, folate, transferrin, peptides or other ligands, which contributed to the prolongation of their circulating time and led to their specific accumulation in a targeted tissue (Chen *et al.*, 2005; Dąbrowski *et al.*, 2016; O'Connor *et al.*, 2009; Song *et al.*, 2017).

Specific lipids or various conditions such as temperature, pH or light may trigger drug release from liposomes (Derycke and De Witte, 2004; Massiot *et al.*, 2017). This approach was also

applied to PSs (Puri, 2014). All these studies demonstrated that liposomes are suitable carriers for overcoming PSs aggregation issue and enhance tumor accumulation (Chen *et al.*, 2005; Huang *et al.*, 2012a; Jin and Zheng, 2011).

I-3.3. Nanoparticles

A wide variety of nanoparticles (NPs) were explored as PSs nanocarriers. NPs exist as nanospheres (solid matrix) or nanocapsules (liquid core surrounded by a solid shell) which can be designed with different features to encapsulate both hydrophobic and/or hydrophilic drugs or multi-drugs (Lee and Kopelman, 2011; Paszko *et al.*, 2011; Vauthier and Bouchemal, 2009). They are obtained using various materials and techniques and generally exhibit higher drug loading and better controlled release than liposomes (Dąbrowski *et al.*, 2016). NPs are classified as (i) biodegradable or not, depending on the nature of materials used for their preparation, and (ii) passive or active NPs according to whether a ligand is attached or not to their surface (Chatterjee *et al.*, 2008). PSs are incorporated inside NPs through hydrophobic or electrostatic interactions with the material used, or by covalent binding to their core or surface.

I-3.3.1. Biodegradable NPs

Biodegradable NPs include NPs prepared from natural or synthetic biodegradable sources such as polymers, polysaccharides, proteins or peptides. The most common polymers used for PSs delivery belong to the aliphatic polyester family, like PLGA (poly(D,L-lactide-co-glycolide)) (Fadel *et al.*, 2010; Konan-Kouakou *et al.*, 2005; Konan *et al.*, 2003) and PLA (poly(D,L-lactic acid)) (Zeisser-Labouèbe *et al.*, 2006, 2009) and are well-known to be biocompatible and biodegradable (Xiao *et al.*, 2006). PSs are loaded inside a nanoparticle core. For example, anti-VEGF (vascular endothelial growth factor)-functionalized PLGA NPs were developed for the treatment of age-related macular degeneration (Zuluaga *et al.*, 2007). Polysaccharides such as xanthan gum (Deda *et al.*, 2009), dextran, chitosan (Sun *et al.*, 2009), alginate (Khdaïr *et al.*, 2008) and hyaluronic acid (Master *et al.*, 2013) were used to form nanoparticles for PSs delivery, but also collagen (Deda *et al.*, 2009), human or bovine serum albumin (HSA or BSA) (Jeong *et al.*, 2011; Rodrigues *et al.*, 2009), and LDLs (Song *et al.*, 2007).

I-3.3.2. Non-biodegradable NPs

Nanocarriers made of inorganic and metallic-based materials were also developed for PSs delivery. They include mesoporous silica NPs (MSNs) (Brevet *et al.*, 2009), quantum dots, gold NPs (AuNPs) (Záruba *et al.*, 2010) and magnetic NPs (iron oxide) (Roblero-Bartolón and Ramón-Gallegos, 2015). Other types of NPs were described by Huang *et al.* (2012a) and Dąbrowski *et al.* (2016). PSs were either loaded or covalently grafted inside these NPs or to their surface (Brevet *et al.*, 2009; Chatterjee *et al.*, 2008; Gary-Bobo *et al.*, 2012; Huang *et al.*, 2012a; Mackowiak *et al.*, 2013; Záruba *et al.*, 2010). PSs covalently linked to the particles were reported to be more effective, as this approach prevented their release from the system (Lee and Kopelman, 2011).

More complex systems, e.g., AuNPs coated with a silica shell entrapping a PS were also proposed (Fales *et al.*, 2011). Some of these NPs were coated by biodegradable materials to improve their biocompatibility and stability (Roblero-Bartolón and Ramón-Gallegos, 2015). There are examples of MSNs coated with a lipid shell (Yang *et al.*, 2010) or magnetic iron oxide NPs coated with a dextran shell (McCarthy *et al.*, 2006). The major issue encountered with PSs encapsulated in nanoparticles is their tendency to self-quench. This phenomenon was mainly observed with high concentrations of PSs, due to tight stacking of PS molecules in the nanoparticles (Konan-Kouakou *et al.*, 2005; Master *et al.*, 2013, 2012a). Self-quenching results in a reduction of the quantum yield of singlet oxygen (Hamblin and Avci, 2015). This observation was also reported to occur in micelles (Ding *et al.*, 2011). As previously mentioned, singlet oxygen generated inside the nanocarriers should diffuse out of them in order to kill cells (Dąbrowski *et al.*, 2016; Simon *et al.*, 2010), but it has a very short lifetime ($< 0.04 \mu\text{s}$). Its diffusion depends on the material constituting the nanoparticle (Jeong *et al.*, 2011). One solution to avoid a PS self-quenching and make singlet oxygen immediately available is to covalently bind the PS to the surface of the nanoparticle (Couleaud *et al.*, 2010; Eshghi *et al.*, 2013; Guo *et al.*, 2010; Sève *et al.*, 2012).

I-3.4. Liponanoparticles (LNP)

Liposomes and NPs are the most popular nanocarriers investigated because of their great potentials as drug delivery systems. As mentioned above, these systems have been proved to solubilize and protect drugs from degrading or inactivating environment. They have succeeded to deliver drugs to tumors by both passive and active pathways, thanks to their functionalization with PEG moieties which prolong their circulation half-time and to grafted ligands allowing specific interaction with the diseased tissue. Moreover, they are biocompatible and can be designed with biodegradable excipients (Paszko *et al.*, 2011). Despite all these interesting features, both liposomes and NPs suffer from important limitations. Liposomes exhibit low hydrophobic drug entrapment and short circulating half-time due to their rapid uptake from the bloodstream by the reticuloendothelial system (RES) (Chen *et al.*, 2005). They are unstable in biological environment because of their rapid interaction with plasma proteins resulting in the leakage of the drug before it reaches its target (Brasseur *et al.*, 1991). They are also unstable during storage and their preparation process is difficult to reproduce from batch to batch (Lapinski *et al.*, 2007; Zhang and Zhang, 2010). Limitations encountered with NPs are the use of toxic organic solvents for their preparation, biocompatibility and/or toxicity of some polymers employed (Raemdonck *et al.*, 2014), fast release of the drug by burst effect (Sengel-Turk and Hascicek, 2017). However, NPs present some advantages over liposomes, like their higher drug loading capacity, better stability (Krishnamurthy *et al.*, 2015), and prolonged release effect (Hadinoto *et al.*, 2013). Furthermore, their design is more controllable and reproducible.

These limitations led researchers to design a new generation of nanoparticles named lipid polymer hybrid nanoparticles (thereafter referred to as liponanoparticles or LNPs). LNPs are core-shell nanocarriers combining both assets of liposomes and nanoparticles while overcoming some of their drawbacks. Their structure is composed of a nanoparticle core coated by a lipid layer. The solid core is believed to bring physical stability to the system, to serve as a matrix for the delivery of both hydrophilic and lipophilic drugs, and to lead to particles with controlled size and reproducible morphology (Chan *et al.*, 2009; Cheow and Hadinoto, 2011; Ma *et al.*, 2017; Troutier and Ladavière, 2007; Von Hoegen, 2001). The lipid shell acts as a membrane barrier preventing drug leakage from the NP core and makes the system more biocompatible (Hadinoto *et al.*, 2013; Sengel-Turk and Hascicek, 2017). It participates to the

controlled release of the encapsulated drugs and can also serve for the loading of even small hydrophobic or hydrophilic molecules and macromolecules such as DNA or antibodies (Asthana *et al.*, 2015; Cao *et al.*, 2018; Sengel-Turk *et al.*, 2018; Troutier-Thuilliez *et al.*, 2009). LNPs surface can be modified to prolong their circulation time in the body, improve their stability, increase their affinity for the diseased tissue, and reduce their recognition and removal by the immune system (Liu *et al.*, 2010; Pramual *et al.*, 2017; Salvador-Morales *et al.*, 2009; Thevenot *et al.*, 2007). LNPs have been largely developed as drug delivery systems (Grigoras, 2017; Mandal *et al.*, 2013; Tan *et al.*, 2013), but also as biomimetic models for investigating the dynamics and surface chemistry of biological membranes (Bayerl and Bloom, 1990; Savarala *et al.*, 2010) or as in vitro biosensors for the diagnosis of some diseases (Eschwège *et al.*, 1996). They were also used as a tool in the biomedical field for the separation and purification of proteins (Bucak *et al.*, 2003). Different methods allow formation of LNPs having lipid monolayer or bilayer/multilayers shell(s) as illustrated in Figure 10.

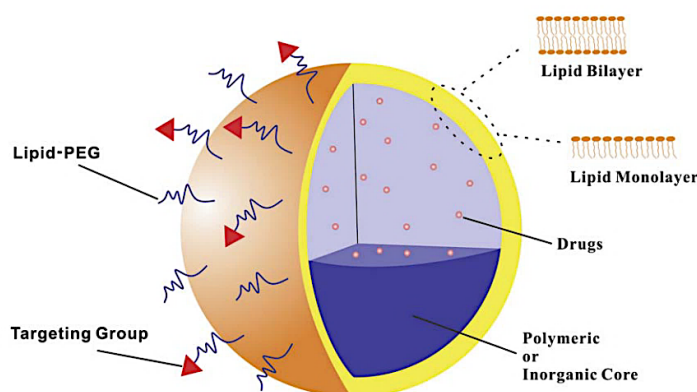


Figure 10: Schematic illustration of the structure of a LNP (from Tan *et al.*, 2013).

I-3.4.1. Preparation methods for LNPs

Single-step and multi-step techniques have been proposed to obtain LNPs with an inner core made of natural, or synthetic organic and inorganic materials (Troutier and Ladavière, 2007;

Zhai *et al.*, 2017). The single-step approach is a relatively recent technique which has been developed to overcome some drawbacks of the multi-step one.

I-3.4.1.1. Single-step technique

LNPs are prepared in one step either by a modified nanoprecipitation method or emulsification-solvent evaporation. In these methods, hydrophobic polymers are employed, which generally leads to LNPs with a lipid monolayer shell (Bose *et al.*, 2016, 2015; Liu *et al.*, 2010; Z. Yang *et al.*, 2013; Zhang *et al.*, 2008; Zhang and Zhang, 2010). Lipid tails spontaneously self-assemble around the polymer core to reduce the free energy of the system via hydrophobic interactions.

I-3.4.1.1.1. Modified nanoprecipitation

This method requires the use of a water-miscible organic solvent and an aqueous phase. The polymer, hydrophobic drug and lipids are dissolved in the organic solvent. Ethanol (Su *et al.*, 2011), acetonitrile (Chan *et al.*, 2009; Dehaini *et al.*, 2016; Salvador-Morales *et al.*, 2009; Zhang *et al.*, 2008), tetrahydrofuran (THF) (Chan *et al.*, 2009), acetone (Chan *et al.*, 2009; Fang *et al.*, 2010) and dimethylformamide (Tahir *et al.*, 2017) can be used. Dropwise dispersion of the organic solution into an aqueous phase is followed by stirring, vortex or sonication of the mixture, during which the organic solvent rapidly diffuses in the aqueous phase, leading to the formation of LNPs (Fang *et al.*, 2010). The organic solvent is then evaporated and LNPs are collected by centrifugation or dialysis (Chan *et al.*, 2009; Dehaini *et al.*, 2016; Zhang *et al.*, 2008).

Another approach consists in dissolving lipids in an aqueous phase containing ethanol and/or methanol as a co-solvent, and then in adding the drug/polymer solution dropwise (Chan *et al.*, 2009; Salvador-Morales *et al.*, 2009; Yang *et al.*, 2012; Zhang *et al.*, 2016, 2008). In another protocol, a lipid film is hydrated with an aqueous phase in which the organic phase was added (Dehaini *et al.*, 2016). The use of a surfactant is not necessarily required in this process since the lipid acts itself as a stabilizer and self-assemble around the polymeric core via hydrophobic interactions (Hadinoto *et al.*, 2013). Some reports, however, mention the addition of a surfactant

to the aqueous phase (Bose *et al.*, 2016; Tahir *et al.*, 2017; Wong *et al.*, 2006). PLGA is the most commonly used polymer (Chan *et al.*, 2009; Salvador-Morales *et al.*, 2009; Tahir *et al.*, 2017; Zhang *et al.*, 2008). LNPs can also be prepared by this method using a scalable microfluidic device which allows controlling LNP characteristics in a reproducible manner (Gdowski *et al.*, 2018; Valencia *et al.*, 2010).

I-3.4.1.1.2. Emulsification-solvent evaporation

Depending on the nature of the drug to be encapsulated, a single (ESE) or double emulsion-solvent evaporation (DESE) process is performed. These methods require the use of a water immiscible organic solvent, such as dichloromethane (DCM) (Bose *et al.*, 2015; Cheow and Hadinoto, 2011), and an aqueous phase. Hydrophilic drugs are dissolved in the aqueous phase while polymer, lipids and hydrophobic drugs are solubilized in the organic phase (Cheow *et al.*, 2011; Dave *et al.*, 2017). In some cases, the lipid is directly dispersed in the aqueous phase under bath sonication (Yan *et al.*, 2010).

The ESE method is applied for the encapsulation of hydrophobic drugs and consists in emulsifying the organic solution in the aqueous one under sonication (Cheow and Hadinoto, 2011; Hu *et al.*, 2015c) or high speed stirring (Dave *et al.*, 2017) forming an oil-in-water emulsion (o/w) that leads to LNPs after evaporation of the organic solvent. PVA is often added to the aqueous phase prior to emulsification (Bose *et al.*, 2015; Cheow *et al.*, 2011; Dave *et al.*, 2017). Cheow and coworkers suggested that the lipids should be dissolved in the aqueous phase instead of the organic one to obtain LNPs that are more stable in physiological environment (Cheow *et al.*, 2011). On the other hand, the DESE method was developed for the encapsulation of hydrophilic compounds and is based on emulsifying the two phases (the aqueous in the organic one) by ultrasonication leading to a single water-in-oil emulsion (w/o), which is subsequently dispersed into a second aqueous phase containing a surfactant, thus forming a water-in-oil-in-water (w/o/w) emulsion. The organic solvent is then evaporated to obtain the final LNP suspension (Cheow and Hadinoto, 2011; Colombo *et al.*, 2015; Hu *et al.*, 2014; Hu *et al.*, 2015c; Jensen *et al.*, 2012; Shi *et al.*, 2011; Thanki *et al.*, 2017). Small molecules as well as macromolecules have been loaded in LNPs using this method.

I-3.4.1.2. Multi-step technique

The multi-step technique was the first common method used to prepare LNPs. It was introduced by Bayerl and Bloom (Bayerl and Bloom, 1990) whose purpose was to conceive an artificial cell membrane by forming a lipid supported bilayer onto a spherical substrate.

LNPs can be obtained by at least two distinct procedures. The first step consists in the formation of nanoparticles and liposomes separately. The polymeric NPs are prepared by nanoprecipitation (Copp *et al.*, 2014; Fang *et al.*, 2014; Thevenot *et al.*, 2008) or emulsification-solvent evaporation as indicated above (Hu *et al.*, 2015a; Sengupta *et al.*, 2005), but also by cross-linking followed by high pressure homogenization (De Miguel *et al.*, 2000; Major *et al.*, 1997) or by surfactant templated aerosol-assisted self-assembly method (Liu *et al.*, 2009b). Liposomes are prepared by the thin lipid hydration method (Conway *et al.*, 2014; Thevenot *et al.*, 2007; Troutier *et al.*, 2005a) or solvent injection method (Carmona-Ribeiro and Midmore, 1992; De Miguel *et al.*, 2000; Rapuano and Carmona-Ribeiro, 2000) followed by subsequent extrusion (Ashley *et al.*, 2011; Liu *et al.*, 2009b; Thevenot *et al.*, 2008), sonication (Conway *et al.*, 2014; De Cuyper and Joniau, 1991; Mornet *et al.*, 2005) or dialysis of the liposomal suspension (Rapuano and Carmona-Ribeiro, 2000) to form large unilamellar vesicles (LUVs) or small unilamellar ones (SUVs). Direct dispersion of the lipids in an aqueous solution was also described (Bayerl and Bloom, 1990; Moura and Carmona-Ribeiro, 2003; Nordlund *et al.*, 2009).

The second step consists in mixing the preformed NPs and liposomes under varied conditions to allow fusion and reorganization of lipid vesicles around NPs core. Different mixing conditions have been proposed depending on the saturation degree of the lipids used (saturated or unsaturated ones).

In most cases, employing saturated lipids requires heating of the liposomal suspension above the gel-to-liquid transition temperature, and lipid fusion to particles is achieved by co-extrusion (Sengupta *et al.*, 2005), dialysis (De Cuyper and Joniau, 1991), vortexing (Bathfield *et al.*,

2008; Rapuano and Carmona-Ribeiro, 2000; Thevenot *et al.*, 2008; Troutier *et al.*, 2005a), mechanical homogenization (Betbeder *et al.*, 2000; Major *et al.*, 1997) and/or simple incubation (Ashley *et al.*, 2011; Bayerl and Bloom, 1990; Rapuano and Carmona-Ribeiro, 2000).

Fusion of unsaturated lipid-based vesicles is achieved at room temperature using soft mixing conditions including co-extrusion, manual pipetting (Liu *et al.*, 2009b) or incubation (Ashley *et al.*, 2011; Bardelle *et al.*, 1993; Buranda *et al.*, 2003; Carmona-Ribeiro and De Moraes Lessa, 1999; Mornet *et al.*, 2005; Obringer *et al.*, 1995). Sometimes, a short-term manual agitation, vortexing or sonication (2 to 5 min) is applied prior to incubation of the mixture at room temperature to favor contact between NPs and liposomes and collapse of the latter (Buranda *et al.*, 2003; Gilbert *et al.*, 1992; Liu *et al.*, 2009b; Obringer *et al.*, 1995).

LNPs can also be prepared by directly hydration of the lipid film with the NPs suspension followed by sonication, stirring, incubation and/or co-extrusion (Eschwège *et al.*, 1996; Messerschmidt *et al.*, 2009; Troutier *et al.*, 2005b; Zhao *et al.*, 2012) to achieve successful lipid coating of the NPs surface. Cell membrane vesicles derived from red blood cells (RBC), platelets (PTL), bacteria and even from a tumor can be used instead of natural or synthetic phospholipids to form LNPs (Copp *et al.*, 2014; Fang *et al.*, 2014; Gao *et al.*, 2015; Hu *et al.*, 2011; Rao *et al.*, 2017) because of their unique characteristics. These membranes are fused with NPs by co-extrusion or sonication.

Finally, in the third step, LNPs are purified by removal of unreacted vesicles by centrifugation.

The multi-step approach promotes the formation of LNPs composed of a NP core, a lipid bilayer and a thin water layer in between (Bayerl and Bloom, 1990; Mornet *et al.*, 2005; Zhang and Zhang, 2010). Electrostatic interactions are usually the driving forces involved in the deposition of a lipid bilayer onto a NP surface (Liu *et al.*, 2009b; Richter *et al.*, 2006; Thevenot *et al.*, 2008; Troutier and Ladavière, 2007) and, in most cases, cationic lipids such as DOTAP (Liu *et al.*, 2009b), DPTAP (Thevenot *et al.*, 2008), DODAC and/or DODAB (Carmona-Ribeiro and Midmore, 1992) are mixed with zwitterionic ones to obtain positively charged lipid vesicles which are then added to negatively charged NPs to form LNPs. LNPs prepared from anionic or neutral lipid vesicles and cationic NPs have also been described (Liu *et al.*, 2009a; Major *et al.*, 1997). One proposed mechanism for lipid bilayer formation is that, once in contact with NP cores, lipid vesicles adhere to them, deform and break, forming bilayer patches that activate decomposition of neighboring adsorbed vesicles and coalescence of lipid patches until complete

coating of the particles (Mornet *et al.*, 2005). Cauda *et al.* proposed a solvent-exchange method based on NPs mixing with an ethanolic solution of a lipid monomer that self-assemble into a defect-free lipid bilayer around the NPs upon addition of a large volume of water (Cauda *et al.*, 2010).

Some issues have been observed with the conventional multi-step procedure such as (i) the possible leakage of drugs loaded in the polymeric cores before lipid coating (Cheow and Hadinoto, 2011), (ii) the complexity of the protocol and (iii) poor control of the characteristics of the produced LNPs (Bose *et al.*, 2015; Zhang *et al.*, 2008).

Recently, non-conventional two-step techniques have been developed, including a particle replication in non-wetting templates (PRINT) technology and spray-drying. PRINT is a large scalable and reproducible manufacturing technique allowing to prepare NPs with precise size, shape and features. Hasan *et al.* (2012) have reported the design of non-spherical lipid-coated PLGA NPs for the delivery of siRNA using this technique. The method consisted in preparing a polymer solution in which siRNA was co-dissolved, and then pouring this solution on a poly(ethylene terephthalate) (PET) sheet. This sheet was then positioned in a PRINT mold followed by heating that led to the formation of copolymer NPs after cooling. The NPs were then transferred to a PVA-coated PET sheet and released in an aqueous DOTAP/DOPE solution. The lipid mass used was 2-fold in excess compared to that of the particles. Excess lipids and PVA were removed by tangential flow filtration. This technique can be used for the encapsulation of a wide variety of hydrophobic and hydrophilic drugs and any shape can be imparted to the particles (DeSimone, 2016; Enlow *et al.*, 2011).

A spray drying technique was developed by Hitzman and co-workers to prepare fluorouracil loaded lipid-coated microparticle using polyglutamic acid, polylysine or lactose for the particle core and egg PC for the lipid shell. The polymer solution containing the drug was first spray-dried. Then, the obtained particles were dispersed in DCM containing the lipids and subsequent freeze-drying was performed to obtain the lipid-coated particles (Hitzman *et al.*, 2006). This technique can be adapted to obtain lipid-polymer hybrid nanoparticles in the nanometric scale (Li *et al.*, 2010).

LNPs with a lipid bilayer shell were also obtained by a two-step microfluidic process (Lu Zhang *et al.*, 2015).

I-3.4.1.3. Critical parameters affecting the characteristics of LNPs

Several factors may impact LNPs characteristics such as their size, polydispersity, surface charge, drug encapsulation and their colloidal stability.

I-3.4.1.3.1. The single-step approach

Two important parameters have been identified in the single-step technique: the lipid-to-polymer mass ratio (L/P) and the lipid/PEG-lipid molar ratio.

L/P was reported to significantly affect the overall characteristics of LNPs. This parameter was also referred to as the membrane-to-core weight ratio. Low L/P ratio (< 15%) led to the formation of large particles (Yang *et al.*, 2013). This was attributed to incomplete coating of oil droplets by the lipids at their surface, leading to coalescence during the process (Fang *et al.*, 2014; Zhang *et al.*, 2008), because lipids are expected to stabilize oil droplets at the oil-water interface. Particles obtained at low L/P ratio spontaneously aggregated in physiological conditions (PBS or cell culture medium containing plasma proteins) (Chan *et al.*, 2009; Fang *et al.*, 2014). Low L/P also resulted in the leakage of encapsulated drug (Zhang *et al.*, 2008). On the other hand, when the L/P ratio was too high, lipids were in excess in the formulation, and self-assembled into liposomes and/or micelles (Yang *et al.*, 2012; Zhang *et al.*, 2008) leading to heterogeneous particles population or deposition of stacked onion-like multilamellar lipid shell around particles core (Bershteyna *et al.*, 2009). Yang *et al.* demonstrated by colorimetric lipid quantification that a minimum of 15% of lipids was required to saturate the NPs surface (Yang *et al.*, 2013). The optimal L/P ratios reported ranged between 15 and 30% (Chan *et al.*, 2009; Cheow and Hadinoto, 2011; Fang *et al.*, 2010; Palange *et al.*, 2014; Zhang *et al.*, 2008).

LNPs appeared to aggregate in physiological conditions even at high L/P ratio, whatever the lipids used (neutral or cationic) (Bose *et al.*, 2015; Chan *et al.*, 2009; Cheow and Hadinoto, 2011; Z. Yang *et al.*, 2013). The good coverage of NP cores by a lipid shell proved to be insufficient to stabilize these particles at high ionic strength because of charge screening. However, the addition of PEG-lipid conjugates to the formulation improved the stability of LNP in PBS or the cell-culture medium containing plasma proteins (Chan *et al.*, 2009; Fang *et*

et al., 2010; Salvador-Morales *et al.*, 2009; Yang *et al.*, 2012). This was attributed to electrostatic and steric repulsive forces induced by the carboxylic end-group and the long chains of the grafted PEG moieties, respectively. Several investigations of the lipid-PEG/lipid ratio required for forming stable non-aggregated LNPs were conducted, which showed that a minimum of 50% of lipid-PEG was necessary to achieve this goal, which is quite high (Fang *et al.*, 2010; Z. Yang *et al.*, 2013; Zhang *et al.*, 2016). Palange *et al.* (2014) reported an increase of about 20% of the size of LNPs in PBS by using 2.5% of DSPE-PEG₂₀₀₀. The use of 10% w/w TPGS (tocopherol polyethylene glycol 1000 succinate) as a stabilizer also proved to prevent LNP aggregation in PBS (Cheow and Hadinoto, 2011). Polymer-PEG conjugates were also proposed as alternative to lipid-PEG for stabilizing LNPs (Yang *et al.*, 2012). For example, PLA-PEG₅₀₀₀ significantly contributed to decrease the adsorption of plasma proteins on particles surface.

I-3.4.1.3.2. The multi-step approach

The aim of using the multi-step technique instead of the single one is to design LNPs with a lipid bilayer shell. Therefore, several aspects such as the nature of lipid and polymer used, the method by which NPs and lipid vesicles are produced, the vesicle-to-particle ratio, the mixing condition performed, and the ionic strength and pH of the aqueous medium should be considered as all these parameters can significantly affect the physico-chemical characteristics of the obtained LNPs.

Various types of NPs and lipids have been used to prepared LNPs. An easy way to favor the formation of a lipid bilayer onto a particle surface by electrostatic attraction is to use oppositely charged vesicles and NPs. Negatively charged NPs such as mesoporous silica, poly(styrene), iron oxide (Fe₃O₄), PLGA and PLA NPs were mixed with positively charged vesicles (Bathfield *et al.*, 2008; Carmona-Ribeiro and De Moraes Lessa, 1999; Cauda *et al.*, 2010; Liu *et al.*, 2009b; Thevenot *et al.*, 2008; Troutier *et al.*, 2005b). Some authors also prepared LNPs by mixing anionic nanoparticles such as silica, PLGA NPs, or positively charged NPs such as polysaccharides, with neutral lipid vesicles (Ashley *et al.*, 2011; Conway *et al.*, 2014; De Miguel *et al.*, 2000; Nordlund *et al.*, 2009; Sengupta *et al.*, 2005). When PLGA NPs were used, LNPs were obtained by co-extrusion of NPs and vesicles (Sengupta *et al.*, 2005). It was also possible to obtain LNPs using both negatively charged NPs and vesicles. This was done either by co-extrusion or sonication of PLGA NPs or Fe₃O₄ magnetic nanoparticles with red blood

cell or platelet ghost membranes which are negatively charged (Copp *et al.*, 2014; Fang *et al.*, 2014; Hu *et al.*, 2011; Rao *et al.*, 2017). Mornet *et al.* (2005) also reported the formation of a continuous lipid bilayer on amorphous silica NPs when the net negative charge of mixed phosphatidylcholine (PC) and phosphatidylserine (PS) lipid vesicles was low (PC/PS ratio $\sim 4/1$ or $3/1$). However, this approach led to unsatisfactory result when the vesicles were highly negatively charged (e.g., PC/PS $1/1$) (Mornet *et al.*, 2005). Carmona-Ribeiro and Moraes Lessa (1999) made the same observation. In their study, they obtained the formation of a lipid monolayer instead of a bilayer by incubating anionic sulfate polystyrene microspheres with negatively charged asolecithin vesicles. This result was explained by the fact that hydrophobic attractions had surpassed the electrostatic repulsion forces normally present between the particles and vesicles because of their surface charge.

The method used to prepare the polymeric core of LNPs can impact the encapsulation efficiency (EE) and drug loading (DL) of entrapped molecules. In general, NPs prepared by nanoprecipitation methods showed lower DL and EE than those obtained by the emulsification techniques (Kamaly *et al.*, 2012). The nature of the drug molecules to be encapsulated was decisive for the choice of the polymer as well as the method used for their encapsulation.

The formation of LNPs requires mixing of polymeric NPs with excess lipid vesicles to ensure complete coverage of the NP surface by a lipid bilayer (Bayerl and Bloom, 1990; Carmona-Ribeiro and Midmore, 1992; Thevenot *et al.*, 2008). This excess is calculated on the basis of the ratio between the total surface area of vesicles (A_v) and that of the particles (A_p) (A_v/A_p) also referred to as the vesicle-to-particle ratio. A_v/A_p was shown to play a critical role in the size and stability of LNPs, mainly when cationic lipid vesicles were mixed with anionic particles (Carmona-Ribeiro and Midmore, 1992; Thevenot *et al.*, 2008; Troutier *et al.*, 2005b). At low A_v/A_p , aggregation occurred, in relation to the proportion of cationic lipids used, while high A_v/A_p led to non-aggregated LNPs having a diameter close to that of bare NPs. LNP aggregation was mainly noticed for formulations containing 10 to 50% of cationic lipids. In such conditions, the lipid vesicles acted as flocculation agents while they contributed to stabilize LNPs at high A_v/A_p ratio. The optimum A_v/A_p ratio at which LNPs aggregation was prevented was ~ 14 (Thevenot *et al.*, 2008; Troutier *et al.*, 2005b).

Lipid vesicles of uniform size are preferred because they contribute to produce LNPs with reproducible size and polydispersity. Lipid vesicles obtained by extrusion appeared as the most suitable for controlling the features of the designed LNPs (Troutier *et al.*, 2005b). It was also

shown that vesicles should be of smaller size than – or at least similar size as – NPs because affinity of the vesicles to the particle surface is influenced by curvature. Adsorption of vesicles to NPs is driven by favorable adhesive energy, while their deformation and rupture results from unfavorable elastic energy (Mandal *et al.*, 2013; Michel and Gradzielski, 2012). In several reports, LNPs were prepared using SUVs because of their high curvature which facilitates their rupture at the surface of the particles (Bathfield *et al.*, 2008; Bayerl and Bloom, 1990; Betbeder *et al.*, 2000; Conway *et al.*, 2014; Mornet *et al.*, 2005). Conversely, using lipid vesicles larger than NPs resulted in the engulfment of several NPs into one liposome (Le Bihan *et al.*, 2009).

As in the one-step approach, the main issue faced with LNPs prepared by the multi-step one was their tendency to aggregate in physiological conditions (Savarala *et al.*, 2011; Thevenot *et al.*, 2008; Troutier *et al.*, 2005b). This was even more critical when cationic lipids were used instead of neutral ones. As the ionic strength of the medium increased, the surface charge of LNPs was screened, therefore weakening repulsive electrostatic interactions initially existing between the LNPs and promoting their aggregation via van der Waals attractive interactions (Carmona-Ribeiro and Midmore, 1992; Savarala *et al.*, 2011; Troutier *et al.*, 2005a; Troutier and Ladavière, 2007). This issue could be resolved by adding long chain PEG covalently conjugated to lipids. Thevenot *et al.* (2007) have demonstrated that the addition of 10% of PEG₁₁₃-lipid conjugates improved the stability of LNPs at high ionic strength.

I-3.4.2. Photosensitizer-loaded LNPs

Few studies were conducted on the use of LNPs as nanocarriers to deliver PSs. Pramual *et al.* (2017) designed LNPs consisting in PLGA or poly(hydroxybutyrate-co-hydroxyvalerate) nanoparticles covered with a lecithin-DSPE-PEG monolayer. Tetrakis(4-hydroxy-phenyl)-21H,23H-porphine (p-THPP) was entrapped in the core of these LNPs. Tayyaba Hasan's team reported the design of theranostic “nanocells” (ie., LNPs) made of benzoporphyrin derivative monoacid (BPD)-loaded PLGA-PEG NPs coated with a liposomal envelop. Avastin, an anti-VEGF agent, was co-encapsulated in the lipid envelop of these LNPs (Spring *et al.*, 2010).

I-4. Conclusion

Different delivery systems have been proposed for the treatment of retinoblastoma by PDT. It was shown that nanocarriers can help solving solubilization, aggregation and specificity issues related to the use of hydrophobic PSs. Indeed, these carriers can be functionalized with PEG moieties to prolong their circulating half-life time and/or with ligands to enhance the specificity of PSs towards tumors, and their cellular uptake. Among these systems, liposomes present the advantage of being biocompatible and to encapsulate both hydrophilic and hydrophobic compounds. Unfortunately, they are highly unstable (Daghildjian, 2013). This might result in the premature leakage of the PS (Massiot *et al.*, 2017; Muehlmann *et al.*, 2011). Premature release of the PS has also been observed with nanoparticles. A more critical issue is that PSs loaded at high concentration into NPs may be inactivated by self-quenching because of the close proximity of the molecules. In addition, diffusion of singlet oxygen outside NPs may be hindered, depending on the nature of the material constituting the nanoparticles. A strategy proposed for solving these issues was to covalently attach PSs inside porous nanostructures of NPs or at their surface (Brevet *et al.*, 2009; Gary-Bobo *et al.*, 2012; Mackowiak *et al.*, 2013).

LNPs combine the attributes and advantages of liposomes and NPs, while suppressing some of their limitations. They can be designed in different ways with a core-shell structure, the shell being composed of a lipid monolayer or bilayer. Their two compartments can serve for the encapsulation of one or more hydrophobic and/or hydrophilic active ingredients. LNPs can also be easily functionalized with ligands to achieve active targeting of tumors cells. We have chosen to form LNPs via electrostatic interactions. For this purpose, an anionic polymer and a mixture of zwitterionic and cationic lipids were used. In these LNPs, β -Lapachone, a cytotoxic agent which has proven to be effective against RB cells (Shah *et al.*, 2008), was co-encapsulated with two PSs, m-THPC (temoporfin, Foscan[®]) or its porphyrin derivative, m-THPP.

In the following chapters, we describe first the formation and characterization of LNPs (Chapter II), a thorough study of the adhesion of a lipid bilayer onto a polymer film surface (Chapter III) and the in vitro evaluation of the antitumor activity of the liponanoparticles on retinoblastoma cells (Chapter IV). The description of these studies will be followed by a general discussion and the conclusion in which some perspectives will be presented for the continuity of this work.

**Chapter II: Formulation and physicochemical characterization of
liponanoparticles**

Chapter II: Formulation and physicochemical characterization of liponanoparticles

II-1. Introduction

Liponanoparticles have emerged as nanocarriers of potential superior interest to liposomes and nanoparticles (Mandal *et al.*, 2013). As described in the previous chapter, several preparation techniques allow formation of LNPs with a lipid monolayer or bilayer covering an inorganic or polymeric core. In our study, we were interested in forming polymeric LNPs with a lipid bilayer shell. As such system is usually formed via electrostatic interactions between preformed oppositely charged vesicles and nanoparticles (De Miguel *et al.*, 2000; Troutier and Ladavière, 2007), we chose the biocompatible and biodegradable poly(D,L-lactic) acid (PLA) for the LNPs core (Xiao *et al.*, 2006), and a mixture of zwitterionic and cationic lipids (POPC and DOTAP), for the lipid shell. We were inspired by the work of Catherine Ladavière and coworkers who prepared LNPs using PLA and DPPC-DPTAP vesicles (Thevenot *et al.*, 2007, 2008). We preferred POPC and DOTAP which are in a fluid liquid-crystalline state at room temperature so that there was no need to heat the system during preparation -which could affect both the polymer and the drugs - and we chose pure PLA over poly(lactide-co-glycolide) (PLGA, the usual favorite for biodegradable nanoparticles in drug delivery systems) because the anticancer drug to deliver, beta-lapachone, was hydrophobic. In this chapter, we describe the preparation and typical characteristics of PLA/POPC-DOTAP liponanoparticles and their stability in a cell culture medium.

II-2. Materials and methods

II-2.1. Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, $M_w = 760.076$ g/mol), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, $M_w = 698.542$ g/mol), and 1-oleoyl-2-{12-

[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphoethanolamine (NBD-PE, $M_w = 839.995$ g/mol) were purchased from Avanti Polar lipids (Alabaster, USA). 5,10,15,20-tetra (m-hydroxyphenyl) porphyrin (m-THPP, $M_w = 678.73$ g/mol) was a gift from Phillipe Maillard (Institut Curie, Orsay, France). Nile red (NR, $M_w = 318.37$ g/mol), formic acid (FAc), Dulbecco Phosphate Buffer Saline (DPBS) and Dulbecco's Modified Eagle's Medium D6429 (DMEM) were provided by Sigma-Aldrich (St Louis, USA). Fetal bovine serum (FBS) was supplied by Gibco. Poly(D,L)-lactic acid (PLA) (Resomer R202H[®], $M_w = 10,000$ -18,000 g/mol, $T_g = 44$ -48°C) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany), and, sodium chloride (NaCl) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, $\geq 99.5\%$) from Carl-Roth Chemicals (Germany). Acetone, chloroform, methanol (all HPLC grade) and water (LC-MS grade) were supplied by VWR Prolabo (Leuven, Belgium). Ultrapure water was produced by a Millipore Milli-Q[®] Direct 8 water purification system. All materials were used without further purification.

II-2.2. Methods

II-2.2.1. Liposomes preparation

POPC-DOTAP liposomes were prepared using Bangham's conventional film hydration method (Bangham *et al.* 1965) followed by extrusion or tip sonication of the vesicle suspension. Briefly, pure POPC or POPC/DOTAP mixtures (90/10, 75/25, 50/50 and 25/75 mol%) were dissolved in a chloroform/methanol (9:1 v/v) solution ($V=3$ mL). The organic solvents were evaporated under reduced pressure for 2 hours to form a thin lipid film. This film was hydrated with ultrapure water (2.8 mg/mL final lipid concentration) then submitted to vigorous vortex mixing leading to a cloudy suspension of multilamellar vesicles (MLV). Large unilamellar vesicles (LUV) were obtained by extrusion of the MLV suspension 11 times through 400 nm and 200 nm polycarbonate membranes (Millipore, USA) using a mini-extruder (Avanti Polar lipids). Small unilamellar vesicles (SUV) were obtained by tip sonication of MLVs using a Vibra-Cell sonicator 75041 (Bioblock Scientific, 750W, 20kHz) for 10 minutes at 20% of amplitude. Sample vials were cooled in an ice bath and 10 s on/off pulse were applied to avoid excessive heating of the lipid. The SUV suspension was then centrifuged three times at 10,000g for 10 min at 4°C to remove titanium particles released from the tip of the sonicator. Sonicated m-

THPP liposomes were prepared as described above by adding 2.5 mol% of m-THPP stock solution (10 mM in chloroform/methanol 9:1 v/v) to a POPC-DOTAP 75/25 mixture solution prior to solvent evaporation. Fluorescent liposomes were also prepared for confocal microscopy experiments by adding 1 or 2 mol% NBD-PE to lipid solutions.

II-2.2.2. Preparation of PLA nanoparticles

Poly(D,L)-lactic acid (PLA) nanoparticles (PLA NPs) were prepared by the nanoprecipitation method (Vauthier and Bouchemal, 2009). Briefly, PLA (50 mg) was dissolved in acetone, a good solvent for the polymer, to achieve a polymer concentration of 10 mg/mL. This solution was added dropwise to ultrapure water, free of surfactant, under moderate magnetic stirring (at 0.36 g). Several parameters were varied to generate nanoparticles with well-controlled size including the initial amount of the polymer (10, 20 and 50 mg) and its concentration in acetone (1.4 and 10 mg/mL), the organic to aqueous phase (OP/PA) ratio (1/1 and 1/2) and the nature of the aqueous phase (ultrapure water or HEPES buffer 10 mM with NaCl 150 mM, pH 7.4). Acetone was removed under reduced pressure using a rotavapor and the concentration of the final suspension was adjusted to 2 mg/mL for the preparation of LNPs. Fluorescent PLA NPs were also obtained as described above, by adding 0.01 or 0.001 % (w/w) Nile red (NR) to PLA/acetone solution prior to nanoprecipitation.

II-2.2.3. Lipo-nanoparticles (LNPs) preparation

Lipo-nanoparticles (LNP) were prepared according to the protocol described by Thevenot *et al.* (2007, 2008), with modification. SUVs in excess were added to nanoparticles. Vesicle excess was expressed as the ratio between the total surface area of vesicles (A_v) and nanoparticles (A_p), respectively. A_v value was estimated from the number of NPs per mg and their mean diameter. The A_v/A_p ratio was 14 in all experiments to ensure full coverage of PLA nanoparticles by a continuous lipid bilayer. The concentration of lipid vesicles required was deducted from the A_p value by calculating the “molecular weight” of a vesicle and the number of lipid molecules per vesicle as described by Clarke and Apell (1989). Four mixing conditions were tested at room temperature including (i) vortex for 1h, (ii) incubation for 1h, (iii) 6 min of sonication followed

by 1h of incubation, and (iv) 2 min vortex followed by 1h of incubation. The obtained LNPs were then separated from free vesicles by centrifugation 2-3 times at 3,000 g for 15 min at 15°C. The pellet was resuspended in pure water. The supernatant (LNP Sup.) was centrifuged one last time at 10,000 g for 10 min to remove residual LNPs and was collected for lipid quantification by HPLC-ELSD. In order to evaluate the stability of LNPs in biological environment, fluorescent LNPs and m-THPP LNPs were also prepared as mentioned above. Fluorescent LNPs were obtained from liposomes and PLA NPs labeled with NBD-PE and NR, respectively, whereas m-THPP LNPs were prepared using m-THPP-loaded liposomes and unloaded PLA NPs.

II-2.2.4. Particles characterization

II-2.2.4.1. Size and zeta potential measurements

Particles size and size distribution were determined by dynamic light scattering (DLS), and zeta potential (ζ) was measured by laser doppler electrophoresis using a zetasizer Nano ZS90 (Malvern). All formulations prepared in water were diluted in 1 mM NaCl solution, while those prepared in buffer were diluted using the same buffer and measurements were carried out in triplicate at 25°C. Zeta potential of samples prepared in buffer could not be performed due to the high ionic strength of medium leading to blackening of the cell electrodes.

II-2.2.4.2. Lipid quantification by RP-HPLC using an evaporative light scattering detector (HPLC-ELSD)

POPC and DOTAP were quantified using the HPLC-ELSD method described by [Zhong et al. \(2010\)](#) with slight modification. The HPLC instrument was an Agilent system, with a 1050 injector and a 1260 pump (Agilent Technologies, Santa Clara, CA, USA). The lipids were separated using a SecurityGuard Cartridges C18 pre-column (4 x 2.0 mm) coupled to a Luna C18(2) column (150 x 3.0 mm, I.D., 3 μ m particle size, 100 Å pore size) from Phenomenex SAS (Le Pecq, France) thermostated at 50°C. The ELSD (Eurosep, Cergy, France) settled parameters were: nebulizer temperature 35°C, drift tube 45°C, photomultiplier 600 and air

pressure 1.5 bar. The signal was acquired with a Chromeleon data station (Thermo Fisher Scientific, Villebon-sur-Yvette, France). The injection volume was 30 μ L. The separation was achieved by binary gradient elution using methanol containing 0.1% formic acid (FAc) (A) and water containing 0.1% FAc (B), starting with 85:15 A:B for 10 min followed by a 6 min plateau. The mobile phase was returned back to initial solvent mixture 10s after the plateau and the column was equilibrated for 7 min. The flow rate was 0.5 mL/min. Standard solutions were prepared by mixing POPC and DOTAP (2 mg/mL) stock solutions in the 20-400 and 10-200 μ g/mL concentration ranges, respectively. The samples (liposomes, LNP Sup. and LNPs) were vacuum dried and directly diluted with methanol to make the lipid concentrations fit in the calibration range. LNP samples were then centrifuged at 10,000 g for 10 min at 4°C to remove the PLA NPs and the supernatant was injected into the HPLC column. Samples and measurements were performed in duplicate.

II-2.2.4.3. Cryo-TEM

The morphology of the nanoparticles and LNPs was evaluated by cryogenic transmission electron microscopy (cryo-TEM). Samples were diluted to 1 mg/mL in 1 mM NaCl solution and 5 μ L of the suspensions were deposited onto a perforated carbon-coated copper grid (TedPella, Inc.). After removal of the excess liquid with a filter paper, the grid was quickly frozen in a liquid ethane bath at -180°C and mounted on the cryo holder (da Cunha *et al.*, 2016). Transmission electron measurements (TEM) measurements were performed just after grid preparation using a JEOL 2200FS (JEOL USA, Inc., Peabody, MA, U.S.A.) equipped with a Gatan Ultrascan 2K camera (Gatan, Evry, France). The obtained images were analyzed using the Image J software.

II-2.2.4.4. Confocal microscopy

Fluorescent LNPs were suspended in an agarose solution at 1% (w/v) to slow down Brownian motion during imaging. The samples were mounted between a microscope slide and a slip cover #1.5. They were imaged by an inverted confocal laser scanning microscope TCS SP8 Leica (Leica, Germany) using a HC PL APO CS2 63x/1.40 oil immersion objective lens, a White

Light Laser at 488 and 555 nm excitation wavelengths for NBD-PE and NR, respectively, and the transmission mode. Green and red fluorescence emission signals were collected with 497-540 nm (PMT2 detector) and 564-702 nm wide emission slits (Hybrid detector), respectively. Transmission images were acquired with a PMT-trans detector. The pinhole was set at 1.0 Airy unit. 12-bit numerical images were obtained with the Leica Application Suite X software (Version 3.1.5; Leica, Germany). The resulting images were treated, and their fluorescence intensity profile plotted using the image J software.

II-2.2.5. Stability of LNPs

Pellets of fluorescent LNPs and *m*-THPP LNPs, previously prepared in water and centrifuged for 15 min at 3,000 g and 15°C, were resuspended in DPBS or DMEM to evaluate their stability in these media. Particles size was measured by DLS and their morphology was observed by cryo-TEM as aforementioned. In another set of experiments, fluorescent LNPs were dispersed in DMEM supplemented or not with 10% v/v FBS, and they were imaged by confocal microscopy under the same conditions as described above.

II-2.2.6. Interaction of LNPs with Y79 cells

Y79 cells were seeded at 30,000 cells/well into a 96-well plate and incubated for 24 h in DMEM supplemented with 10% v/v FBS. The cells were treated with fluorescent LNPs (7.5 µg/µL of LNPs) or *m*-THPP LNPs (13 µg/µL of LNPs corresponding to 0.01 µg/µL of *m*-THPP) and incubated for various times (1, 2, 4, 24 and 48h). The cells were then transferred into eppendorfs, centrifuged at 200g for 2 min in a miniSpin® centrifuge and washed in DPBS for removing LNPs. The pellet obtained was resuspended in DPBS and cells were observed with an inverted confocal laser scanning microscope LSM 510-Meta (Carl Zeiss, Germany) using a Plan-Apochromat 63X/1.4 objective lens, equipped with an argon (488 nm excitation wavelength) and a helium neon laser (543 nm excitation wavelength). The green and the red fluorescence emissions were collected with a 505-550 nm band-pass and a 650 nm long pass emission filter respectively, under a sequential mode. The pinhole was set at 1.0 Airy unit. 12-

bit numerical images were acquired with LSM 510 software version 3.2 and the resulting images were analyzed using the image J software.

II-3. Results and discussion

II-3.1. Nanoprecipitation conditions influencing NP characteristics

In the nanoprecipitation process, surfactants are often used to control the particles size and prevent their aggregation. Commonly used surfactants are non-ionic polyvinyl alcohol (PVA), poloxamer 188 (Pluronic F68) and Tween 80 at concentrations varying from 0.1 to 2.5% w/v (Chorny *et al.*, 2002; Maaz *et al.*, 2015; Almoustafa *et al.* 2017). Higher surfactant concentrations may be required, depending on the nature of the polymer (Khan and Schneider, 2013). Surfactants adsorb to the surface of the NPs thus modifying their physicochemical properties. An increase in size and decrease in ζ -potential were reported for PLA NPs prepared with Poloxamer 188, compared to surfactant-free NPs (Hirsjärvi, 2008). Because surfactants can be potentially toxic and carcinogenic (Dwivedi *et al.*, 2016; Jeong *et al.*, 2005; Menon *et al.*, 2012) and their use might affect the adhesion of the phospholipid bilayer, we prepared PLA NPs without adding any surfactant. Table 2 summarizes the protocols and conditions tested for PLA NPs formation, and the size and ζ -potential values measured. The results show that the polymer concentration, organic-to-aqueous phase ratio, and nature of the aqueous phase had a significant impact on particle size. The use of HEPES buffer instead of water induced a significant increase in particle size and size distribution (F1 vs F2, F3 vs F5 and F6 vs F8). The increase in size of nanoparticles in saline has previously been reported in the literature, for PLGA NPs for example, when increasing NaCl concentration (Huang and Zhang, 2018) or for BSA NPs in PBS with ionic strengths ranging from 10 to 100 mM (Tarhini *et al.* 2018). This increase in size could be attributed to (i) particle aggregation resulting from the screening of PLA negative charges by buffer ions, and (ii) the absence of surfactant. PLA NPs prepared in water showed reproducible size and ζ -potential. They exhibited a narrow size distribution centered on 170 ± 3 nm, with a polydispersity index (PDI) of 0.08 ± 0.02 and a negative zeta potential value of -46 ± 1 mV (Figure 11A, C and D). This zeta potential value was of the same

order of magnitude as that reported for PLA NPs in the literature. As an indication, Riley and coworkers, Troutier-Thuilliez *et al.* and Pavot *et al.* have obtained zeta potential values of -50 ± 1 , -58 and -60 ± 5 mV, respectively, for PLA NPs prepared in water without surfactant (Pavot *et al.*, 2013; Riley *et al.*, 1999; Troutier-Thuilliez *et al.*, 2009). The pH of the NPs suspension in water was 4.51, higher than the pKa of the lactic acid unit (3.8) of the polymer. Therefore, the negative surface charge of the NPs can be explained by the ionization of the carboxylic end groups of the polymer at this pH. Since the zeta potential of NPs was higher than ± 30 mV, good stability was expected (Dwivedi *et al.*, 2016).

Formulation	m_{PLA} (mg)	C_{orgPLA} (mg/mL)	Aqueous Phase (AP)	OP/AP ratio (v/v)	Size (nm)	PDI	ζ -potential (mV)
F1	50	10	Water	1/2	170 ± 3	0.08 ± 0.02	-46 ± 0
F2			Buffer	1/2	237 ± 5	0.15 ± 0.02	nd
F3	20	10	Water	1/2	173 ± 2	0.09 ± 0.02	-45 ± 1
F4				1/1	171 ± 2	0.09 ± 0.03	-45 ± 0
F5			Buffer	1/2	257 ± 3	0.19 ± 0.01	nd
F6	10	1.4	Water	1/2	86 ± 1	0.13 ± 0.02	-43 ± 1
F7				1/1	89 ± 1	0.08 ± 0.01	-39 ± 1
F8		1.4	Buffer	1/2	104 ± 0	0.19 ± 0.02	nd
F9				1/1	408 ± 7	0.17 ± 0.06	nd

Table 2: Effect of nanoprecipitation conditions on the characteristics of PLA NPs. m_{PLA} is the mass of PLA initially used; C_{orgPLA} is the polymer concentration in the organic solvent; OP/AP ratio is the organic-to-aqueous phase ratio; PDI is the polydispersity index and ζ the zeta potential. Nd: not determined.

The impact of the polymer mass on the particle size is related to polymer concentration in the organic phase. Increasing the polymer mass did not lead to any significant change in

nanoparticles characteristics when the other parameters were conserved, including polymer concentration (F1 vs F3). On the other hand, the increase in polymer concentration from 1.4 to 10 mg/ml resulted in an increase in particle size (F1 vs F6, F3 vs F6 and F4 vs F7). This was expected as [Pandey *et al.* \(2015\)](#) showed a size increase for quercetin-loaded PLA NPs for PLA concentrations varying from 10 to 40 mg/mL, and [Legrand and coworkers \(Legrand *et al.*, 2007\)](#) made the same observation when varying PLA concentration from 5 to 20 mg/mL. Also, a size increase was reported for PLGA NPs by [Huang and Zhang \(2018\)](#) when copolymer concentration was increased from 1 to 20 mg/mL. Larger particles were obtained because the viscosity of the polymer solution increased with its concentration, thus decreasing the diffusion rate of the organic solvent into the aqueous phase ([Dwivedi *et al.*, 2016](#)), and limiting droplet breaking during stirring ([Tahir *et al.*, 2017](#)).

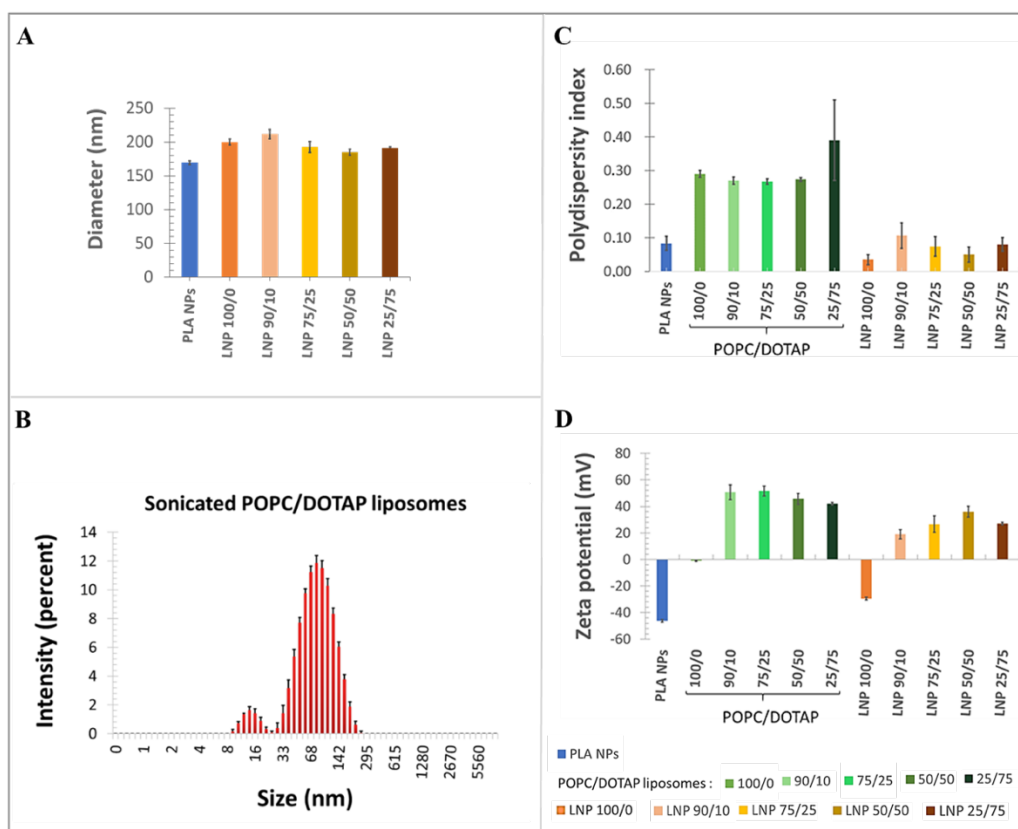


Figure 11: Particles size (A, B), polydispersity index (C) and zeta potential (D) for PLA NPs, sonicated liposomes and LNPs prepared under condition *iii*.

A reduction of the particle size was expected when doubling the volume of the aqueous phase (OP/AP: 1/2) as it might facilitate the diffusion of the organic phase in the aqueous one (Miladi *et al.*, 2016). However, no difference was observed between NPs prepared by using 1/1 or 1/2 OP/AP ratio (F3 vs F4, F6 vs F7), except for those prepared in buffer from low concentrated PLA organic solution. In this case, smaller particles were indeed obtained with the 1/2 OP/AP ratio.

Since the size of NPs can significantly impact their biodistribution within the body (Duan *et al.*, 2017; He *et al.*, 2010; Kadam *et al.*, 2012) and negative surface charge is required for the formation of LNPs via electrostatic interactions, it was important to determine the optimal conditions for preparing PLA NPs with desired size ≤ 200 nm and high negative zeta potential. It appears from our results that PLA NPs prepared in pure water with a polymer concentration of 10 mg/mL and the 1/2 OP/AP ratio (F1) exhibited reproducible characteristics in terms of size, PDI and ζ -potential, agreeing with expectation. Therefore, this formulation was used for the next experiments.

II-3.2. Liposomes

Liposomes were tip-sonicated or extruded to obtain vesicles with equivalent (LUVs) or smaller (SUVs) size than PLA NPs. The mean diameters of vesicles obtained by the two methods are given in

Table 3. Extruded liposomes had a mean diameter of 160 nm, similar to that of PLA NPs, and a narrow size distribution ($PDI \leq 0.1$). No significant difference was noticed in their size and PDI when the DOTAP ratio was increased from 10 to 25%. Sonicated liposomes were smaller, but exhibited a bimodal size distribution (Figure 11B) and were polydisperse with a $PDI > 0.2$ (Figure 11C). These features are consistent with those reported in the literature for tip-sonicated vesicles (Woodbury *et al.*, 2006) (

Table 3). As pointed out by Lapinski and coworkers, the main drawback of tip-sonication is its lack of reproducibility from batch to batch (Lapinski *et al.*, 2007). However, as for extruded vesicles, variation of the DOTAP ratio did not significantly affect the size of the liposomes.

POPC/DOTAP liposomes	Average diameter (nm)	
	Extrusion	Sonication
100/0	—	38 and 142
90/10	164	18 and 83 ± 13
75/25	166 ± 10 nm	16 ± 2 and 90 ± 22
50/50	—	16 ± 2 and 75 ± 6
25/75	—	21 and 51

Table 3: Mean size of extruded and tip-sonicated POPC/DOTAP liposomes.

As expected, liposomes prepared with DOTAP were positively charged with a zeta potential value higher than ± 30 mV, which theoretically ensures the good stability of colloids. Surprisingly, an increase of the DOTAP content in vesicles did not significantly increase the zeta potential value (Figure 11D). In fact, ζ -potential even decreased at molar ratios higher than 25 mol%. Indeed, the zeta potential of liposomes prepared with 10, 25, 50 and 75 mol% DOTAP were $+51 \pm 6$, $+52 \pm 4$, $+46 \pm 4$ and $+42 \pm 1$ mV, respectively.

Blakeston *et al.* (2015) prepared POPC/DOTAP liposomes in a phosphate buffer (10 mM, pH 7.1) to form a lipid bilayer on polymer brushes. They observed an increase in the zeta potential up to +60 mV by varying DOTAP concentration (from 10 to 75 mol%). Interestingly, their results showed a decrease in zeta potential for POPC/DOTAP 25/75 vesicles from +60 mV to around +40 mV by increasing the pH from 2 to 9. Martens *et al.*, (2017) obtained a zeta potential of $+54 \pm 1$ mV for DOPE/DOTAP liposomes (50:50) in HEPES buffer (25 mM, pH 7.2). However, in our experiments, the liposomes were prepared in water and not in a buffer. So, the slight decrease in the zeta potential that we observed is likely due to a pH rather than a salt effect. The pH measured for the POPC/DOTAP 75/25 liposomes suspension was 6.33 for a zeta potential of +56 mV.

POPC liposomes exhibited a null zeta potential (Figure 11D). In the literature, the zeta potential of POPC vesicles has been reported to vary between -1 and -10 mV from pH 4 to pH 9, which is in agreement with our result (Cho and Frank, 2010).

II-3.3. LNPs

II-3.3.1. Evaluation of various protocols for LNPs formation

LNPs were prepared by a two-step protocol, the first step consisting in the preparation of NPs and liposomes separately, and the second step, to the mixture of the two colloids under different conditions. Table 4 reports the size distribution and zeta potential of the prepared LNPs depending on the mixing conditions (vortex, sonication and/or incubation). In all tested conditions, we observed a significant increase in particles size, with similar polydispersity, compared to that of PLA NPs. The results suggest that the mixing conditions had a significant impact on the size of the new particles. The smallest LNPs were obtained after 6 min sonication and 1h incubation of vesicles and nanoparticles (**iii**) at room temperature. Conversely, those with the largest sizes were obtained by vortexing the system for 1h at room temperature (**i**). Particles with intermediate sizes were formed after 1 h incubation (**ii**) or 2 min vortex followed by 1h incubation at room temperature (**iv**).

Concentration of DOTAP in the formulation also affected the particles size. LNPs prepared with 10% DOTAP were the largest. A slight decrease in particle size was obtained by increasing DOTAP content up to 50%. No further size decrease was observed beyond 50% of DOTAP. A similar trend has been reported by Ladavière's group for liponanoparticles prepared with polystyrene NPs and DPPC/DPTAP vesicles in a Tris buffer (10 mM, pH 7.4) (Troutier *et al.*, 2005b). They observed aggregated structures with 10% of DPTAP and a decrease in particle size with the increase of the cationic lipid concentration.

Our goal was to form LNPs composed of a solid nanoparticle core coated by a lipid bilayer. In Figure 12 is illustrated the theoretical cross-section of our LNPs having three distinct compartments, namely the NP core, a thin water layer and the lipid bilayer. The thickness of the water layer has been measured by various authors, using lipid bilayers formed on planar and spherical supports, and different techniques such as neutron reflectivity (Gutberlet *et al.*, 2004; Johnson *et al.*, 1991) or ¹H-NMR (Bayerl and Bloom, 1990). It was found in the 0.6 – 3 nm range (Bayerl and Bloom, 1990; Dolainsky *et al.*, 1992; Gutberlet *et al.*, 2004; Johnson *et al.*, 1991; Koenig *et al.*, 1996; White *et al.*, 2006). For a lipid bilayer surrounding a spherical substrate, a thickness of ~2 nm was reported (Bayerl and Bloom, 1990). A lipid bilayer is known

to be ~4 nm thick (Ashley *et al.*, 2011; Johnson *et al.*, 1991; Tahara and Fujiyoshi, 1994; Vasilca *et al.*, 2018). In chapter III, we describe the study of the formation of a POPC-DOTAP bilayer onto a PLA monolayer. The thickness of the bilayer deduced from atomic force microscopy height pictures was 3.9 ± 0.4 nm. So, we expected an increase in the particle size of about 12 nm corresponding to 2[lipid bilayer thickness + water layer thickness], compared to PLA NPs.

Formulation	Mixing conditions	ΔHD (nm)	PDI	ζ - potential (mV)	Estimated number of adsorbed lipid bilayers	Cryo-TEM images
LNP 90/10 (i)	1h vortex (i)	98 ± 1	0.07 ± 0.02	$+ 39 \pm 1$	8.2	Figure 4
LNP 75/25 (i)		69 ± 1	0.09 ± 0.01	$+ 50 \pm 1$	5.8	Figure 5
LNP 75/25 (ii)	1h incubation (ii)	37 ± 3	0.17 ± 0.03	$+ 22 \pm 1$	3.1	Figure 7
LNP 100/0 (iii)	6 min sonication + 1h incubation (iii)	31 ± 1	0.04 ± 0.02	$- 30 \pm 1$	2.6	Figure 6A
LNP 90/10 (iii)		42 ± 4	0.11 ± 0.04	$+ 19 \pm 4$	3.5	—
LNP 75/25 (iii)		23 ± 5	0.07 ± 0.03	$+ 27 \pm 6$	1.9	Figure 6B
LNP 50/50 (iii)		16 ± 2	0.05 ± 0.02	$+ 36 \pm 4$	1.3	Figure 6C
LNP 25/75 (iii)		18 ± 0	0.08 ± 0.02	$+ 27 \pm 1$	1.5	Figure 6 D
LNP 75/25 (iv)	2 min vortex + 1h incubation (iv)	46 ± 1	0.20 ± 0.02	$+ 26 \pm 0$	3.8	—

Table 4: LNP physicochemical characterization by DLS and zeta potential depending on the mixing condition. ΔHD is the difference in the hydrodynamic diameter (HD) between LNP and bare PLA NPs ($\Delta HD = HD_{LNPs} - HD_{NPs}$).

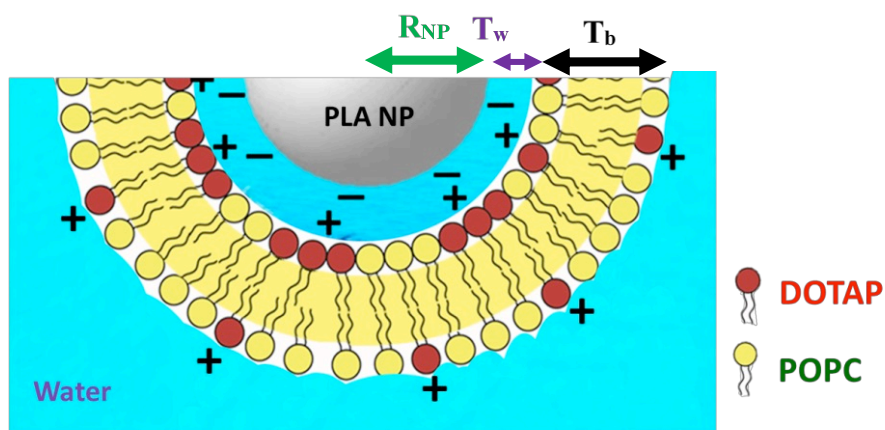


Figure 12: Illustration of the cross-section of a LNP; R_{NP} is the radius of the PLA NP; T_w is the thickness of the water layer lying between the NP and lipid bilayer, and T_b is the thickness of the lipid bilayer. The theoretical diameter of LNP expected is calculated as $D_{LNP} = 2R_{NP} + 2T_w + 2T_b$, where T_b is ~ 4 nm and $T_w \sim 2$ (Bayerl and Bloom, 1990; Mornet *et al.*, 2005; Savarala *et al.*, 2011).

An equivalent of at least 1 lipid bilayer was estimated to be adsorbed on the PLA NP for LNPs obtained after 6 min of sonication and 1h of incubation except for LNPs made of pure POPC and those containing 10 mol% of DOTAP which were apparently covered by 2 to 3 lipid bilayers. The calculated number of lipid bilayers was as high as 8 for LNPs obtained with the conditions **i** (Table 4).

Regarding the zeta potential, a reversal of the LNP surface charge was observed for all formulations containing the cationic lipid. This observation confirmed the adsorption of lipid molecules onto the PLA surface. However, the zeta potential values were lower than +30 mV, predicting a short-term stability. There was no significant difference between the zeta potential of LNPs composed of 25% or 75% of the cationic phospholipid. For LNPs prepared with zwitterionic POPC vesicles, the zeta potential values also decreased, but remained negative. This suggests that PLA NPs were only partially coated with the neutral phospholipid bilayer. These results agree with those published by Troutier *et al.* (2005b) for polystyrene-DPTAP particles. They reported a charge reversal of cationic lipoparticles prepared with pure DPTAP compared to bare polystyrene particles (from -58 to +22 mV) and a slight decrease in the zeta potential of lipoparticles obtained with zwitterionic DPPC (from -58 to -40) (Troutier *et al.*, 2005b).

All these results suggest that LNPs formed after 6 min of sonication and 1h of incubation presented the best features. The increase in the cationic lipid proportion did not significantly influence the physicochemical properties of the LNPs. Since a high amount of cationic lipids could cause a high cytotoxic effect, the formulation containing 25 % of DOTAP represented a good compromise.

Cryo-TEM images of PLA NPs are shown in Figure 13. As expected, NPs were spherical with a smooth surface. Figure 14 and Figure 15 present cryo-TEM images of LNPs obtained after 1h vortexing (i). We observed a distinct contrast at the surface of PLA NPs suggesting the presence of one or more lipid bilayers. LNPs 90/10(i) exhibited a rough surface with adsorption of either intact or broken vesicles (Figure 14). LNPs 75/25(i) showed similar morphology, except that the intact vesicles were smaller and less numerous (Figure 15). These findings are coherent with the significant size increase measured by dynamic light scattering (Δ HD values in Table 4) and suggest that lipid organization around the NP core was perturbed by vortex intensity.

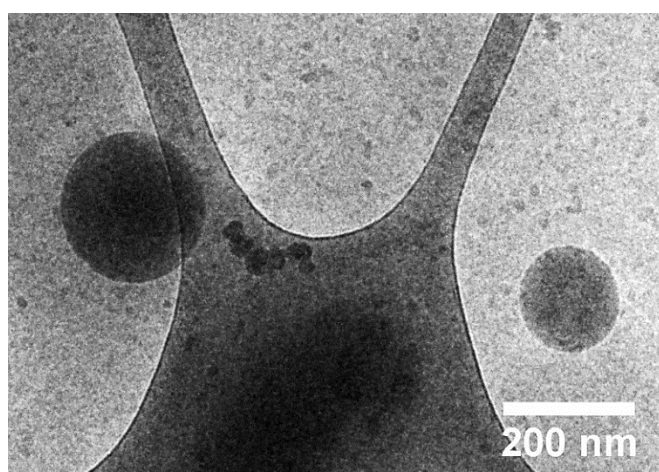


Figure 13: Cryo-TEM image of PLA NPs.

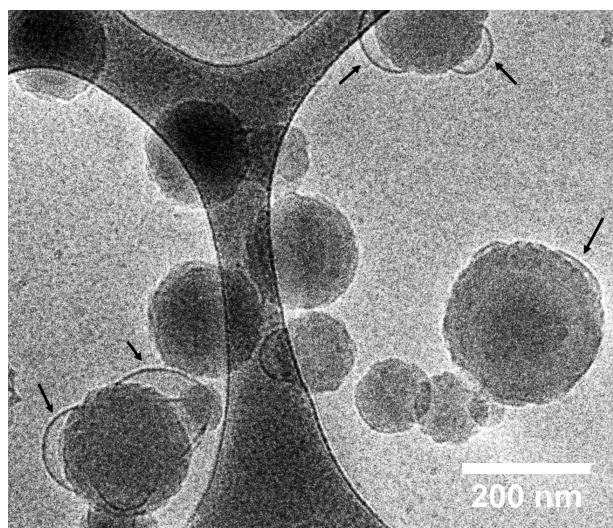


Figure 14: Cryo-TEM image of LNPs 90/10(i) obtained after 1h vortexing. Black arrows show the presence of some unbroken vesicles or lipid membranes adsorbed on the NPs core.

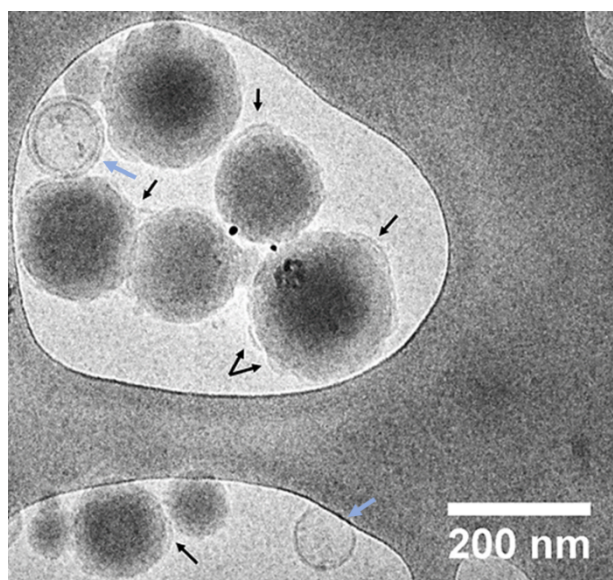


Figure 15: Cryo-TEM image of LNPs 75/25 (i) obtained after 1h vortexing. Black arrows show the presence of lipid membranes adsorbed on the NPs core which confer roughness to the surface. Some non-adsorbed lipid vesicles (blue arrows) are also visible in the suspensions.

LNPs obtained with 25, 50 and 75% DOTAP (Figure 16 B-D), after 6 min of sonication and 1h of incubation (iii), exhibited a smooth surface. It was difficult to visualize the lipid bilayer. No difference could be detected between these formulations. Conversely, LNPs formed of pure

POPC showed a rough surface and some small vesicles adsorbed at their surface (Figure 16A). Finally, LNP 75/25(ii) obtained by incubating the particles mixture for 1h exhibited an irregular surface due to the presence of some adsorbed vesicles, as shown in Figure 17. However, their surface was smoother than that of LNPs prepared under the condition i (Figure 14 and Figure 15). Some lipid bridges were also observed. Cryo-TEM experiments suggest that the short sonication step performed prior to incubation favored the reorganization of lipids around the NPs. Therefore, our best formulation is that containing 25% of DOTAP, obtained after 6 min sonication in a bath sonicator and 1h incubation at room temperature.

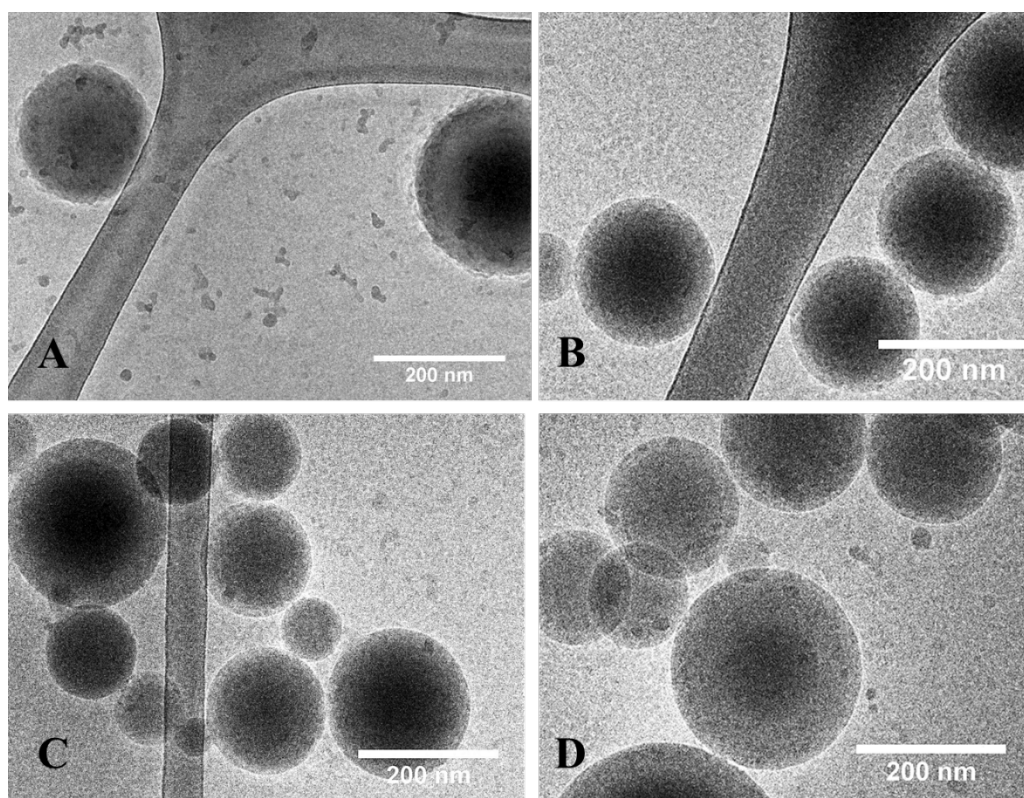


Figure 16: Cryo-TEM images of LNPs 100/0(iii) (A), LNPs 75/25(iii) (B), LNPs 50/50(iii) (C) and LNPs 25/75(iii) (D) obtained after 6 min of sonication followed by 1h of incubation. The black arrows indicate the presence of adsorbed vesicles onto the NPs surface.

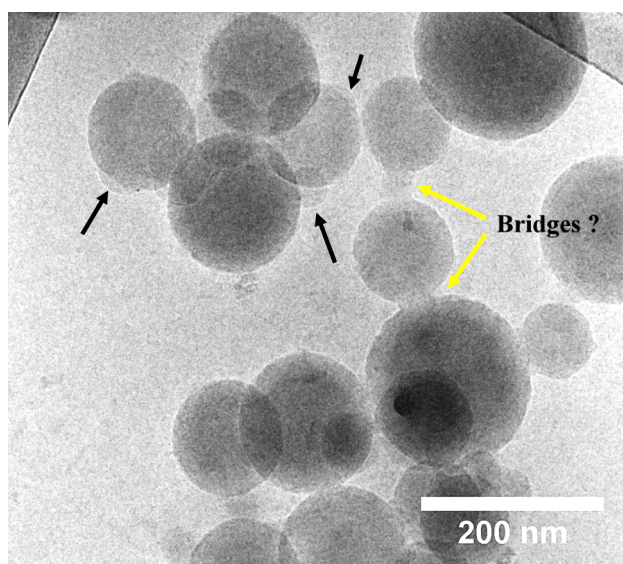


Figure 17: Cryo-TEM image of LNPs 75/25 (ii) obtained after 1h incubation. Black arrows show the presence of lipid membranes adsorbed on the NPs core which confer roughness to the surface. The yellow arrows indicate the presence of some lipid bridges.

II-3.3.2. Lipid assay

We have indirectly estimated the number of lipid bilayers from the LNPs diameter. However, this estimation is probably not accurate because in some cases (when pure POPC or a mixture with 10% of DOTAP were used, or depending on the mixing conditions), cryo-TEM images showed the presence of unbroken vesicles adsorbed onto LNPs surface, while in other cases, the presence of the lipid bilayer was difficult to distinguish at the surface of NPs. Several methods have been used to quantify the adsorbed lipids, in order to estimate the number of lipid layers adsorbed on NPs surface. They include indirect methods such as fluorescence quenching measurements (Major *et al.*, 1997) and thermogravimetric analysis (TGA) (Troutier *et al.*, 2005a; Ahmed *et al.*, 2011) and direct methods like ^1H -NMR (Thevenot *et al.*, 2008), chemical assays and MALDI-TOF MS analysis (Troutier *et al.*, 2005b). Lipid quantification by chemical assays varies depending on the nature of the lipid - inorganic phosphorus or enzymatic titration for neutral lipids such as POPC, and colorimetric titration by complexation with orange G for cationic ones like DOTAP (Rapuano and Carmona-Ribeiro, 2000; Troutier *et al.*, 2005a).

Chemical assays are time-consuming when a mixture of lipids is used and the presence of one lipid may hamper the quantification of the others.

We have thus quantified POPC-DOTAP lipids using HPLC-ELSD. HPLC is an effective tool already used to separate lipid molecules according to their physico-chemical properties including their polarities, the nature of the head group, etc. (Lesnefsky *et al.*, 2000). ELSD (Figure 18) is useful for the quantification of non-volatile compounds that do not have chromophoric groups in their molecular structure. Therefore, HPLC-ELSD can allow the simultaneous quantification of both neutral and ionic lipids.

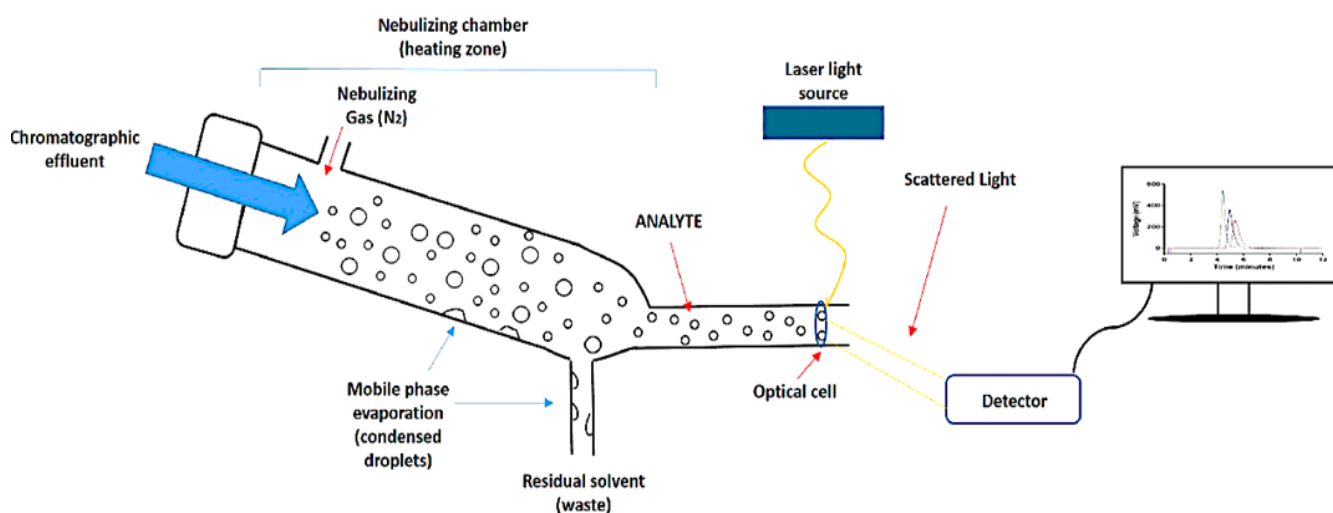


Figure 18: Mechanism of analyte detection by an evaporative light scattering detector (ELSD). Following the effective separation of molecules in the HPLC column, the eluate is mixed with N_2 gas in the nebulizing chamber leading to a dispersion of droplets; then the solvents are evaporated, condensed and removed leading to solid analytes which cross an optical cell illuminated by a light laser source. Analytes absorb the laser beam and scatter the light which is detected and converted into a signal (from Rocés *et al.*, 2016).

First, both lipids were analyzed separately and simultaneously, and no difference was noticed in their retention times. DOTAP and POPC were efficiently eluted at 7.4 ± 0.2 min and 15.7 ± 0.1 min, respectively. These retention times were consistent with expectations (Zhong *et al.*,

2010) since DOTAP is more polar than POPC. A slight variation of the retention time of DOTAP was observed from day to day analysis (Figure 19) - as reported by Zhong *et al.* - that was attributed to small changes in FAc concentration in the mobile phase, but also to the temperature, while that of POPC remained stable. Exponential calibration curves of the lipids were plotted as the mean height of the ELSD response related to the lipid concentration (C) by the following relationship $y = aC^b$ (y is the response, a and b are constants depending on the experimental conditions) (Zhong *et al.*, 2010). The obtained correlation coefficients R^2 were 0.988 and 0.989, for DOTAP and POPC, respectively.

All samples were dried and dissolved in methanol since PLA is insoluble in this solvent (Sato *et al.*, 2013). We ensured that undissolved PLA NPs were removed from LNP samples by centrifugation before injecting the supernatant containing the lipids into the HPLC column. Lipid recovery from liposomal suspension was calculated from the mass of lipids quantified by HPLC-ELSD (m_q) and the initial amount of lipid added (m_i) as shown in equation (1).

$$\% \text{ lipid recovery} = \frac{m_q \times 100}{m_i} \quad (1)$$

At least 95% of the lipids initially incorporated in our formulations were recovered by HPLC-ELSD (Table 5). Lipid recovery higher than 100% can be due to an increase in lipid stock solutions concentration following solvent evaporation during storage. We found that about 5 to 10 % of the total lipid used in the formulation was effectively adsorbed onto LNPs. LNPs prepared with 10% of DOTAP exhibited the highest proportion of adsorbed lipids. This lipid proportion decreased with increase of DOTAP content in the formulation. This is coherent with cryo-TEM images which showed that at 10% DOTAP, the NPs surface was covered with intact vesicles that did not break and fuse in a continuous lipid bilayer. The increase in cationic lipid content resulted in a stronger electrostatic interaction between particles and vesicles, followed by vesicles breaking and fusion in a lipid bilayer (Thevenot *et al.*, 2008). Whether this lipid bilayer was continuous or not is an open question.

Unexpectedly, the peak corresponding to DOTAP was found partially overlapped by a large peak, and there were two more unidentified peaks (Figure 19). By injecting a sample prepared from PLA NPs in the same condition as the LNPs, we deduced that these peaks belonged to the polymer. This meant that contrarily to literature reports, PLA had a certain solubility in

methanol. The large peak was attributed to PLA while the two other peaks were assigned to impurities, such as lactic acid moieties and/or polymerization initiators present in the supplied polymer batch.

	POPC/DOTAP Liposome			LNP		
Theoretical POPC-DOTAP ratio	90/10	75/25	50/50	90/10	75/25	50/50
Experimental POPC/DOTAP ratio	90/10 89/11	78/22 71/29	55/45 52/48	83/17 84/16	65/35 66/34	39/61 46/54
% lipid recovery	105 108	109 114	97 96			
% adsorbed lipids				9.5 9.7	6.8 6.3	5.4 4.5
Number of lipid bilayers*				1.4 1.4	1.1 1.0	0.7 0.6

*Table 5: Quantification of POPC and DOTAP in two liposome and LNP samples by HPLC-ELSD; expression of the experimental ratio of POPC/DOTAP, lipid recovery as function of the initial amount of lipids used, proportion of lipid and number of bilayers covering NPs surface. * Calculated using equation (2 to 7).*

Despite the overlapping of DOTAP peak with that of PLA, a good correlation could be obtained between the signal of both lipid peaks and their concentration in the formulations. The height of the DOTAP peak increased linearly with its concentration in the formulation, and conversely, that of POPC linearly decreased (Figure 19). Moreover, as shown in Table 5, the ratio of DOTAP really present in LNPs shell increased with the increase of the initial amount of DOTAP added to the formulation. This result agrees with expectations since DOTAP would preferentially adsorb to the negatively charged NPs surface via an electrostatic interactions (Ahmed *et al.*, 2012; Rapuano and Carmona-Ribeiro, 2000; Thevenot *et al.*, 2008).

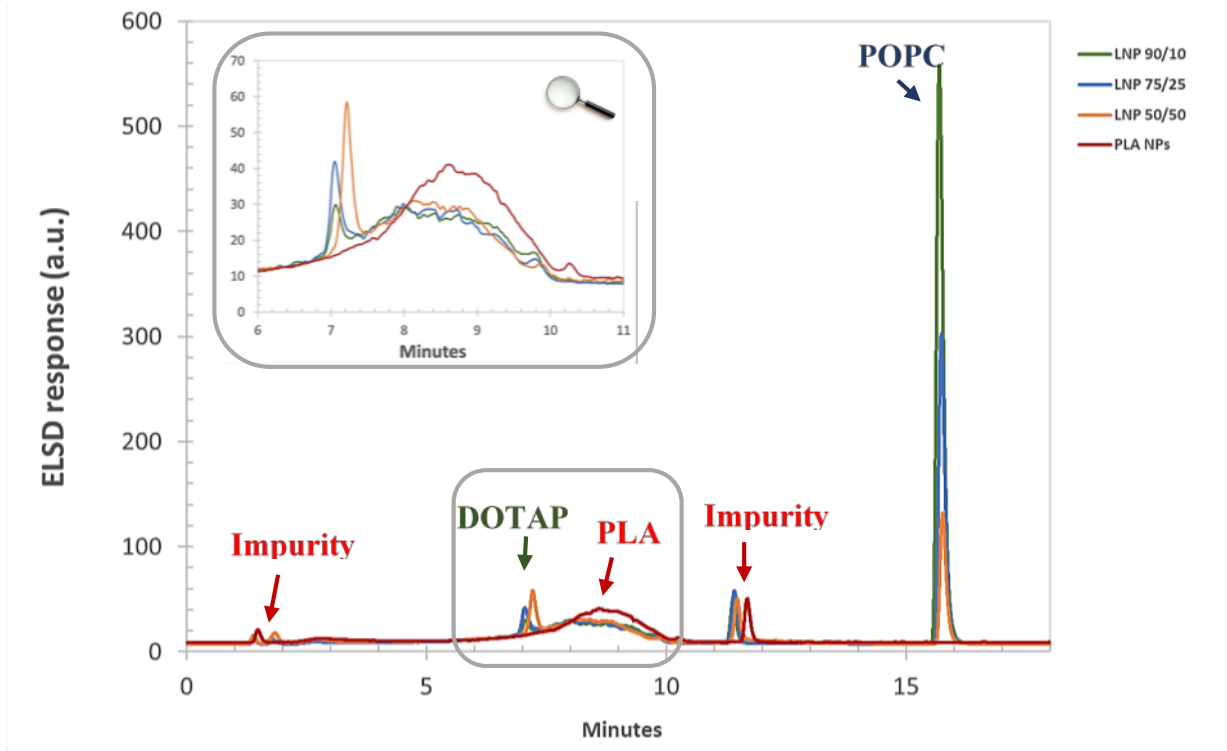


Figure 19: HPLC-ELSD chromatogram for LNPs (90/10, 75/25 and 50/50 mol%) (iii) and PLA NPs.

The equivalent number of lipid bilayers found on LNPs surface was estimated by assuming that a lipid bilayer fully covering the NPs had a total surface area (A_v) equal to that of the NPs (i.e., $A_v/A_p = 1$) (Savarala *et al.*, 2010). Based on this theory, the total surface area of the vesicles (A_v , equation 4) was calculated from the mass of dried PLA NPs used to prepare the LNPs (m_{NP}), the surface of one NP (S_{NP}), NP volume (V_{NP}) and PLA density (ρ_{PLA}). S_{NP} and V_{NP} were calculated from the mean diameter of the NPs using equations (2) and (3). ρ_{PLA} is 1.25 g.cm⁻³ (Thevenot *et al.*, 2008).

$$S_{NP} = \pi \left(\frac{HD_{NP}}{2} \right)^2 \quad (2)$$

$$V_{NP} = \frac{4}{3} \pi \left(\frac{HD_{NP}}{2} \right)^3 \quad (3)$$

$$A_v = \frac{m_{PLA} \times S_{NP}}{V_{NP} \times \rho_{PLA}} \quad (4)$$

The “molecular weight” (M_v) of one vesicle was then determined, which allowed calculating the number of lipids per vesicle and, thus, the required lipid concentration, using Clarke and Apell’s equation (Clarke and Apell, 1989).

$$M_v = \frac{\mathcal{N} \left[\frac{4}{3} \pi (r_o^3 - r_i^3) \right]}{\bar{v}} \quad (5)$$

Where $\mathcal{N}$ is Avogadro’s constant, r_o and r_i , the external and internal radii of the lipid bilayer - considering the thin water layer (T_w) of about 2 nm and lipid bilayer thickness (T_b) of ~ 4 nm as illustrated in Figure 12 – and $\bar{v}$, the partial specific volume of the lipid used. r_o and r_i , were estimated to be 90 ($r_o = R_{NP} + T_w + T_b$) and 86 nm ($r_i = R_{NP} + T_w$) respectively. The value of the partial specific volume of POPC ($\bar{v}_{POPC}$) is 0.987 – 1.0 mL/g (Heerklotz and Seelig, 2002; Hianik *et al.*, 1998). Since $\bar{v}_{DOTAP}$ was not found, M_v was calculated with $\bar{v}_{POPC} = 1$ mL/g.

Then, the number of lipid molecules per vesicle (N_{LV}) was determined from M_v and the molecular weight of POPC and DOTAP mixture (M_{PL}) taking into account their molar ratio a and b , respectively (equation 6).

$$N_{LV} = M_v \left[\left(\frac{a}{M_{POPC}} \right) + \left(\frac{b}{M_{DOTAP}} \right) \right] \quad (6)$$

N_{LV} is related to the number of moles of lipid required for covering a nanoparticle with one bilayer (n_{LB}) by the following equation (equation 7):

$$n_{LB} = \frac{N_{LV} \times A_v}{S_v \times \mathcal{N}} \quad (7)$$

Where S_v is the surface of one vesicle calculated assuming the diameter of a lipid bilayer to be equal to $2r_o$ ($HD_v = 180$ nm).

The theoretical concentration of lipid (C_{TL}) needed to cover the NP surface by a lipid bilayer is expressed as function of n_{LB} , the volume of the suspension (V_s), the molecular weight of both POPC and DOTAP and their molar ratio, a and b (equation 8).

$$C_{TL} = \frac{n_{LB}}{V_s \left(\frac{a}{M_{POPC}} + \frac{b}{M_{DOTAP}} \right)} \quad (8)$$

Finally, the equivalent number of lipid bilayers was estimated from the lipid concentration (C_{TL}) and that quantified by HPLC-ELSD (C_Q) (equation 9).

$$\text{Equivalent number of lipid bilayer} = \frac{C_Q}{C_{TL}} \quad (9)$$

This method for determining the number of bilayers may have some biases. Firstly, we didn't know the real value of $\bar{v}_{DOTAP}$. However, since the partial specific volume of DOPC is 0.999 mL/g (Nagle *et al.*, 2009) and DOTAP headgroup is believed to be less bulky than that of POPC (Gurtovenko *et al.*, 2004), $\bar{v}_{DOTAP}$ is unlikely to significantly impact the result. Secondly, our calculation assumed that all NPs in the suspension had a unique size of 170 nm which was not true, although DLS analysis indicated that the particles were monodisperse. Cryo-TEM images clearly showed particles with various sizes. Nevertheless, HPLC-ELSD allowed us to show that PLA NPs were coated by one lipid bilayer only (Table 5). No significant difference was observed between the number of lipid bilayers estimated by dynamic light scattering and HPLC-ELSD, except for LNPs 90/10(iii) for which the number of bilayers determined from DLS measurements seemed overestimated. HPLC-ELSD appears as a more accurate technique than DLS. The increase in DOTAP percentage in the formulation led to a decrease in the number of lipid bilayers adsorbed onto the particle surface. This observation also confirms the better reorganization of lipids around NPs core driven by attractive electrostatic interactions.

II-3.3.3. Assessment of LNP core-shell organization by confocal microscopy

Confocal microscopy experiments were performed with LNPs prepared with pure POPC (**iii**) (Figure 21) or POPC-DOTAP 75/25 (**iii**) vesicles (Figure 20). Colocalization of green and red fluorescence was observed for LNPs 75/25 (**iii**), as confirmed by the superimposition of the fluorescence intensity profiles (Figure 20A-D). For LNPs obtained with pure POPC (Figure 21A-D), a colocalization of green and red fluorescence was also observed, but the fluorescence signal of lipids was very weak as compared to LNPs 75/25. Indeed, the green peak for POPC LNPs had an intensity 4 times lower than that for LNPs 75/25 (Figure 21D). This result suggests partial coating of the particles surface by POPC liposomes and is supported by the zeta potential value for these LNPs, which is less negative than that of PLA NPs, but still negative.

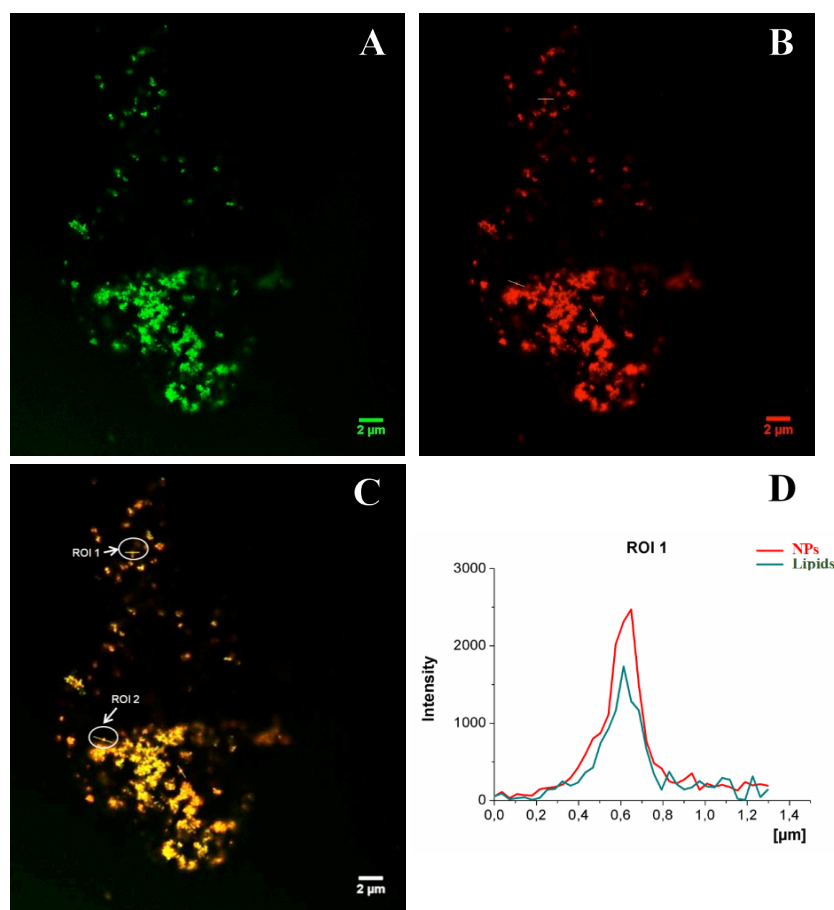


Figure 20: Confocal microscopy images of LNPs 75/25(**iii**). The NP core was labelled with 0.01% NR (red dye) and the lipid shell with 1% NBD-PE (green dye). The green and red fluorescence signals of the LNPs are shown in A and B respectively. C corresponds to the

overlay of both fluorescence and D shows the plotted fluorescence intensity profiles of a selected region of interest (ROI).

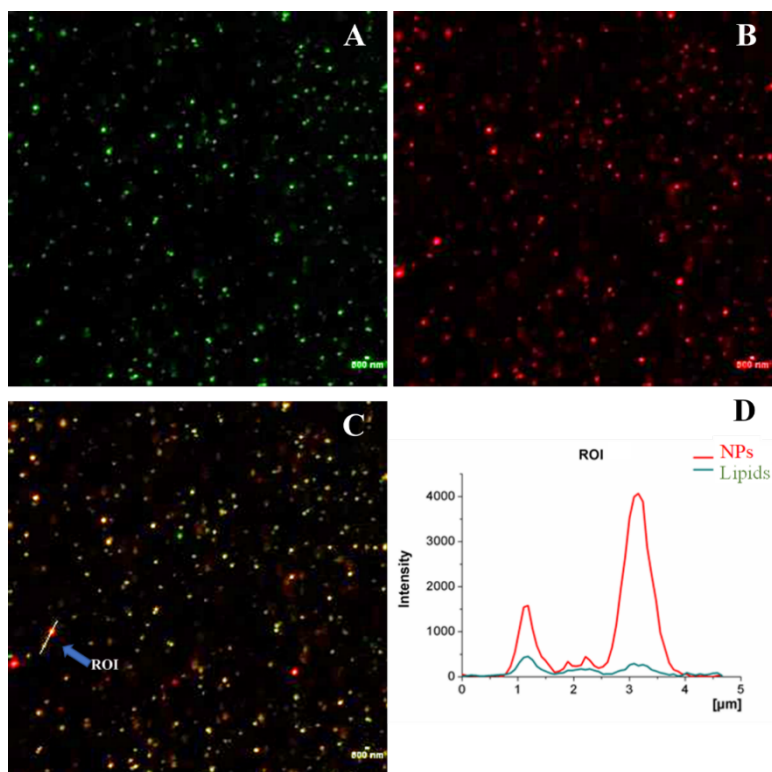


Figure 21: Confocal microscopy images of LNPs 100/0(iii). The NP core was labelled with 0.01% NR (red dye) and the lipid shell with 1% NBD-PE (green dye). The green and red fluorescence signals of the LNPs are shown in A and B respectively. C corresponds to the overlay of both fluorescence and D shows the fluorescence intensity profiles of a selected region of interest (ROI).

II-3.3.4. Stability of the lipo-nanoparticles

The stability of LNPs 75/25 in DMEM and DPBS was evaluated by dynamic light scattering (DLS). Spontaneous aggregation of the LNPs occurred after these media were added. Large and polydisperse particles were measured by DLS ($HD > 1 \mu m$ and $PDI \sim 0.3$) in both suspensions compared to LNPs prepared in pure water (Figure 22). Such aggregation has already been reported for LNPs prepared from PLA NPs and DPPC/DPTAP (100/0, 50/50 and

0/100 %) lipid mixtures (Thevenot *et al.*, 2008). In all cases, an aggregation was noticed with 10 mM NaCl. It was explained by the screening of particles charge at high ionic strength (Pfeiffer *et al.*, 2014), but also by the high density of PLA which accelerates the sedimentation of particles (Thevenot *et al.*, 2008). Aggregation was also observed for LNPs made of polystyrene NPs and cationic lipids (Bathfield *et al.*, 2008), or of PLGA and DOPC/DOTAP 1/1. For the latter, aggregates in PBS were larger than 1 μM (Cao *et al.*, 2018).

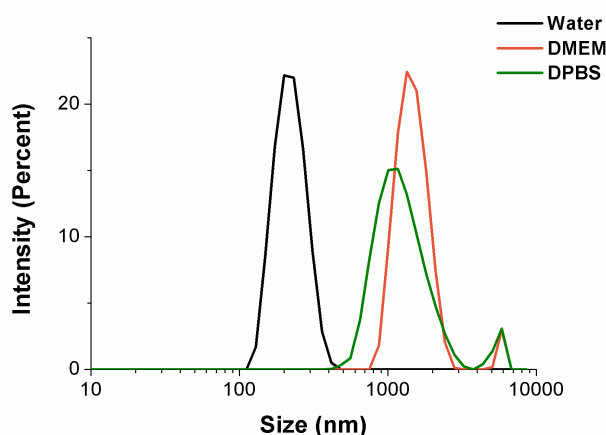


Figure 22: Evaluation of the stability of LNP 75/25(iii) in DMEM and DPBS by DLS.

Cryo-TEM images of our aggregated LNPs showed the presence of lipid vesicles or membranes at their surface (Figure 23). It means that, although aggregation occurred at high ionic strength, the lipid shell remained attached to the nanoparticles core. However, it reorganized differently. The screening of particles charge probably gave rise to attractive van der Waals interactions between lipids and NPs, leading to aggregation (Moura and Carmona-Ribeiro, 2005; Pfeiffer *et al.*, 2014). The surface of the particles became rough contrarily to LNPs suspended in water. Moreover, the formation of lipid bridges between the particles was observed (Figure 23). Presence of lipid bridges was also reported by Savarala *et al.* (2011) following the aggregation of DMPC lipid bilayer-coated SiO_2 nanoparticles at high ionic strength, but it was attributed to the partial coverage of the SiO_2 nanoparticles by the neutral lipid bilayer. Their system was different from ours. They had inorganic NPs while ours were polymeric. Even though our NPs were entirely covered by a lipid bilayer, we still observed the formation of bridges.

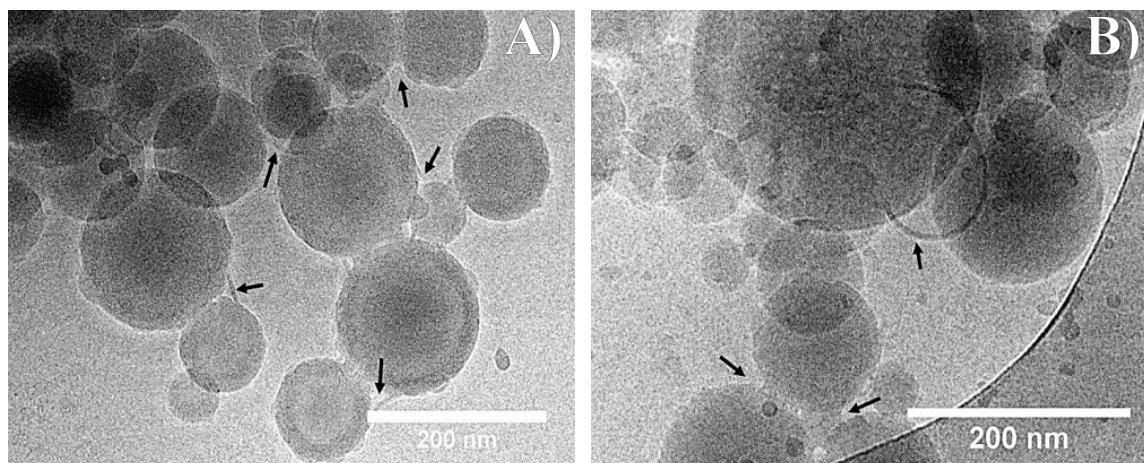


Figure 23: Evaluation of the stability of LNPs 75/25(iii) by cryo-TEM in DPBS (A) and DMEM (B). Lipid bridges are visible between particles (black arrows). These bridges probably formed due to the screening of DOTAP charge at high ionic strength, and consecutive closer contact between liponanoparticles.

Confocal microscopy analysis of fluorescent LNPs pellets suspended in DMEM with or without 10% of FBS also showed aggregates. However, we still observed an overlay of both green and red dyes fluorescence which confirmed the colocalization of the lipid and nanoparticles (Figure 24). This is a good point since the PS is to be incorporated in the lipid shell of the LNPs. Confocal microscopy also showed an interesting effect of fetal bovine serum. In DMEM supplemented with FBS, aggregates were smaller than in DMEM. The reason for the formation of smaller aggregates in the presence of FBS is still unclear. However, colocalization of lipids and NPs is maintained and smaller aggregates are preferable in cell cultures, if aggregation cannot be avoided.

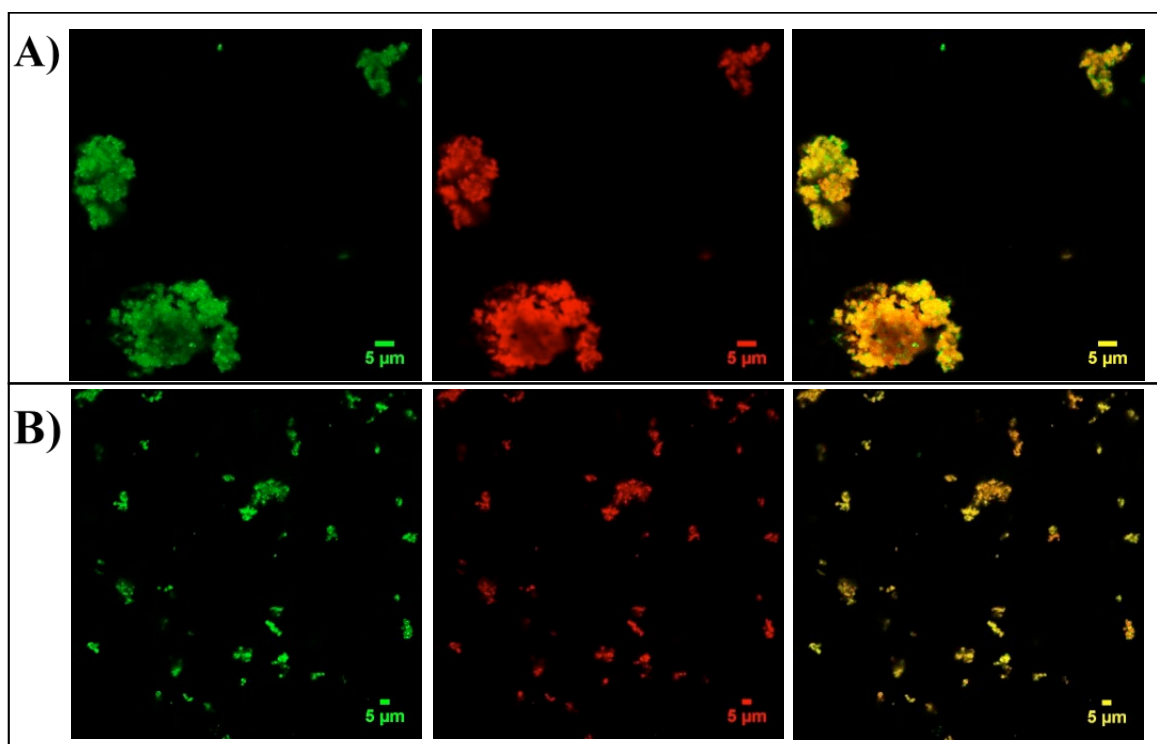


Figure 24: Confocal microscopy images of fluorescent LNPs labelled with 0.001 mol% NR and 2 mol% NBD-PE suspended in DMEM (A) and DMEM supplemented with 10% FBS (B); the green fluorescence reveals the presence of lipids and the red fluorescence that of the NPs. Overlay of both fluorescence leads to a yellow colour which confirms the colocalization of lipid shells with polymer cores.

m-THPP was incorporated in the lipid shell of LNPs and the stability of the colloidal suspension was also evaluated in DMEM supplemented or not with 10% FBS. As illustrated in Figure 25, m-THPP LNPs (red) showed the same behavior as LNPs colored by NR and NBD-PE.

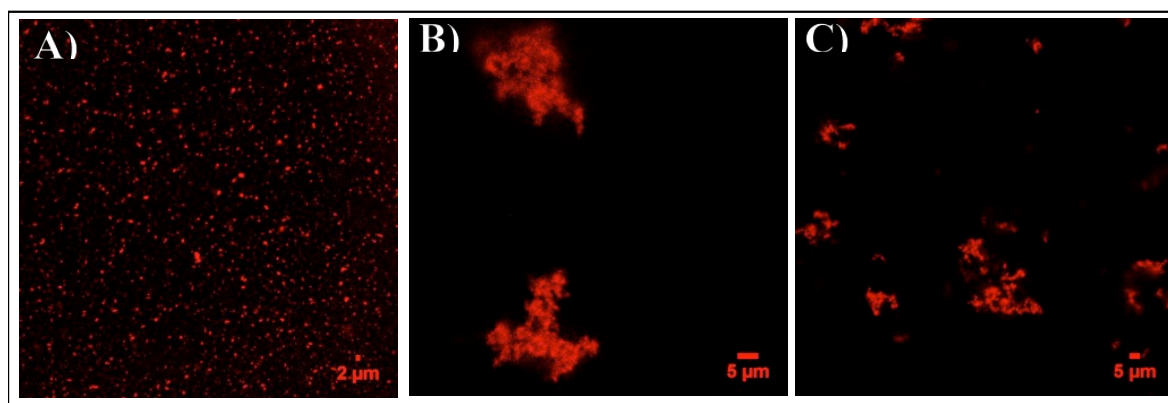


Figure 25: Confocal microscopy images of *m*-THPP LNPs dispersed in pure water (A), DMEM (B) and DMEM supplemented with 10% FBS (C). The red fluorescence confirms the presence of *m*-THPP in the lipid shell of the LNPs.

Several strategies have been adopted to prevent LNPs aggregation at high ionic strength, including grafting of long PEG chains (Hu *et al.*, 2015c; Thevenot *et al.*, 2007) or poly(N-acryloylmorpholine) (poly(NAM)) ones (Bathfield *et al.*, 2008) to lipid vesicles. Such approaches could be tested in new experiments.

II-3.3.5. Determination of the mechanism of interaction of PSs and LNPs with cells

Y79 cells were incubated with fluorescent LNPs, free *m*-THPP and *m*-THPP-loaded LNPs at different times and their kinetics of internalization was assessed by confocal microscopy as shown in Figure 26, Figure 27 and Figure 28. No fluorescence was observed in untreated cells (Figure 26A). The LNPs aggregates were found in the cell culture medium, as mentioned above, but we still observed the colocalization of lipid shells and NP cores (yellow fluorescent aggregates in Figure 26). This observation was confirmed by the red fluorescence of *m*-THPP-loaded LNPs (Figure 27). Aggregates adsorbed to the cells surface, due to electrostatic interactions occurring between cationic LNPs and anionic cell membranes. This is in agreement with studies reported in the literature (Bose *et al.*, 2015; Hu *et al.*, 2015c; Koo *et al.*, 2012).

Confocal microscopy images show that fluorescent LNPs were internalized by the cells within one hour of incubation (Figure 26B). The maximum uptake seemed to occur within the first 4h

of incubation as observed by others (Hasan *et al.*, 2012). From 24 to 48h incubation, the green fluorescence appeared more intense, which can be attributed to possible lipid mixing between LNPs and cell membranes (Wrobel and Collins, 1995). LNPs were distributed all over the cytoplasm of cells, as previously observed (Bose *et al.*, 2015).

Cellular uptake was also observed with m-THPP-loaded LNPs (Figure 27). A weak red fluorescence signal was observed inside the cells incubated with these LNPs for 4h, which progressively increased with the length of incubation time (Figure 27). Similar feature was obtained when Y79 cells were incubated with free m-THPP solubilized in DMSO (Figure 28), although the internalization mechanism of free PSs is expected to be different from that of NPs. In both cases, the maximum accumulation occurred between 24h and 48h of incubation (Peng *et al.*, 1995). The red fluorescence was also homogeneously distributed in the cytoplasm. According to literature, cell death will probably occur by apoptosis (O'Connor *et al.*, 2009).

Our results are consistent with previous studies showing the fast uptake of cationic LNPs (Dombu *et al.*, 2010; Harush-Frenkel *et al.*, 2008). It was suggested this cellular uptake occurred by clathrin-mediated endocytosis (Duan *et al.*, 2017; Liu *et al.*, 2009b; Rejman *et al.*, 2004) and transcytosis (Harush-Frenkel *et al.*, 2008).

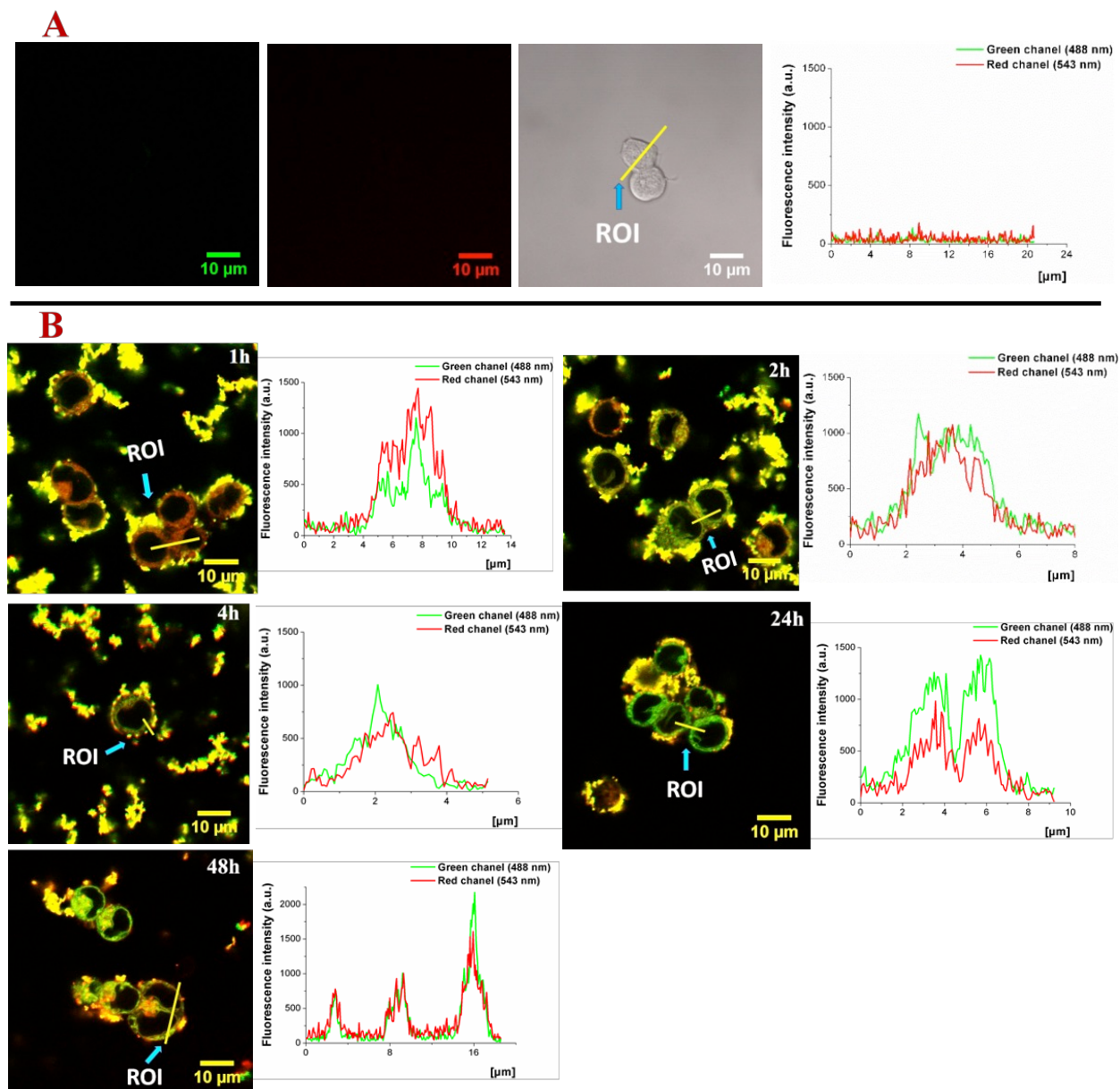


Figure 26: Confocal microscopy images depicting the interaction and internalization of fluorescent LNPs (iii) in Y79 cells. NPs were labelled with 0.001% NR (red dye) and lipid shells by 2 mol% NBD-PE (green dye). The autofluorescence of cells was assessed at 488 nm and 543 nm (A). Cells were incubated with fluorescent LNPs at 37°C at different times (B). Fluorescence overlays in the images and intensity profiles confirm the penetration of both NPs and lipid shells in cells.

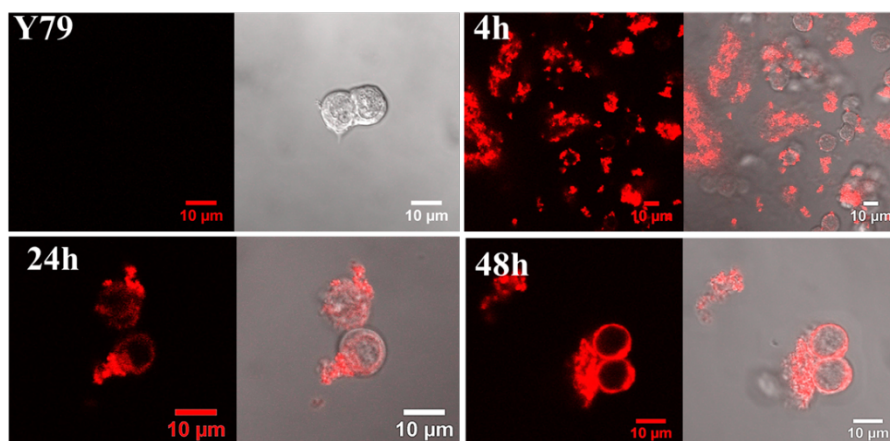


Figure 27: Confocal microscopy images of Y79 cells incubated with *m*-THPP LNPs at different times (4h, 24h and 48h). For more clarity, the red fluorescence of *m*-THPP at 633 nm (left) is also shown on transmitted light images (right). No autofluorescence of Y79 cells was observed at 633 nm.

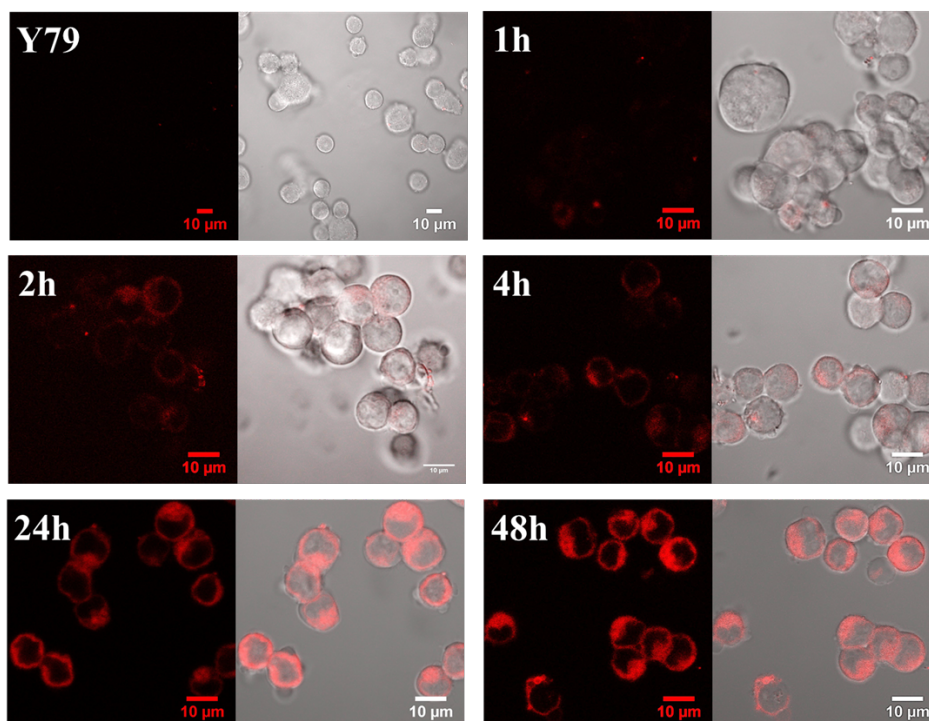


Figure 28: Confocal microscopy images of Y79 cells incubated with free *m*-THPP at different times (1h, 2h, 4h, 24h and 48h). For more clarity, the red fluorescence of *m*-THPP at 633 nm (left) is also shown on transmitted light images (right). No autofluorescence of Y79 cells was observed at 633 nm.

II-4. Conclusion

We have prepared LNPs from preformed PLA NPs and POPC/DOTAP liposomes. Various parameters such as the POPC/DOTAP ratio and NPs and vesicles mixing conditions were tested to ensure successful coverage of the nanoparticles by a lipid bilayer. The tested proportions of DOTAP were 10, 25, 50 and 75 mol%. NPs and liposomes were mixed either by **i)** 1h vortexing, **ii)** 1h incubation, **iii)** 6 min of sonication followed by 1 h incubation or **iv)** 2 min vortexing and 1h incubation. The obtained LNPs were characterized by different methods which allowed us to identify the condition **iii)** as optimal for forming our system. A minimum of 25% DOTAP was necessary to achieve good coating of the NPs. Otherwise, lipid vesicles tended to adsorb on the particle surface without reorganizing into a continuous lipid bilayer. Since we didn't observe any significant difference by increasing the amount of DOTAP up to 75% in the formulation, and because cationic lipids are known to be toxic in a dose-dependent manner, we chose LNPs obtained by a short-time sonication of POPC/DOTAP 75/25 liposomes and PLA NPs followed by an incubation of 1h. These LNPs were called LNP 75/25 (**iii**). Lipid quantification showed evidence of the formation of one lipid bilayer. We also demonstrated that, although our LNPs aggregated in the cell culture medium, they still preserved a core-shell structure. Moreover, we found that they strongly bound to the surface of retinoblastoma cells and were internalized within few hours. We finally showed that a PS loaded into these LNPs accumulated into the cells.

The next steps of our work consisted in a further characterization of the interactions between POPC/DOTAP vesicles and PLA nanoparticles (Chapter III), and the evaluation of the cytotoxic/phototoxic efficacy of LNP co-encapsulated anticancer drug and PS against retinoblastoma cells (Chapter IV).

Chapter III: Combined QCM-D and AFM experiments for deciphering the mechanism of fusion of lipid vesicles with polymer nanoparticles

Chapter III: Combined QCM-D and AFM experiments for deciphering the mechanism of fusion of lipid vesicles with polymer nanoparticles

III-1. Introduction

Construction of polymer-supported lipid bilayers modeling plasma membranes has been widely described in the literature ([Ábrahám *et al.*, 2017](#); [Majewski *et al.*, 1998](#); [Nirasay *et al.*, 2012](#); [Shao *et al.*, 2017](#)). In general, the objective of the reported studies was to separate a planar lipid bilayer from a solid support by a layer of soft hydrated polymer, so that the interaction between the membrane and the rigid solid substrate was minimized, while mimicking the constraining effect of the cytoskeleton on the plasma membrane ([Lenne *et al.*, 2006](#); [Majewski *et al.*, 1998](#); [Wagner and Tamm, 2000](#)). The underlying polymer layers were in the form of (i) polymer coils, grafted to the solid support, (ii) mono- or multi-layers adsorbed to the substrate ([Andersson and Köper, 2016](#); [Majewski *et al.*, 1998](#); [Nirasay *et al.*, 2012](#); [Wlodek *et al.*, 2015](#)) or (iii) polymer brushes ([Blakeston *et al.*, 2015](#)). Following polymer adsorption or grafting to the substrate, the phospholipid bilayer was formed by liposome fusion ([Nirasay *et al.*, 2012](#); [Shao *et al.*, 2017](#)), or Langmuir-Blodgett (LB)/Langmuir-Schaefer (LS) transfer of phospholipid monolayers. Numerous articles reported simulations works ([Dimitrievski and Kasemo, 2008](#); [Kong *et al.*, 2016](#); [Shao *et al.*, 2017](#)). The uncoupling of the membrane from the solid substrate was achieved to a greater or lesser extent, depending on the degree of swelling of the polymer ([Shao *et al.*, 2017](#)). The characterization of these systems was usually carried out by atomic force microscopy (AFM) and quartz crystal microbalance (QCM-D) experiments ([Mingeot-Leclercq *et al.*, 2008](#); [Richter and Brisson, 2005](#)). We have used this approach to model the adsorption-fusion of POPC and POPC-DOTAP vesicles on PLA nanoparticles.

We spread a PLA monolayer at the air-water interface, transferred it onto a solid substrate and followed vesicles adsorption-rupture of neutral or cationic phospholipid vesicles onto this film, using AFM and QCM-D.

III-2. Materials and methods

III-2.1 Materials

Glass surfaces and sulfuric acid (H_2SO_4) were supplied by VWR Prolabo (Leuven, Belgium) and sodium chloride (NaCl) from Carl-Roth chemicals (Germany). 1,1,1,3,3,3-Hexamethyldisilazane (HMDS, $M_w = 161.39$ g/mol) and perdrogenTM. H_2O_2 , 30% w/w, were obtained from Sigma-Aldrich (St Louis, USA). All other materials used in the following experiments are described in Chapter II.

III-2.2 Methods

III-2.2.1. Surface pressure-area (π -A) measurements

The Langmuir trough (Figure 29) is an instrument that measures the surface pressure as a function of the area occupied by the molecules forming a monolayer. It consists of a very shallow tank (< 1 cm) with a large surface area. When filled with water or a buffer, it is possible to deposit on its surface insoluble amphiphilic molecules that organize at the interface. Two movable barriers in contact with the surface can move towards each other, compressing the formed monolayer. As the available surface decreases, the initially dispersed amphiphilic molecules come closer together and interact. They rearrange, producing a surface pressure π that increases as the surface area decreases (Figure 29). When the limiting molecular area is reached, the monolayer collapses. Molecular area and surface pressure at collapse are characteristic values of a given pure compound as a function of temperature. The isotherms obtained are called compression isotherms.

Surface pressure-area (π -A) measurements were performed using a thermostated Langmuir film trough (775.75 cm², Biolin Scientific, Finland) enclosed into a Plexiglas box to limit surface contamination (Figure 29). Prior to experiments, the pure water surface was cleaned by suction. Solutions of PLA in chloroform (1 mg/mL) were spread onto the aqueous surface and left for

15 minutes to allow complete evaporation of the solvent. Compression was then performed at a speed of $15 \text{ cm}^2 \cdot \text{min}^{-1}$. All experiments were run at $22 \pm 1^\circ\text{C}$. The results reported are mean values of at least three measurements. The experimental uncertainty was estimated to 0.2 mN/m . The surface compressional moduli (C_s^{-1}) were calculated using Equation (10):

$$C_s^{-1} = -A \frac{d\pi}{dA} \quad (10)$$

Where π is the surface pressure and A is the molecular area. In this case, as the molecular weight of the polymer was not known with accuracy, the area was expressed in m^2/mg .

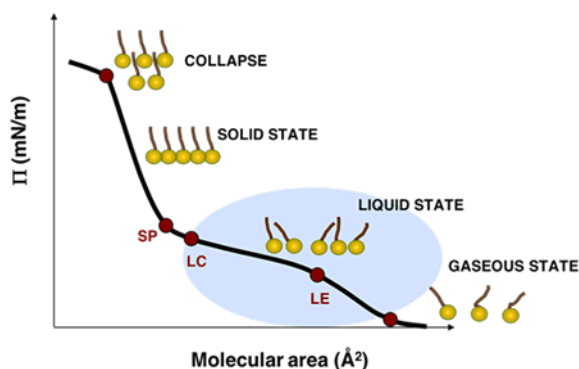
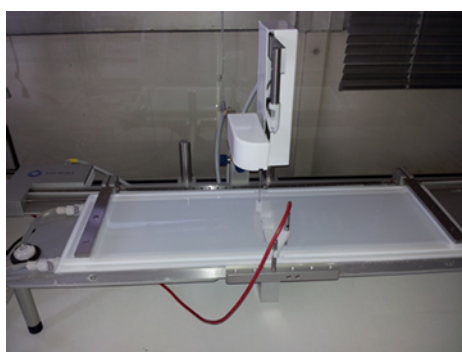


Figure 29: (Left) Langmuir trough. (Right) Langmuir isotherm. LE: liquid-expanded; LC: liquid condensed; SP: solid phase.

III-2.2.2. Surface potential measurements

Surface potential (ΔV) measurements were performed using a home-made Langmuir trough (217.2 cm^2 surface area) coupled to a SPOT1 instrument (KSV, Finland). Surface potential changes were continuously measured during monolayer compression (Ambike *et al.*, 2011). Each experiment was performed at least two times. The standard error was 2 mV . The surface potential ΔV results from the contribution of the double-layer potential (Ψ_0) and the non-Coulombian potential related to the dipole moment, $\mu_\perp$ of molecules (Equation 11).

$$\Delta V = \frac{2kT}{e} \sinh \left[\frac{e\alpha}{A(5.88 \times 10^7 c \epsilon T)} \right] + \frac{\mu_{\perp}}{A \epsilon_0 \epsilon} \quad (11)$$

with k the Boltzmann's constant, T the absolute temperature, e the electronic charge, α , the degree of ionization, c the ionic concentration, A , the molecular area, and ϵ_0 the permittivity of the free space. The apparent relative permittivity of the monolayer (ϵ) was assumed to be equal to 1 in the gaseous state and 2 in the condensed state (Taylor, 2000).

III-2.2.3. Brewster Angle Microscopy (BAM)

Brewster Angle microscopy (BAM) allows the visualization of the inhomogeneities of a film of molecular thickness at the air/water interface. In order to be able to visualize the light reflection from the air/water interface, it is necessary to get rid of the signal coming from the water subphase itself. This is achieved using Brewster incidence and polarized light in the plane of incidence. The incidence of Brewster between an optical index medium n_1 and an optical index medium n_2 is $\text{Arctg}(n_2/n_1)$. For the air/water interface ($n_1 = 1$, $n_2 = 1.33$), the Brewster angle is 53° (Figure 30). Under these conditions the reflection coefficient of a perfectly flat and thin interface between two transparent media is canceled. In the presence of a PLA film spread at the interface, the reflected light is expected to be more intense if the film is thick or dense. It is thus possible to visualize the optical inhomogeneities of the film, for example phase coexistences, with a resolution of the order of the wavelength.

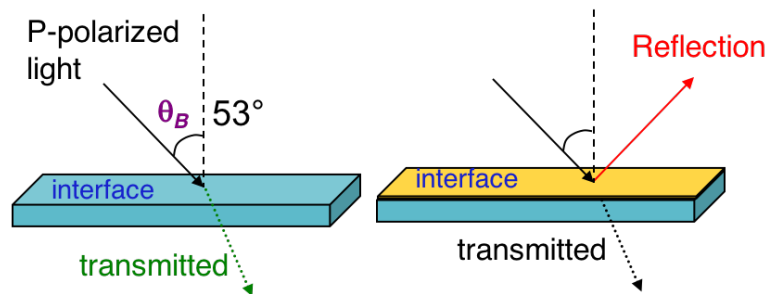


Figure 30: Principle of the Brewster Angle Microscope.

The morphology changes of the PLA film at air-water interface during compression were analyzed using a Brewster angle microscope (MicroBAM 3, NimaTechnology Ltd., Coventry, U.K.) connected to an auto-recording Langmuir trough (MCN Lauda, Germany). The microscope was equipped with a frequency laser diode ($\lambda = 659$ nm, 30 mW optical power) generating a collimated beam of approximately 6 mm diameter, with a p-polarizer, analyzer, and a USB camera. The spatial resolution of the BAM was about 6 μm per pixel, with a field of view of 3.6×4.1 mm², resolved over 640×480 pixels. Image size was 2.0 mm² after rescaling. PLA films were compressed in the same experiment conditions as previously described.

III-2.2.4. Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) transfer of PLA monolayers

LB transfers of PLA monolayers were performed at 22°C on freshly cleaved mica slides for AFM analysis in the air of the PLA topography by atomic force microscopy (AFM).

For QCM-D and AFM measurements in water, LS transfers were performed using hydrophobically modified substrates. 1 square-cm glass surfaces were first cleaned by immersion in a fresh acidic piranha bath for 15 minutes, then thoroughly rinsed with ultrapure water and dried under nitrogen stream. The glass surfaces were then exposed to UV/ozone treatment for 45 minutes. Hydrophobic modification of the substrates with HMDS in vapor phase was performed for 12 hours in a glove box filled with argon. HMDS-functionalized glass surfaces were stored in a closed box.

LB or LS transfers of a PLA layer on mica or HMDS-covered glass plates were performed with the thermostated Langmuir film trough fitted with a well. The LB transfer was obtained by pulling out (under constant surface pressure, and at a rate of 1 mm/min) the mica surface that had been immersed in the well prior to PLA spreading (Figure 31A). For the LS transfer, a teflon home-made sample holder was used to insert the HMDS-functionalized glass surface in the well. After PLA monolayer formation at the air-water interface the compression was performed until the desired surface pressure was reached. Regulation of surface pressure at a constant value allowed the LS transfer of the PLA film, by maintaining by suction directly above the air-liquid interface a purpose-built device able to align the HMDS-covered glass substrate parallel to the buffer surface. The surface was then dipped through the interface and

lowered into the sample holder placed in the well of the trough (Figure 31B). All LS transfers were deposited at 22°C and kept in ultrapure water until analysis.

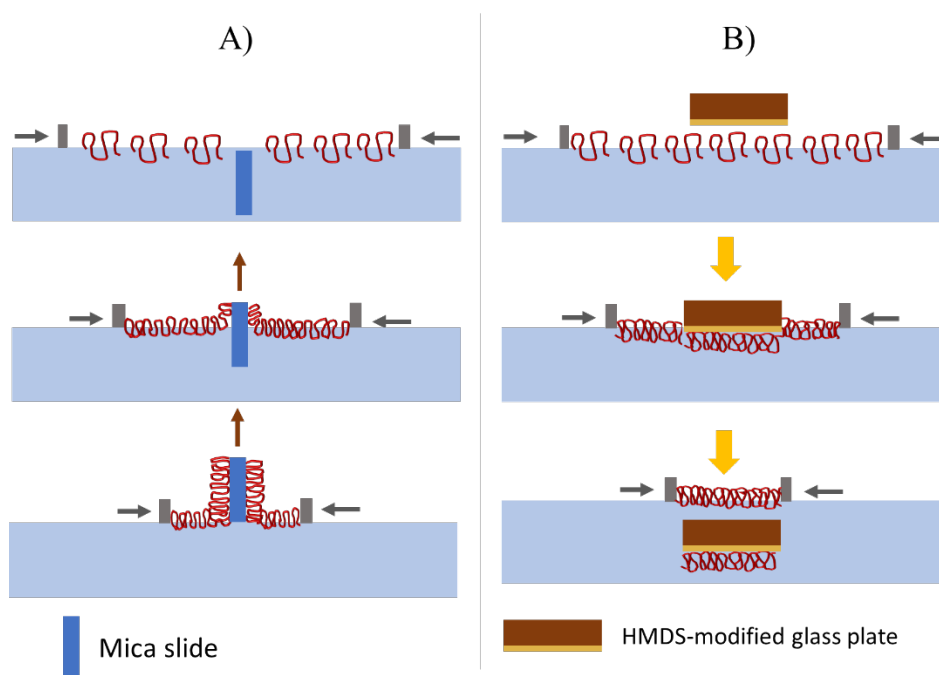


Figure 31: LB (A) and LS (B) transfers of a PLA the monolayer onto a solid substrate.

III-2.2.5. Quartz Crystal Microbalance with Dissipation (QCM-D) measurements

QCM-D is a technique that allows the quantification of deposited masses and characterization of their viscoelastic properties onto a vibrating surface. This technique is based on measuring the resonance behavior of a quartz crystal oscillator operating in shear mode. The mechanical oscillation of the piezoelectric quartz can be excited by applying an oscillating electric field. The resonance frequency is related to the mass of the crystal and any mass adsorbed to or removed from the sensor induces a frequency shift (Δf) corresponding to a change in adsorbed or removed mass. As the electrical power is turned off sequentially, QCM-D allows the simultaneous measurement of the energy dissipation changes (ΔD) obtained from the rate of decay of the oscillation amplitude. ΔD values provide information on the viscoelastic properties of the adsorbed material.

AT-cut silicon dioxide-coated quartz crystals with a fundamental frequency of 5 MHz were provided by Q-Sense AB (Gothenburg, Sweden). Prior to use, they were cleaned in a 10 mM sodium dodecyl sulfate solution for 30 min. They were then thoroughly rinsed with ultrapure water and dried under a stream of N₂. The QCM SiO₂ surface was then exposed to UV/ozone treatment for 45 minutes. Functionalization with HMDS in vapor phase was performed for 12 hours in a glove box filled with argon. Hydrophobically-modified QCM surfaces were further used for LS transfer of PLA. Experiments using the quartz crystal microbalance with dissipation monitoring were performed at room temperature (22°C), using the QCM-D E4 from Q-Sense (Gothenburg, Sweden). The QCM-D cell loaded with the crystals was first immersed in aqueous medium. When stable baselines for both oscillation frequency shift (Δf) and energy dissipation change (ΔD) signals were obtained, extruded POPC or POPC-DOTAP liposomes (0.4 or 4 mM) were injected into the cell (Figure 32). A constant flow rate of 50 $\mu\text{l}/\text{min}$ was used for the experiments. Frequency shift and dissipation were measured and recorded at 3rd, 5th, 7th, 9th, 11th overtones. When both Δf and ΔD signals reached equilibrium, a water rinse at the same flow rate was finally performed. Incubation with the liposomes was maintained for 1h. Rinsing with ultrapure water was performed at the end of each experiment. For experiments performed with POPC and unmodified SiO₂ surfaces, pre-treatment of the supports with CaCl₂ enriched buffer was performed after UV/ozone treatment, before injection of liposomes. All liposome suspensions were in pure water.

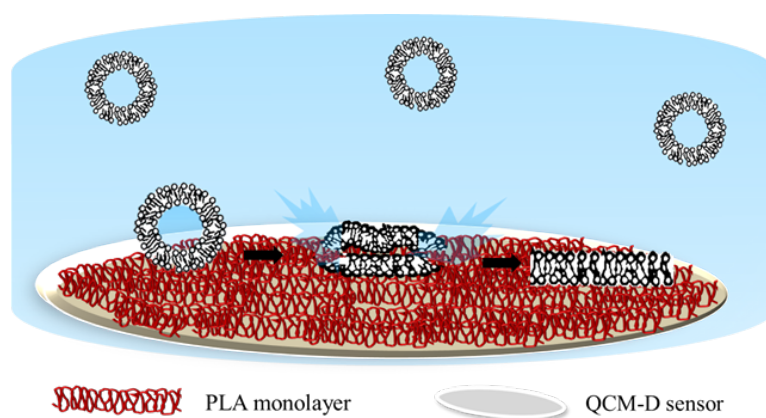


Figure 32: Adsorption-rupture of phospholipid vesicles onto the PLA film.

III-2.2.6. Atomic force microscopy experiments

Atomic force microscopy is a technique that makes possible to explore the topography and mechanical properties of a solid surface thanks to the force exerted by an oscillating cantilever. Its principle is based on the interactions between the existing molecules on the surface and the cantilever oscillating in a controlled manner. A laser beam measures this oscillation and its variations due to the interactions between the tip and the surface. By working at cantilever deflection, the microscope uses a feedback loop that modulates the height of the tip, which creates a topographic contrast. A photodetector makes it possible to detect the variations of position of the reflected beam on the cantilever. AFM experiments were performed using a JPK Nanowizard 3 UltraSpeed (JPK Instruments, Berlin, Germany, www.jpk.com) standing on an air-buffered table coupled to a dynamic anti-vibration device and enclosed in an acoustic box. AFM imaging was performed in AC mode (amplitude modulation) with gold coated silicon cantilevers of 0.6 ± 0.2 N/m nominal spring constant and 125 ± 35 kHz resonance frequency (Bruker, Santa Barbara, USA). The pyramid-shaped tips had a radius of curvature around 20 nm. Images were taken at the scan rate of 1 Hz. Image processing (flatten, plane fit, edge and line detection) was performed with the JPK Data Processing software (JPK Instruments). HMDS modified glass and QCM surfaces were imaged in air in order to check the good quality of HMDS grafting. PLA-modified surfaces were all imaged in the air after LB transfer and in pure water conditions after LS transfers. Further incubation with POPC or POPC-DOTAP 75/25 liposomes (0.4 and 4.0 mM) suspensions was performed for 1h before a final abundant rinsing step with ultrapure water. The system elasticity was quantified by the determination of apparent Young's modulus using force measurements that were performed on $5 \mu\text{m}^2$ surface areas at a fixed velocity ($1 \mu\text{m/s}$) over a Z length of 1300 nm to record the cantilever deflection. The linear portion of the extension curves was fitted to a Hertz-Sneddon model to extract the apparent Young modulus with the JPK Data Processing software. The tip radius provided by the manufacturer was applied (20 nm). A force curve was captured on bare mica surface to estimate the deflection sensitivity found between 20 and 30 nm/V.

III-3. Results and discussion

III-3.1. Interfacial behavior of PLA film at the air-water interface

The π -A isotherm of a PLA film spread at the air-water interface is shown in Figure 33. It is similar to that published by Boury *et al.* (1994) and Kiss *et al.* (2004) and shows the same three successive states: (i) a highly compressible region up to 13 mN/m where the film is homogeneous in a liquid-expanded (LE) state (Figure 33a,b); (ii) a plateau corresponding to transition from the liquid-expanded to liquid-condensed (LE-LC) phase of PLA (13-16 mN/m), with coexistence of PLA domains and fluid phase (Figure 33c), and (iii) a poorly compressible region where the PLA film is in a condensed homogeneous state (Figure 33f,g,h).

In the plateau region at 15 mN/m, nanodomains are clearly visible by AFM with elongated structures on top of them, resembling 3-dimensional nanocrystals (Figure 33d,e). The height of the layer is about 2.5 nm as deduced from the line profile. Large circular crystallites with diameters close to 0.5 μ m are also observed. At 25 mN/m, the mean thickness of the PLA layer is 5 ± 0.5 nm, as determined from cross sections analysis in AFM pictures. These results are very similar to those obtained by Boury *et al.* (1994) who studied a PLA layer transferred onto a solid substrate. The area considered in their work (2.3 $\text{\AA}^2$ /per lactic acid segment) corresponds to a surface pressure close to 25 mN/m. By X-ray reflectivity, in similar conditions of polymer layer hydration, they measured a thickness of 2.6 nm for the expanded PLA monolayer and 4.4 ± 0.2 nm for the condensed one.

The surface potential isotherm reaches its maximum value (558 mV) at the beginning of the LE-LC phase transition, suggesting that reorientation of PLA charged groups at the interface mainly occurs before this transition (Figure 34A). Similar results were reported for pure PLA enantiomers (Klass *et al.*, 2003). However, in general, comparison of PLA with poly (L)-lactic acid or poly (D)-lactic acid showed differences in interfacial organization of these polymers. Indeed, pure enantiomers are crystalline (Tsuji and Ikada, 1997) and organize as helices with specific unit cells (Ni *et al.*, 2006; Pelletier and P  zolet, 2004), whereas PLA is an amorphous polymer (Tsuji and Ikada, 1997). Due to random arrangement of L- and D-repeating units, the formation of helix structures is hindered. PLA organizes in random coils at all concentrations (Cheng *et al.*, 2017).

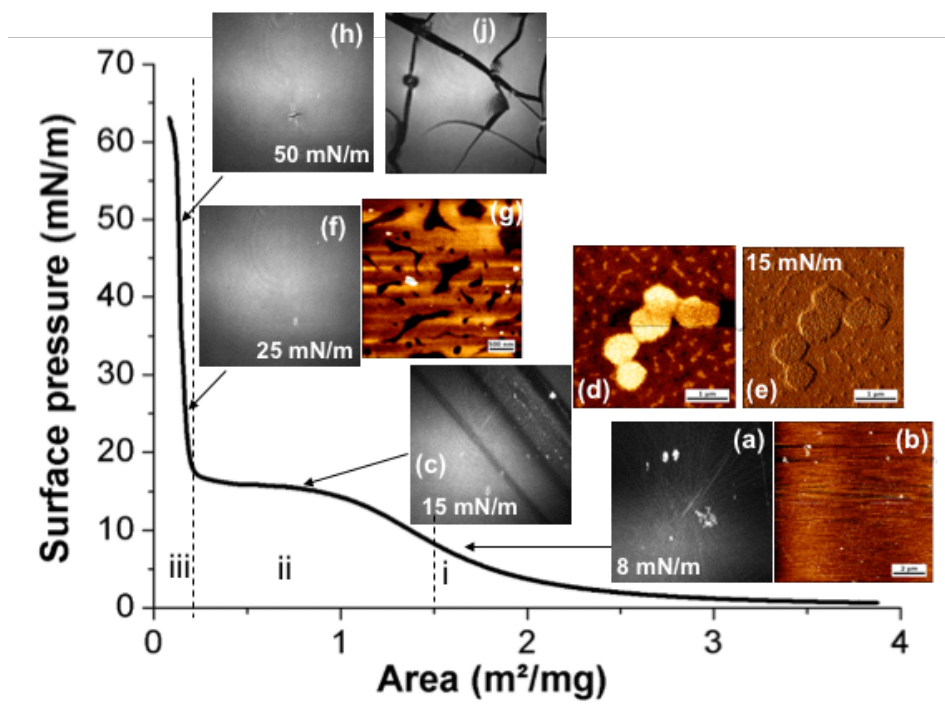


Figure 33: Surface pressure-area isotherm and Brewster angle (BAM) microscope images of a PLA film spread at the air-water interface (22°C): (a) 8 mN/m; (c) 15 mN/m; (f) 25 mN/m; (h) 50 mN/m; (j) expansion of the monolayer. The film was LB-transferred onto mica and imaged by atomic force microscopy (AFM) in the air: (b) 8 mN/m (height); (d,e) height and phase images at 15 mN/m; (g) 25 mN/m (height).

Compressibility modulus values show two maxima, one at 9 mN/m ($C_s^{-1} = 21$ mN/m) and the other around 40 mN/m ($C_s^{-1} = 160$ mN/m) (Figure 34B). At surface pressures above 30 mN/m ($C_s^{-1} \geq 100$ mN/m), the PLA film breaks under expansion (Figure 33j) due to cohesive interactions between polymer chains. For QCM-D and AFM experiments, the PLA film was transferred on the solid substrates at 25 mN/m ($C_s^{-1} = 65$ mN/m), to avoid film breakage during Langmuir-Schaefer transfer.

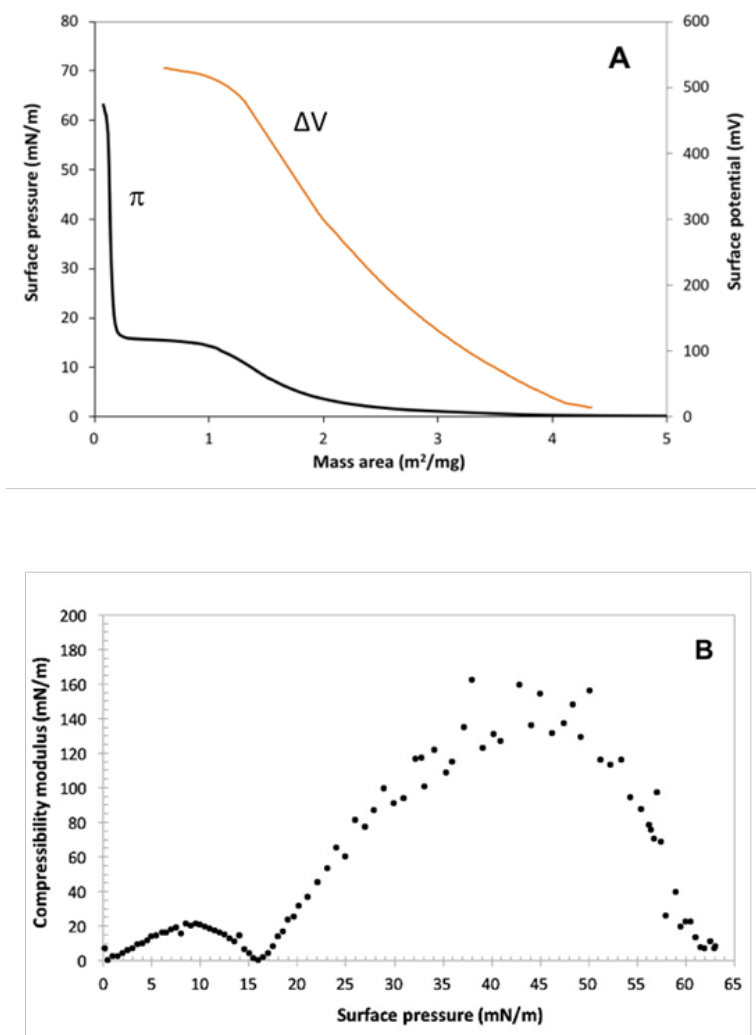


Figure 34: (A) Surface pressure and surface potential-area isotherms; (B) Compressibility moduli deduced from the PLA π -A isotherm in (A).

III-3.2. Analysis by QCM-D and AFM of liposome interaction with a PLA layer adsorbed onto a HMDS-modified surface

The transfer efficiency of a monolayer from a water surface to a solid substrate immersed in water depends on the choice of the surface pressure and the nature of the chosen surface for transfer. The surface pressure at which transfer is performed is important to maintain the organization and cohesion of the film during transfer and obtain uniform substrate coverage.

The polarity of the substrate is crucial to keep the orientation of the monolayer as it was at the air-water interface.

When PLA is adsorbed at the air-water interface, the polymer is organized in such a way that its hydrophilic groups are in contact with water. These groups must also be in contact with water in the QCM or AFM chamber. So, in order to transfer the polymer layer without modifying its organization, the Langmuir-Schaefer method was applied, using a glass surface covered by a layer of hydrophobic hexamethyldisilazane (HMDS). We first verified the quality of the surface functionalization by an AFM analysis. We then performed PLA monolayer transfer LS at various surface pressures. Once a satisfactory protocol was found, interaction of POPC and POPC-DOTAP liposomes with the PLA-coated surface was monitored by QCM-D. The PLA covered surfaces were examined by AFM after incubation with vesicles and subsequent rinsing.

III-3.2.1. Transfer of the PLA monolayer onto a HMDS-functionalized glass plate

The glass surfaces were modified by HMDS as described in the experimental section. We verified that after 12h of exposure to HMDS vapor phase, the glass surface was fully covered by a homogeneous layer of silane (Figure 35).

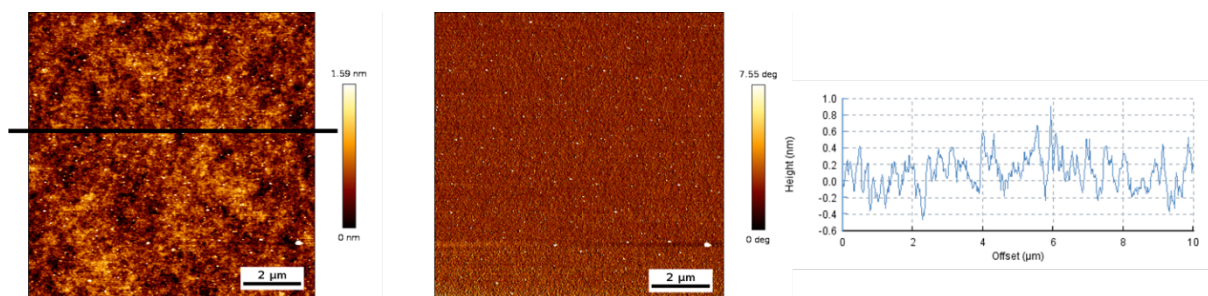


Figure 35: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS. Height cross section performed along the black line is shown on the right side.

The thickness of the hydrophobic layer was ~ 1 nm with a roughness ranging between 250 and 350 pm (Table 6). Water deposited on the HMDS surface displayed a full droplet shape confirming the hydrophobic nature of the HMDS modified glass surface. LS transfers of PLA films from the air-water interface onto HMDS surfaces were performed at different surface pressures. The goal was to obtain continuous PLA layers with uniform thickness, without any polymer aggregates or cracks in the structure. According to Figure 33 and the compressibility moduli plot in Figure 34B, the LE-LC transition ends at 16 mN/m. Thus, PLA films were transferred at 18, 25, and 35 mN/m. At 18 mN/m, the PLA layer was discontinuous with disconnected domains of different heights (Figure 36). Many voids (darker areas) between the polymer domains were observed due to incomplete coverage of the surface.

At 35 mN/m, the surface was almost completely covered with stacked polymer domains. Many white patches could also be detected onto the PLA layer, very likely polymer aggregates. This surface pressure value was considered as too high to obtain a PLA layer of uniform thickness. Large variations of film thickness were observed (Figure 36).

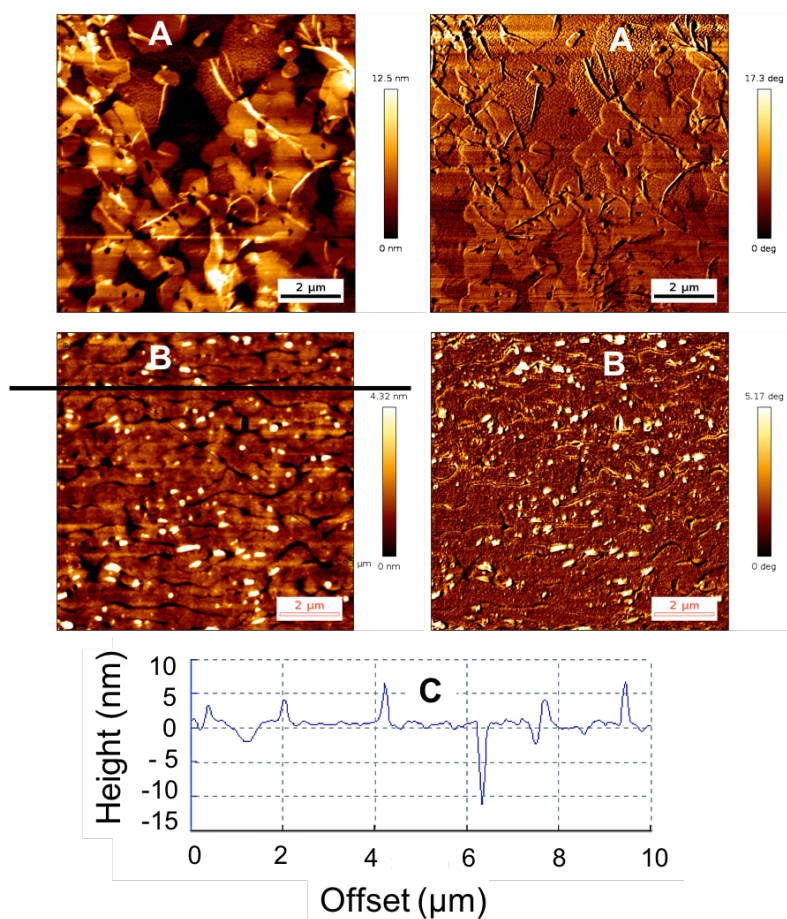


Figure 36: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS after LS transfer of PLA monolayers at 18 (A) and 35 mN/m (B). Height cross section performed along black line depicted on height picture at 35 mN/m is shown in (C).

Following transfer of the PLA monolayer at 25 mN/m, polymer domains covered most of the surface area (Figure 37). However, some holes were detected (dark areas on height pictures), and their lateral size varied between tens and several hundreds of nm. The total surface coverage by the polymer was $87 \pm 2 \%$, as deduced from the examination of the dark areas/polymer domain areas ratio in height pictures. The thickness of the PLA layer was uniform: 5 ± 0.5 nm as determined from cross sections analysis. Surface roughness of PLA surface was estimated to range between 150 and 200 pm (Table 6). Phase imaging could discriminate the polymer from the HMDS layer, although involved phase shifts remained small, indicating a relatively rigid polymer layer. Force-distance measurements performed on the PLA layer allowed calculation of the apparent elastic moduli from the fit of the linear part in approach curves using

the Hertz-Sneddon model (Hertz, 1895): values between 15 and 30 MPa were measured on different PLA surfaces (Figure 38, Table 6). We kept the surface pressure value of 25 mN/m for the LS transfer of PLA onto HMDS surface, before incubation with liposomes.

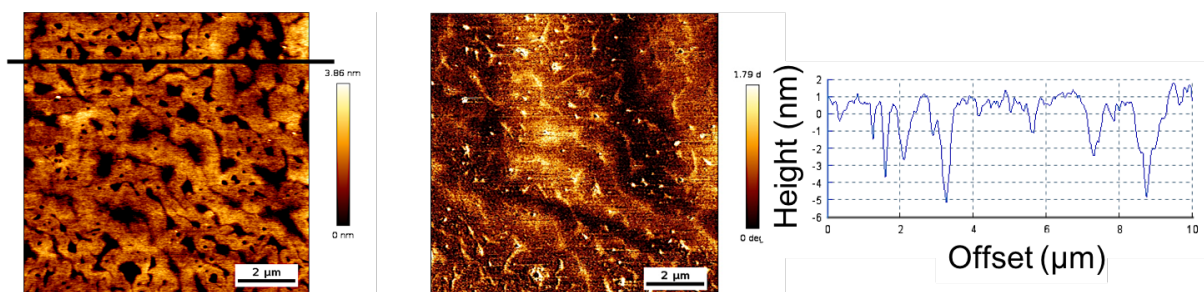


Figure 37: AFM height (left) and phase (right) pictures in water of the glass surface functionalized with HMDS following LS transfer of PLA at the surface pressure of 25 mN/m. Height cross sections performed along black line depicted on height pictures are shown on the right.

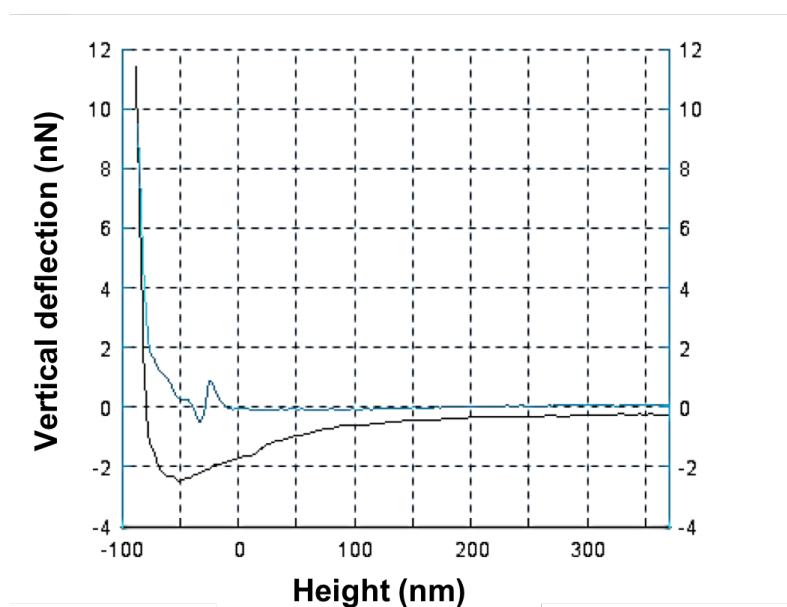


Figure 38: Force-distance measurement performed in water on the PLA film transferred at 25 mN/m. Approach and retract curves are displayed as vertical deflection (in nN) of the cantilever versus the AFM head height (in nm).

Surface	LayerCoverage	PLA surface coverage (%)	vesicle	Bilayer surface coverage (%)	Thickness (nm)	RMS (pm)	Young modulus (MPa)
Glass	HMDS	-	-	-	1	250-350	-
Glass-HMDS	PLA	87 ± 2	-	-	5 ± 0.5 *	150-200	15-30
Glass-HMDS	PLA	87 ± 2	POPC (4 mM)	-	no bilayer	-	-
Glass-HMDS	PLA	87 ± 2	POPC-DOTAP (0.4 mM)	26.7	3.9	680	75 ± 18
Glass-HMDS	PLA	87 ± 2	POPC-DOTAP (4 mM)	69.2	nd	nd	nd

*Table 6: Characteristics of the various studied surfaces in contact with liposomes, as deduced from AFM measurements in water. RMS: roughness. *The thickness of the PLA monolayer was measured 1.5h after spreading at the air-water interface and transfer at 25 mN/m. No change in thickness was observed afterwards. The bilayer surface coverage was deduced from the surface coverage of PLA (87%), and the final ΔF value obtained in the QCM-D experiment.*

III-3.2.2. Interaction of POPC and POPC-DOTAP liposomes with a SiO₂ surface

Liposome adsorption and breaking onto solid surfaces immersed in water is a well-known process. Many authors have described the mechanism of fusion of vesicles and formation of supported bilayers onto various surfaces (glass, silicon dioxide, silica, mica, etc.) and listed factors involved in bilayer formation such as electrostatic interactions, or polarity and roughness of the solid support (Brian and McConnell, 1984; Reimhult *et al.*, 2009; Richter *et al.*, 2006; Richter and Brisson, 2005; Vermette, 2009). Other parameters are liposome charge, phase and size, composition of the aqueous medium (buffer, pH, ionic strength, calcium ions, sodium chloride), temperature and osmotic pressure (Ferhan *et al.*, 2017; Jing *et al.*, 2014; Nirasay *et al.*, 2012; Reimhult *et al.*, 2003; Sundh *et al.*, 2010). Cho *et al.* (2013) also pointed out differences in behavior of vesicles prepared by extrusion or sonication, and they concluded that sonicated vesicles formed lipid bilayers with better controlled properties.

Previous works in our laboratory dealt with the formation of complex symmetric and asymmetric lipid bilayers onto SiO₂ and mica substrates (Makky *et al.*, 2011; Michel *et al.*, 2017). In this work, we compared the adsorption-rupture process of sonicated POPC and POPC-DOTAP vesicles onto PLA, but also on SiO₂ surfaces. Both surfaces were negatively charged but had different surface properties.

According to Ábrahám *et al.* (2017), the full coverage of a SiO₂-coated quartz crystal QCM sensor surface by lipid vesicles with a diameter of 100 nm gives ΔF values of about -320 Hz. None of our liposome compositions and substrates allowed to reach such values.

For pure POPC liposomes (0.4 mM, 170 nm), we observed a maximal ΔF lowering of -100 Hz (Figure 39A and Table 7). The corresponding ΔD value was 16×10^{-6} . The final ΔF and ΔD values after rinsing were -26 Hz and 0.8×10^{-6} , respectively. These values agree with those published in the literature for a phospholipid bilayer (Ábrahám *et al.*, 2017; Diamanti *et al.*, 2016; Gromelski *et al.*, 2009; Makky *et al.*, 2011). According to Dimitrievski and Kasemo (Dimitrievski and Kasemo, 2008), the ΔF -time and ΔD -time profiles account for the adsorption of intact vesicles at low coverage, then formation of the bilayer by rupture of vesicles after critical coverage has been reached.

When POPC-DOTAP vesicles (4 mM) were injected in the QCM measurement cell, the above adsorption-breaking process did not occur. A faster but much less significant frequency change was observed (-25 Hz) (Figure 39B). The ΔF values then increased slightly for about an hour and continued to increase after rinsing (Figure 39C). Liposomes broke very early, probably as soon as they reached the surface. This was due to the electrostatic interaction between the negatively charged SiO₂ surface and the positively charged vesicles (Dimitrievski and Kasemo, 2008). This hypothesis is supported by the fast decrease of ΔF and the low ΔD values which did not exceed 1.2×10^{-6} , accounting for a rigid lipid layer. However, the final ΔF value after rinsing was -17 Hz, a much lower value than expected for a bilayer (-26 Hz). If 100% coverage of the SiO₂ surface corresponds to $\Delta F = -26$ Hz, then a value of -17 Hz would indicate that the coverage of the surface by 4 mM POPC-DOTAP was 65.4% only.

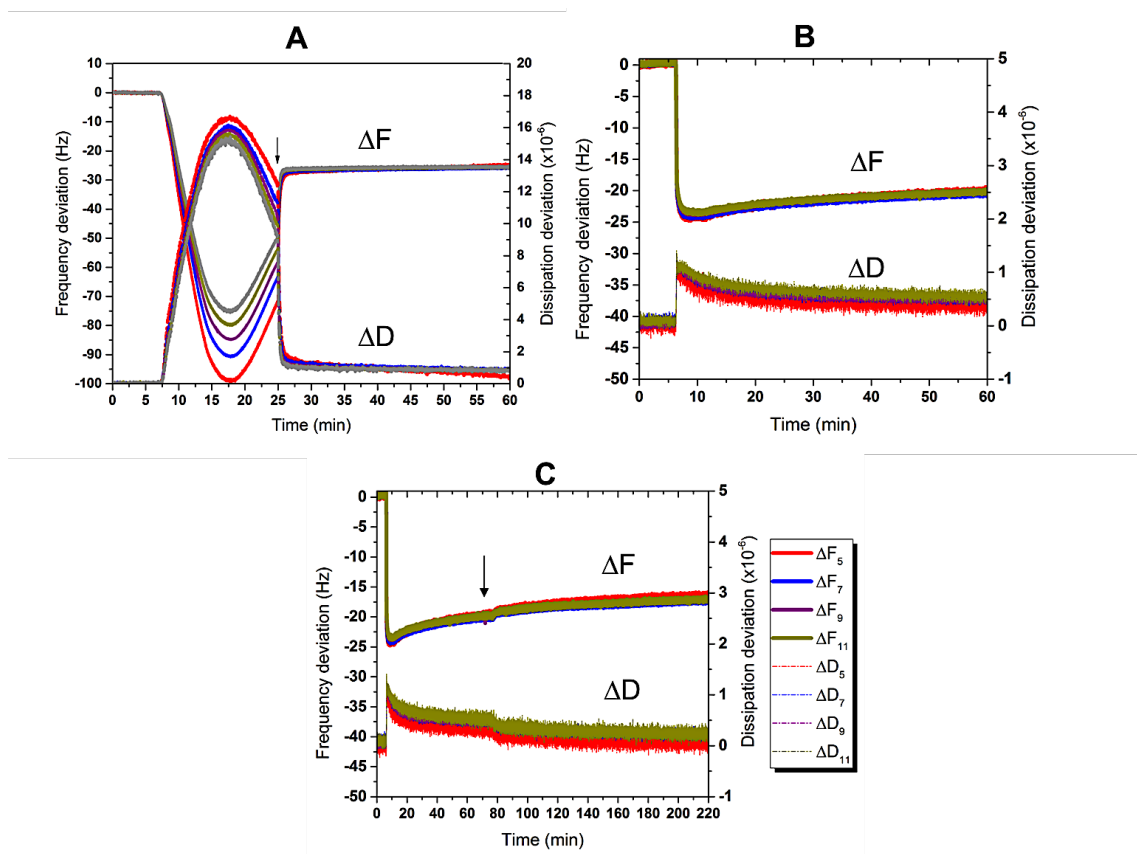


Figure 39: ΔF and ΔD values for (A) POPC (0.4 mM), (B) and (C) POPC-DOTAP (75:25) liposomes (4 mM) on the SiO_2 -coated quartz crystal QCM-D sensor. Whereas (B) is an enlargement of the plot for the first hour, (C) shows the whole QCM-D experiment. Arrows indicate the rinsing step.

III-3.2.3. Interaction of POPC and POPC-DOTAP liposomes with a PLA surface

After LS transfer at 25 mN/m of a PLA film onto the HMDS-modified QCM sensor surface, the sensor was transferred into the QCM cell in conditions ensuring its continuous immersion in water. Figure 40A shows the kinetics of interaction between the PLA surface and POPC liposomes over 110 minutes. At 4 minutes, the liposomal suspension was injected into the cell. A rapid decrease of low amplitude (between -3 and -6 Hz) was observed in the following minutes, with a dispersion of overtone, indicating a weak adsorption of POPC liposomes onto the PLA surface. The corresponding dissipation shifts were low (below 0.5×10^{-6}) and

dispersed. After 10 minutes, all signals were stable. After 1h incubation with liposomes, frequency and dissipation harmonic signals were still dispersed but almost stable in amplitude, demonstrating that no further adsorption of liposomes occurred onto the PLA surface. Upon water rinsing a loss of 1 Hz in frequency occurred for each harmonic, whereas dissipation signals narrowed by staying below 0.5×10^{-6} . After 30 min rinsing, frequency shift stabilized between -3 and -5 Hz, with corresponding low dissipation shifts (-0.25×10^{-6}) (Table 7).

Bilayer formation was also assessed by AFM, in the same conditions as in QCM-D experiments. Figure 40B shows the PLA surface after 1h incubation with 4 mM POPC liposomes, and water rinsing. Despite the high lipid concentration studied, the pictures look very similar to those obtained before incubation with liposomes, suggesting a lack of interaction between the anionic PLA surface and the zwitterionic POPC liposomes. They may have adsorbed onto the PLA surface, but the rinsing step washed them away. However, a few spots are noticed at the edges of polymer domains. They are more visible on the phase image (see white arrows in Figure 40C). Liposomes adsorption in the voids between PLA domains is also observed, indicating nonspecific adsorption of POPC liposomes on the HMDS surface.

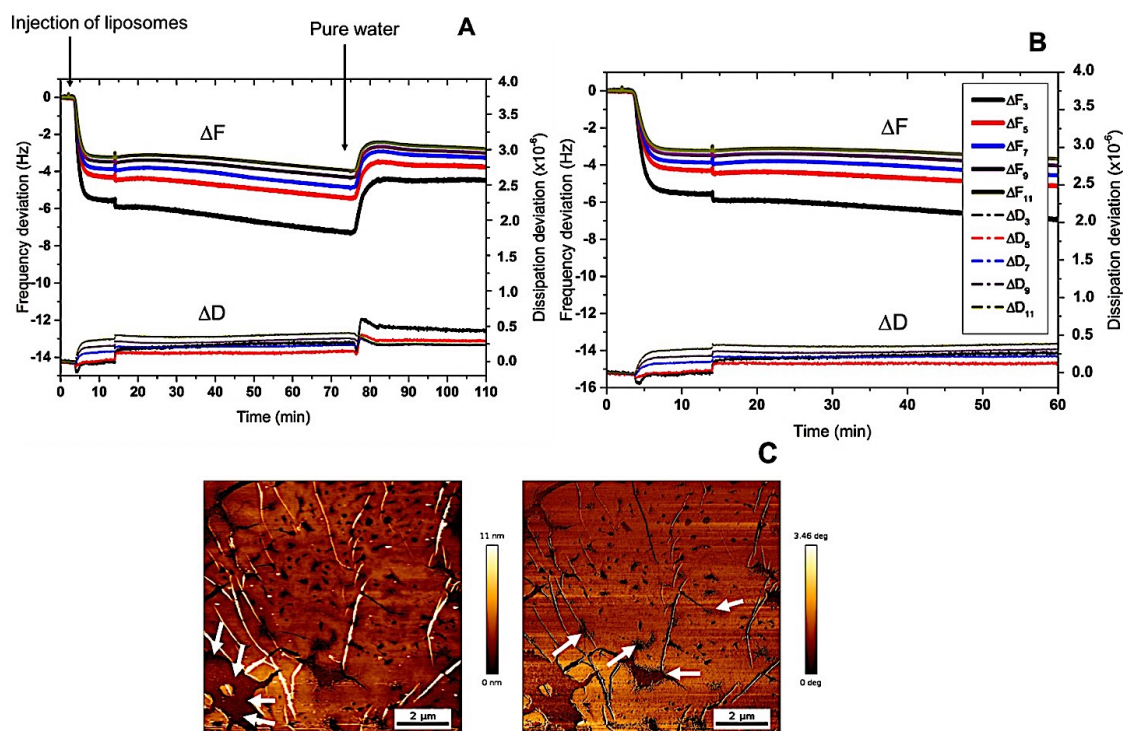


Figure 40: Interaction between 4 mM pure POPC liposomes and the PLA monolayer transferred at 25 mN/m: (A) ΔF -time and ΔD -time relationships monitored by QCM-D; (B) is

the enlargement of the QCM plot in (A); (C) height (left) and phase (right) pictures of the PLA surface after 1h incubation with the liposomes and abundant water rinsing.

Overall, this experiment demonstrated the very weak interaction of POPC liposomes with the PLA monolayer. Only a few vesicles adsorbed and apparently remained intact onto the PLA-covered surface.

III-3.2.4. Interaction with POPC-DOTAP liposomes with the PLA surface

The kinetics of interaction between the PLA surface and 0.4 mM POPC-DOTAP 75/25 liposomes (i.e., a concentration 10 times lower compared to POPC liposomes in the previous experiment) were monitored over 100 minutes, as presented in Figure 41A and Figure 41B and Table 7. At $t = 6$ minutes, the liposomal suspension was injected into the microfluidic cell. A sharp decrease in frequency amplitude (until -18 Hz) was observed during the following minutes, with no dispersion of overtones, indicating the adsorption of POPC-DOTAP liposomes onto the PLA surface, although the ΔF value was higher than that observed for the adsorption of the same vesicles onto the silicon dioxide surface (Figure 39B). As previously observed with the SiO_2 surface, vesicles broke spontaneously as soon as they reached the sensor surface. The dissipation shifts were relatively high (reaching 1.5×10^{-6}) and converging. After 1h incubation with liposomes, frequency and dissipation harmonic signals were still evolving, with a slow decrease in frequency (until -8 Hz) and in dissipation (until 0.15×10^{-6}), indicating the progressive rearrangement of the POPC-DOTAP bilayer onto the OLA surface (Table 7). Upon water rinsing a loss of 1 Hz in frequency occurred, whereas dissipation signals were almost down to 0. After 30 min rinsing, all signals were stable. Overall, this experiment demonstrated the attractive interaction between POPC-DOTAP liposomes and the PLA surface, with the formation of a bilayer. This interaction was stronger than with the SiO_2 surface, also a negatively charged surface. However, the shape of the QCM-D curve and values of ΔF and ΔD suggest in both cases the formation of bilayer patches, rather than that of a continuous bilayer (Richter and Brisson, 2005).

Surface	Coating	Lipid	Lipid concentration (mM)	ΔF_{ads} (Hz) (time)	ΔD_{ads} ($\times 10^{-6}$) (time)	ΔF_{final} (Hz) (time)	ΔD_{final} ($\times 10^{-6}$) (time)
SiO ₂	-	POPC	0.4	[-70; -100] (10 min)	16 (10 min)	-26 (25 min)	0.8 (25 min)
SiO ₂	-	POPC-DOTAP	4	-25 (2 min)	1.2 (2 min)	-17 (> 220 min)	0.5 (> 220 min)
SiO ₂ -HMDS	PLA	POPC	4	[-3; -6] (2 min)	0.5 (2 min)	[-3; -5] (90 min)	[0.25; 0.5] (90 min)
SiO ₂ -HMDS	PLA	POPC-DOTAP	0.4	-18 (0 min)	1.5 (0 min)	-8 (> 60 min)	0.15 (> 60 min)
SiO ₂ -HMDS	PLA	POPC-DOTAP	4	-35 (0 min)	3 (0 min)	[-17; -19] (200 min)	[0.5; 1.5] (200 min)

Table 7: QCM-D characteristics values for various studied samples. The time refers to the duration of the frequency and dissipation changes for the adsorption and following steps.

Figure 41C and Figure 41D show the AFM height and phase pictures of PLA surface before and after 1h incubation with 0.4 mM POPC-DOTAP 75/25 liposomes suspension. After rinsing, the morphology of the PLA surface appeared strongly modified by the interaction with the cationic liposomes. Polymer domains that were visible before the interaction could merely be distinguished. Dark areas almost disappeared indicating that material had adsorbed and filled the voids separating the polymer domains before incubation. Comparison of height cross sections performed along the same line on the picture before and after interaction demonstrated the addition of 3 to 4 nm lipid layer onto the PLA surface, a value roughly corresponding to that of a phospholipid bilayer patch (Figure 41C). The whole PLA surface was covered by similar patches. Phase images indicate a 10° increase in phase shift that can be related to their presence on the polymer surface (Figure 41D). In Figure 41E, low scale AFM height and phase images of PLA surface after interaction with POPC/DOTAP liposomes show lipid patches covering the whole polymer surface, which are particularly visible on the phase image.

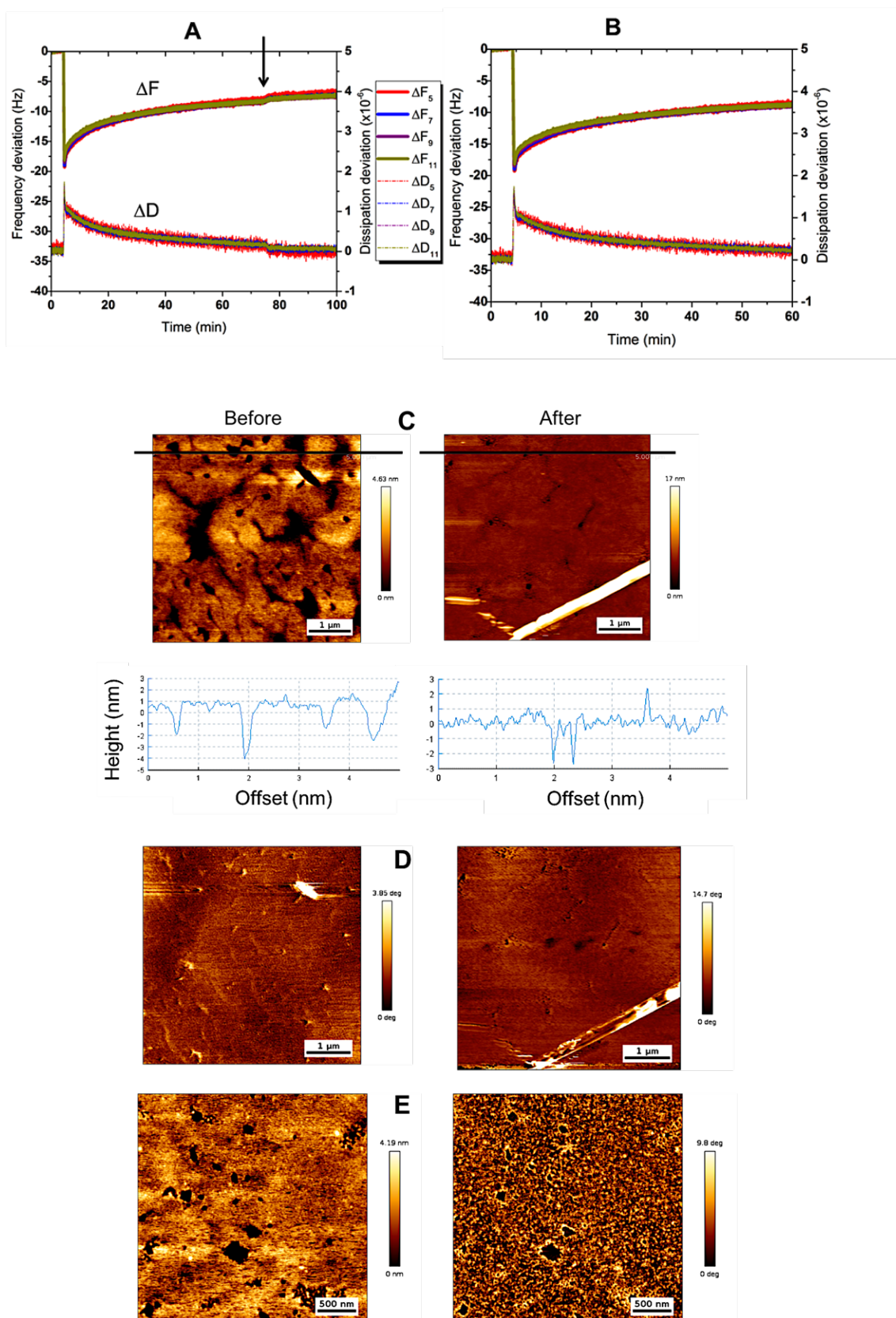


Figure 41: Interaction between 0.4 mM POPC-DOTAP (75:25) liposomes and the PLA monolayer transferred at 25 mN/m: (A) ΔF -time and ΔD -time relationships monitored by

QCM-D. (B) close-up on the 60 first minutes of ΔF -time and ΔD -time relationships. (C) height and (D) phase pictures of the PLA surface before and after 1h incubation with the liposomes and abundant water rinsing (same area scanned). Height cross sections performed along black and white lines are shown below C. (E) Enlargement of height and phase pictures showing evidence of large holes in the lipid bilayer covering the PLA monolayer.

As mentioned previously, the adsorption-rupture process of vesicles onto a solid surface is dependent upon different parameters related to the surface, the vesicles and the aqueous medium. The mechanism includes four steps: (i) adsorption, (ii) shape deformation (flattening) and critical coverage, (iii) rupture and (iv) bilayer patch fusion (Ferhan *et al.*, 2017; Shao *et al.*, 2017) (Figure 42).

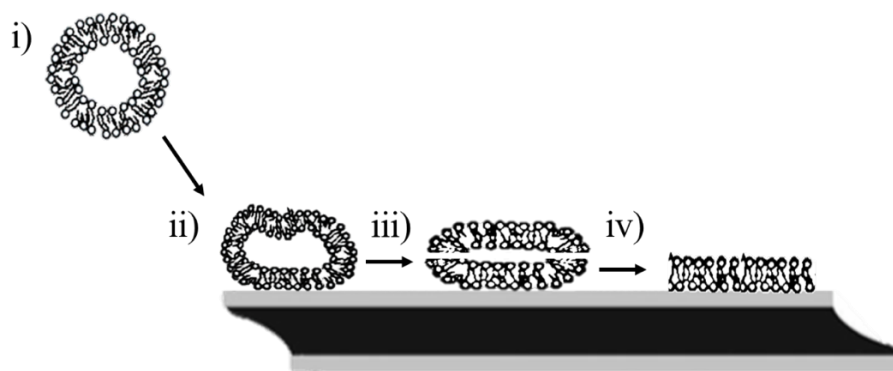


Figure 42: The four steps of phospholipid bilayer formation (adapted from Sundh *et al.*, 2010).

QCM-D measures the mass of material adsorbed onto the surface of the sensor. The initial ΔF value lowering is thus related to the size of vesicles (Ferhan *et al.*, 2017; Jing *et al.*, 2014). The larger the liposome, the lower the ΔF value. As POPC and POPC-DOTAP vesicles were of similar diameter, the size is not a relevant parameter here.

Effect of vesicles concentration

In the absence of electrostatic interactions, rupture occurs at a critical vesicle coverage and is progressive. Surface coverage is dependent upon vesicle concentration in the aqueous medium (Anderson *et al.*, 2009). So, one factor that could explain the discontinuous covering of the PLA monolayer by the POPC-DOTAP bilayer could be the low concentration of POPC-DOTAP used in our experiments (0.4 mM, instead of 4 mM for pure POPC). However, the same experiment performed with 4 mM POPC-DOTAP led to limited improvement (Figure 43). The maximal ΔF decrease was -35 Hz. Then ΔF values increased progressively up to -19 Hz after 200 min. This value was closer to that expected for a full bilayer, but still the profiles of the plots were very different from those of usual adsorption-rupture processes and showed a very fast first step (< 1 min) corresponding to spontaneous breaking of the vesicles (Table 7). Calculation of the surface coverage by the bilayer gave a value of 69.2%, if one considers 87% PLA coverage of the surface of the sensor (Table 6).

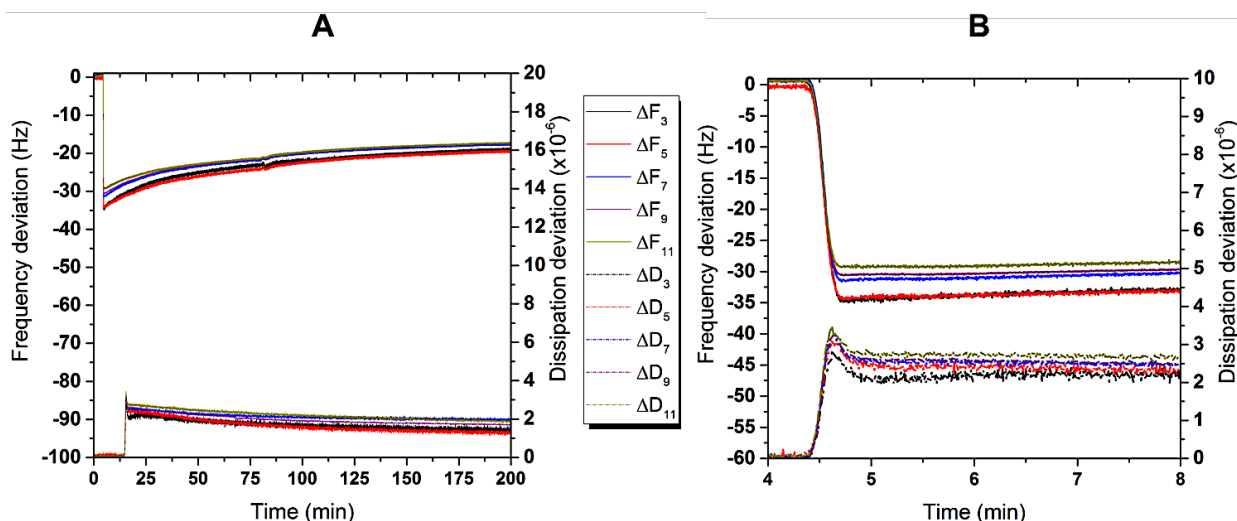


Figure 43: ΔF -time and ΔD -time relationships monitored by QCM-D for the interaction of 4 mM POPC-DOTAP (75:25) liposomes with a PLA monolayer transferred at 25 mN/m : (A) Full experiment with rinsing at $t = 80$ min; (B) Close-up on the initial times of the experiment.

There is another argument minimizing the effect of concentration. Indeed, we observed previously that POPC vesicles at 0.4 mM broke easily onto the SiO₂ surface and formed a uniform continuous bilayer (Figure 39A). So, increasing liposome concentration certainly improves the quality of the bilayer, but it does not impact the vesicle adsorption-rupture process as much as physical interactions do.

Effect of the electrostatic interactions

It is well-known that vesicle adsorption is controlled by van der Waals and electrostatic interactions (Anderson *et al.*, 2009; Blakeston *et al.*, 2015). In our case, the lipo-nanoparticles were designed so that the coating of the PLA core by lipid vesicles was driven by an electrostatic interaction. We have shown that POPC vesicles did not form a bilayer around the nanoparticles. The QCM-D plots and AFM pictures indicate that POPC vesicles adsorbed onto the SiO₂ surface (Figure 39A), but that they did not adsorb much at the surface of the polymer layer. Even if some vesicles adsorbed, they did not break (Figure 40 AB). Conversely a ΔF lowering was observed with POPC-DOTAP vesicles when interacting with both SiO₂ and PLA surfaces. Both surfaces were negatively charged. So, electrostatic interactions favored vesicles adsorption.

The rupture process may initiate after crowding (i) at the place where the vesicle and the support interact or (ii) at the points of highest curvatures (Reimhult *et al.*, 2009). Depending on the mechanism, the bilayer unfolds with its inner (mechanism i) or outer leaflet (mechanism ii) against the substrate. In some cases, bilayer patches with the inner or outer leaflet in contact with the support coexist. Anderson *et al.* (2009) also proposed a mechanism in which vesicle rupture is induced after crowding by the deformation stress at the edge of the points of contact of the vesicle and support. Dimitrievski and Kasemo (Dimitrievski and Kasemo, 2008) described the diffusion of cationic lipids in two-lipid vesicles towards the SiO₂ substrate surface, maximizing the adhesion and contributing to the flattening of the vesicles. The zwitterionic POPC molecules were more weakly attracted to the surface. When the critical deformation energy was reached, vesicles ruptured (Dimitrievski and Kasemo, 2008). According to Fuhrmans and Muller's simulations (Fuhrmans and Müller, 2013), if potential

strength is kept sufficiently low to avoid vesicle rupture due to “collision” between the vesicles and the surface, a “parachute” adsorption would be observed instead of the “receding-top” (flattening) process usually described (Figure 44). Kong *et al.* (2016) and Shao *et al.* (2017) also considered the “parachute” model for vesicles interaction with soft polyelectrolytes.

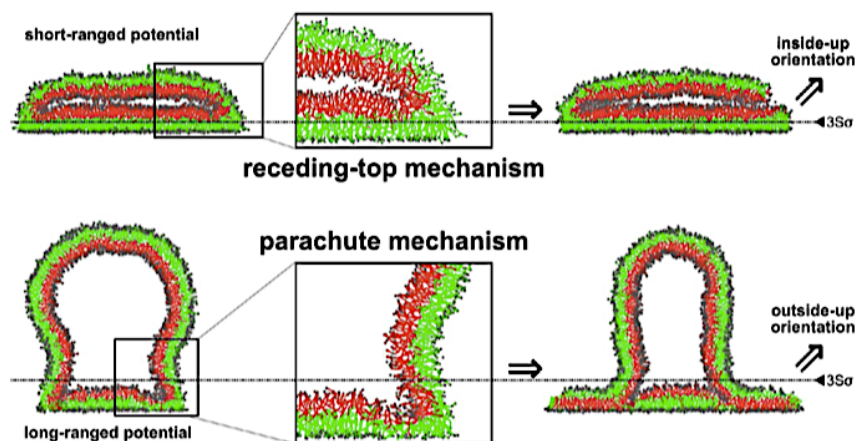


Figure 44: The receding-top and parachute models (from Fuhrmans and Müller, 2013).

In our experiment, both the limited ΔF lowering and dissipation increase observed with POPC-DOTAP liposomes are explained by the spontaneous rupture of vesicles immediately after contact with the surface, due to the deformation induced by the strong interaction of DOTAP molecules with the negative charges of the SiO₂ or polymer substrates. Thus, with cationic vesicles there was apparently no vesicle accumulation at the surface, no crowding period, no rupture induced by critical coverage. The time during which the initial ΔF lowering occurred was shorter when POPC-DOTAP vesicles were in contact with PLA, compared to the same vesicles in contact with SiO₂, or compared to POPC liposomes in contact with SiO₂ (Table 7). The kinetics of the breaking step were also faster with POPC-DOTAP vesicles.

As mentioned above, upon vesicle adsorption a reorganization of the lipids may occur due to the preferential interaction of DOTAP molecules with the negative charges of the support. Lateral diffusion (intraleaflet) and flip-flop (transbilayer or interleaflet movement) of lipids are observed in membranes. Lateral diffusion of molecules is much faster than interleaflet diffusion. Flip flop depends on the nature of the lipid and the presence of other lipids, the temperature, the thickness of the bilayer, etc. It is energy driven and may last hours or days

(Holthuis and Levine, 2005; Marquardt *et al.*, 2015; Nakano *et al.*, 2009). According to Nakano and coworkers' study (2009) using small-angle neutron scattering, the spontaneous flip-flop half-life for free-floating unlabeled POPC vesicles with a bilayer thickness of 39.1 Å would be close to 1000 h at 37°C (pH 7.4). Flip-flop of polar lipids such as DOTAP would take longer time than that of a neutral phosphatidylcholine (Holthuis and Levine, 2005). Although interleaflet diffusion was observed in several simulation studies (Dimitrievski and Kasemo, 2008; Kong *et al.*, 2016) flip-flop appears improbable in our time scale. Conversely, lateral electrostatic interaction-driven lipid diffusion would lead to a local asymmetry between the two phospholipid leaflets and to the deformation of the vesicles driven by the adhesion of DOTAP molecules to the polymer surface (Dimitrievski and Kasemo, 2008; Kong *et al.*, 2016). Nirasay *et al.* (2012) showed that a polydopamine support preserved the lateral mobility of lipids. Could this reorganization of local lipid composition really occur for both POPC and DOTAP on the PLA surface?

Under usual conditions, after rupture has been initiated, intact vesicles coexist with the forming bilayer and bare surface for a certain period of time (Reimhult *et al.*, 2009; Richter *et al.*, 2006; Wlodek *et al.*, 2015). It is probable that in the absence of crowding, this period of time is particularly long. The kinetics of ΔF increases in Figure 39B, Figure 41 and Figure 43 tends to confirm it. It is possible that repulsive electrostatic interactions also occurred between the adsorbed bilayer patches and new vesicles approaching the surface, therefore limiting the adsorption of these new vesicles.

The results described above confirm that the negative charge of the support is responsible for electrostatic interactions which accelerate cationic vesicle adsorption-rupture process but seem to lengthen the fusion of bilayer patches, compared to neutral vesicles. Under these conditions, the fast kinetics prevent the formation of a continuous bilayer. On the one hand, this would explain the similar QCM-D plots for POPC-DOTAP vesicles in contact with SiO₂ or PLA substrates, although the kinetics were faster when the cationic vesicles adsorbed to the PLA layer. On the other hand, it does not explain why POPC vesicles did not behave in the same way in contact with the SiO₂ or PLA surfaces.

Effect of support softness

Shao *et al.* (2017) simulated the adsorption-rupture process of anionic vesicles on a solid support and a soft polyelectrolyte one. They showed evidence of much faster kinetics when vesicles were in contact with the soft material. In our study, as previously mentioned, we observed faster kinetics for POPC-DOTAP vesicles in contact with PLA compared to SiO₂. We also observed the faster adsorption of POPC vesicles at the surface of PLA, but these vesicles did not break at the surface. Therefore, the charge of the support was not the only predominant factor, and the effect of the support was not only a kinetic effect.

To analyze the QCM-D plots independently of kinetics, we have plotted in Figure 45 the $\Delta D = f(\Delta F)$ relationships for all studied systems. They clearly show different profiles between POPC/PLA and POPC-DOTAP/PLA systems, but also between POPC-DOTAP/PLA and POPC-DOTAP/SiO₂ ones. When comparing Figure 45A and Figure 45B, one observes that neutral and cationic vesicles do not adsorb and break on SiO₂ via the same mechanism. Also, the results in Figure 45A and Figure 45C show that neutral vesicles do not behave in the same way in contact with a solid or relatively soft support. The dispersion of overtones in Figure 45C is certainly related to the different behavior of vesicles in contact with the soft polymer surface, compared to the solid SiO₂ surface. The adhesion of POPC vesicles to the polymer surface is very weak, and this does not favor vesicles breaking.

Overtones are also slightly more dispersed in Figure 45D compared to Figure 45B. Even if it is less dramatic than in Figure 45C, this overtone dispersion accounts for a weaker interaction of the cationic vesicles with the polymer surface than with the solid support.

We have studied the mechanical properties of the bilayer by AFM. The mean elastic modulus determined for the POPC-DOTAP/PLA system from 10 force measurements was 75 MPa, a value deduced from the fit of the linear part of the force curve. This value is much lower than that of a SiO₂ surface, 70 GPa, as reported by Ashcroft and Mermin (1976). Even if one considers the presence of 1 nm-thick HMDS layer below PLA, the value of the elastic modulus should be lower but close to 70 GPa. However, the value of the elastic modulus measured after transfer of the PLA layer was in the 15-30 MPa range accounting for a much softer surface than SiO₂. The presence of lipid patches after injection of the vesicles slightly rigidified the system,

but the elastic modulus remained in the same order of magnitude with and without the lipids adsorbed onto the PLA layer.

It is interesting to note that despite the dispersion of the overtones, the ΔD - ΔF relationships in Figure 45D resemble more to those expected for a system forming a bilayer than those in Figure 45B (Makky *et al.* 2011). In order to better see the ΔD - ΔF relationship in Figure 45B, the 7th overtone has been plotted in a separate figure (Figure 46). It depicts an unusual behavior after adsorption of the vesicles than we are not able to explain yet.

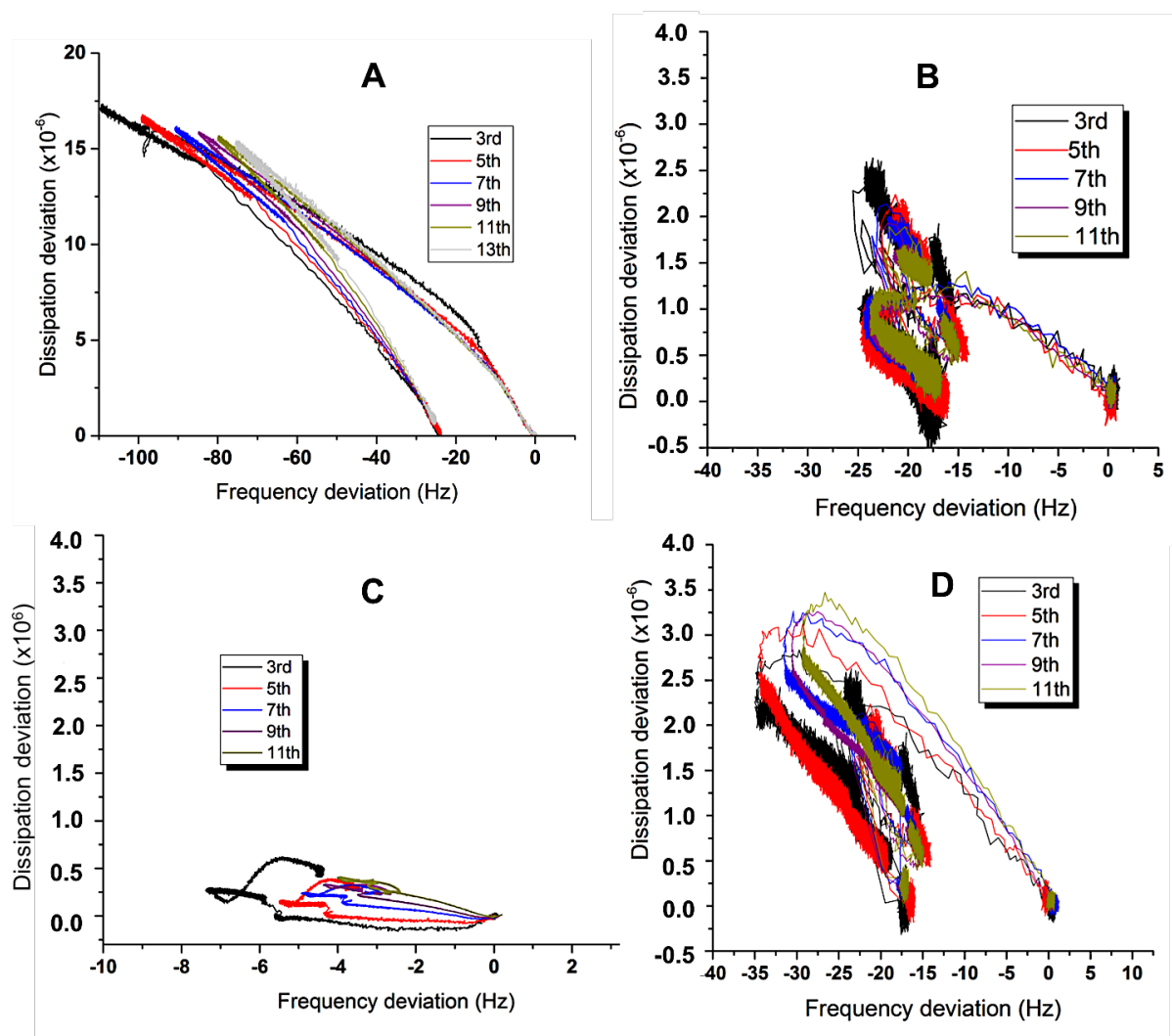


Figure 45: ΔD - ΔF relationships for (A) POPC/SiO₂; (B) POPC-DOTAP/SiO₂ (4 mM); (C) POPC/PLA (4 mM); (D) POPC-DOTAP/PLA (4 mM).

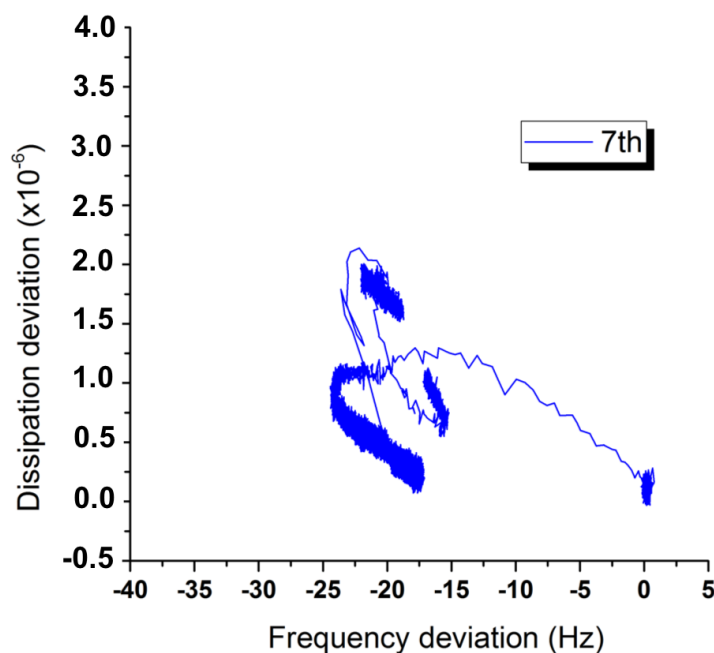


Figure 46: 7th overtone of the ΔD - ΔF relationships for POPC-DOTAP/SiO₂ (4 mM).

Roughness effect

Another reason for the accelerated rupture process and consequent imperfect coverage could be the roughness of the PLA monolayer (Blachon *et al.*, 2017). Indeed, the PLA monolayer adsorbed onto the HMDS layer had a roughness of 150-200 pm. However, this RMS is not significant. Ohsawa *et al.* (2009) measured a roughness of 120 pm for a SiO₂ surface with a 10 nm-oxide layer. Furthermore, our QCM-D experiment performed with POPC-DOTAP vesicles in contact with the negatively charged SiO₂ surface also led to the formation of bilayer patches instead of a uniform continuous bilayer, although the surface was much less rough. Richter *et al.* (2006) concluded that roughness in the nanometer range did not strongly affect the formation of supported bilayers. Nevertheless, the softness and roughness of the support could be enhanced by polymer swelling in the aqueous medium. Although we did not observe any swelling of the PLA layer by AFM, Jäger *et al.* (2018) and Shao *et al.* (2017) both reported polymer swelling during their experiments. This eventuality should be considered.

Control of the vesicle adhesion-fusion process

Blakeston *et al.* (2015) compared the interaction of POPC and POPC-DOTAP vesicles (with increasing DOTAP concentrations) with polymer brushes of different (negative) zeta potentials, and much higher RMS roughnesses than our PLA layers. Like us, they observed a weak interaction of POPC vesicles with the polymer brushes and no bilayer formation. For POPC-DOTAP (90:10) vesicles, they reported a slight improvement in vesicle adsorption onto the polymer surfaces, but still no bilayer formation. This agrees with our own results when we attempted to combine nanoparticles with POPC-DOTAP (90:10) vesicles (Chapter II). Higher DOTAP contents were necessary to observe the formation of a bilayer. Interestingly, the authors noted that to induce vesicle rupture and bilayer formation, the system had to be incubated for 1h, at least at 37°C, even better above 50°C. These conditions led in some cases to the initial coexistence of double bilayers with one-bilayer patches, which fused in a single bilayer during another 48h incubation at room temperature (Dimitrievski and Kasemo, 2008; Reimhult *et al.*, 2003). We did not heat our system during lipo-nanoparticle preparation, because we considered that the phase transitions of our lipids were sufficiently low to ensure the fluidity and plasticity of lipid bilayers. Anyway, this would be worth a try.

III-4. Conclusion

In summary, like many authors before us, we observed that neutral and cationic vesicles do not adsorb and break in the same way onto solid and soft anionic surfaces. On the one hand, neutral vesicles show typical bilayer formation onto a solid SiO₂ surface, with a 4-step mechanism including vesicle adsorption and flattening up to critical coverage, vesicle breaking and fusion of bilayer patches. The presence of the polymer prevents the formation of this bilayer. This observation confirms our previous results (Chapter II) and those of other authors showing that lipo-nanoparticles are not formed when neutral vesicles are incubated with polymer nanoparticles. The weak physical interactions and the softness of the polymer opposes the adhesion of the vesicles. In turn, the weak adhesion of the vesicles prevents their rupture. Conversely, neutral vesicles can form bilayers at the surface of silica particles (Ahmed *et al.*,

2012; Mornet *et al.*, 2005). Attractive electrostatic interaction, on the other hand, appear as a powerful force for inducing liposome breaking onto a solid or polymeric support. The zeta potentials of the PLA surface and POPC-DOTAP (75:25) vesicles were high: -46 and +53 mV, respectively. So, the electrostatic interaction could be strong.

Similar adsorption-breaking kinetics were observed for cationic vesicles in contact with both the solid SiO₂ surface and the soft PLA one. However, the kinetics of the QCM-D plots showed that vesicles broke immediately at the surface. It was more a spontaneous rupture mechanism than a critical coverage one. This spontaneous rupture affected the formation of the lipid bilayer, leading to the deposition of bilayer patches instead of a continuous bilayer.

The low ΔF and ΔD values account for the formation of rigid bilayer patches but could also account for the formation of a monolayer. However, we observed that ΔF values decreased with the increase in POPC-DOTAP concentration (and the final ΔF values were closer to those expected for a bilayer) which should not be the case if a monolayer was formed. Also, AFM height pictures showed that the thickness of the lipid patches corresponded to one bilayer.

The electrostatic interaction was obviously too strong, but it could be controlled by the ionic strength and pH of the aqueous medium. The POPC-DOTAP 75/25 vesicles suspension could also be more concentrated as we have noticed an improvement of bilayer formation when the concentration was increased from 0.4 to 4 mM. Another important parameter is the softness of the polymer layer. Although the behavior of the vesicles is controlled by the electrostatic interaction, the rigidity/softness of the polymer surface also plays an important role on the adhesion and kinetics of rupture of the vesicles. Under the present conditions, the impact of the rigidity of the support was minimized by the electrostatic interactions, but if the latter were diminished, the viscoelastic properties of the polymer layer could become the main issue. Fine tuning would be necessary to balance the two effects while improving the adsorption-rupture process for obtaining a defect-free bilayer.

All these results confirm the formation of a POPC-DOTAP bilayer at the surface of PLA nanoparticles as concluded from our experiments in Chapter II. They also indicate that the bilayer is not continuous and that our experimental conditions could be improved by a better control of the electrostatic interactions between the vesicles and nanoparticles, and by heating the system during nanoparticle-vesicle fusion.

Chapter IV: Co-encapsulation of a photosensitizer and beta-lapachone in liponanoparticles and in vitro evaluation of their cytotoxicity and phototoxicity for dual PDT/chemotherapy

Chapter IV: Co-encapsulation of a photosensitizer and beta-lapachone in liponanoparticles and *in vitro* evaluation of their cytotoxicity and phototoxicity for dual PDT/chemotherapy.

IV.1. Introduction

In this chapter, we describe the encapsulation of an anticancer drug and two photosensitizers for combined therapy against retinoblastoma.

Beta-lapachone (β -Lap) is a natural naphthoquinone derivative extracted from the bark of the lapacho tree (*Tabebuia avellanedae*). This molecule has earned many attentions because of its wide range of pharmacological activities. Indeed, it was reported to be anti-parasitic, anti-fungal, antiviral, and wound healer. More importantly, it is recognized to exhibit an anti-tumor effect against several types of cancers, including retinoblastoma (Kung, 2014; Lee *et al.*, 2012; Shah *et al.*, 2008). β -Lap exerts its antitumoral activity by various mechanisms and signaling pathways resulting from its reduction by the enzyme NAD(P)H:quinone oxidoreductase (NQO1) through a futile redox cycle. Beta-Lapachone induces the production of reactive oxygen species (ROS) and superoxide anion resulting in a massive release of Ca^{2+} from endoplasmic reticulum, activation of poly ADP ribose polymerase (PARP) and caspase cascade. It interacts directly with topoisomerase I and II, and is also responsible for the breakage of DNA strands, activation of Ca^{2+} -dependent calpain and cell cycle arrest. All these mechanisms lead to apoptosis (Kung, 2014). The cytotoxic effect of the drug is potentiated in tumor cells which overexpress NQO1. Interestingly, the association of β -Lap with other treatments may result in a synergistic effect. Indeed, β -Lap was reported to sensitize the cells to ionizing radiations as it inhibits DNA repair while, on the other hand, cancer cells are more sensitive to β -Lap due to increased level of NQO1 induced by radiations (Kung, 2014).

Beta-Lapachone has been found effective against retinoblastoma cells by a mechanism considered as NQO1-dependent (Shah *et al.*, 2008). Since PDT can boost the expression of

NQO1 in cancer cells 24h post-illumination (Lamberti *et al.*, 2013), it may be interesting to combine β -Lap with PDT for a synergistic effect.

Despite the promising potential of beta-lapachone as a drug, its development is limited due to its unfavorable physico-chemical properties. Beta-lapachone is poorly water soluble (0.16 mM or 0.04 μ g/mL) and rapidly crystallizes in aqueous medium (Ling Zhang *et al.*, 2015). Furthermore, β -Lap is sensitive to oxidation (Kim *et al.*, 2017). Therefore, it requires to be encapsulated into carriers to facilitate its delivery, improve its bioavailability and ensure its protection against degradation. To our knowledge, β -lap has been encapsulated in polymeric PEG-PLA (Blanco *et al.*, 2007; Zhang *et al.*, 2017) and PEG-b-PCL micelles (Sutton *et al.*, 2007), in poly(methyl methacrylate)/polystyrene microcapsules (Kim *et al.*, 2017), in PLGA ones (Costa *et al.*, 2016), and in lecithin-chitosan NPs (Moreno *et al.*, 2015). It has also been solubilized by formation of a complex with hydroxypropyl- β -cyclodextrin (HP β -CD) (Nasongkla *et al.*, 2003) and incorporated into liposomes (Cavalcanti *et al.*, 2011; Wu, 2018) or in PLGA millirods (Wang *et al.*, 2006). Another strategy consisted in synthesizing its diester prodrug derivative and solubilize it in PEG-b-PLA micelles (Ma *et al.*, 2015).

m-THPC (temoporfin, Foscan[®]) is one of the most effective photosensitizer on the market. It is active at very low concentrations ($\sim$ 0.1-0.15 mg/kg). It absorbs light in the red spectrum region, at 652 nm (Bonnett *et al.*, 1999). At this wavelength, the light can penetrate deep in the tissues (Josefsen and Boyle, 2008). It has a molar extinction coefficient of 29,600 mol⁻¹.dm³.cm⁻¹, a quantum yield of triplet state formation (Φ_T) of 0.89 with a lifetime (τ_T) of 50 μ s, and a quantum yield of singlet oxygen formation (Φ_Δ) of 0.43 (Bonnett *et al.*, 1999).

The tetraphenyl porphyrin derivative m-THPP has a similar chemical structure as m-THPC, with only one additional double bond. Like m-THPC, it absorbs light in the red region but at a wavelength slightly lower than that of m-THPC (644 nm). It also has a lower quantum yield of triplet state formation (Φ_T : 0.69), a longer lifetime (τ_T : 120 μ s), but a similar quantum yield of singlet oxygen formation (Φ_Δ : 0.46) (Bonnett *et al.*, 1999).

The PDT effect generated by m-THPC is superior to that of m-THPP. This has been demonstrated in vivo (Bonnett *et al.*, 1999; Peng *et al.*, 1995). In vitro, the two PSs have shown the same efficiency in the photodynamic treatment of retinoblastoma (Maillard *et al.*, 2007).

The rationale for the study of both photosensitizers, instead of just one, was that (i) they had almost the same chemical structure and in vitro efficacy against retinoblastoma, (ii) one was

already on the market and could be considered as relevant for a proof-of-concept of our strategy, and (iii) the other was the reference precursor for the synthesis of tetraphenylporphyrin derivatives modified with various glycodendrimeric branches (Ballut *et al.*, 2012) or with glycodimeric structures (Chen *et al.*, 2018) that have been shown to specifically interact with mannose receptors overexpressed at the surface of retinoblastoma cells (Gallud *et al.*, 2015; Makky *et al.*, 2011). In case of success, m-THPP could be easily replaced by one of these derivatives which have been already inserted into liposome bilayers. However, considering the low lipid recovery % in LNPs, it was impossible to directly test the synthetic tetraphenylporphyrins as they were available in very low quantities.

Both m-THPP and m-THPC are hydrophobic and aggregate in aqueous medium, which results in a loss of their phototoxic capacity. Their incorporation into nanocarriers may contribute, at least, to preserve their pharmacological activity and improve their accumulation in tumors.

In this study we have co-encapsulated β -Lap with each PS (m-THPP or m-THPC) in our optimal LNP formulation, LNP 75/25 (described in chapter II). β -Lapachone was loaded in the core and the PS was incorporated in the lipid shell. We have evaluated i) the effects of the co-encapsulation on the physicochemical properties of formed LNPs, ii) the cytotoxicity and phototoxicity of the systems, and, iii) the possible synergistic effect of the combined chemo/photodynamic therapies.

IV.2. Materials and methods

IV.2.1. Materials

m-THPP (Mw = 678.73 g/mol) and m-THPC (Mw = 681.2 g/mol) were gifts from Dr. Philippe Maillard (Institut Curie, Orsay, France) and β -Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione, Mw = 242.27 g/mol) (β -Lap) from Prof. N. Santos Magalhaes (Federal University of Pernambuco, Recife, Brazil). Tetrahydrofuran (THF) was purchased from Carlo Erba reagents. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was provided by Sigma-Aldrich (St Louis, USA). A streptomycin-penicillin solution

(P4333) was bought from Sigma-Aldrich (St Louis, USA). All other chemical reagents and solvents (HPLC grade) used in the following experiments are described in Chapter II.

IV.2.2. Methods

IV.2.2.1. Preparation of PS-loaded liposomes

m-THPP and m-THPC based liposomes were prepared by the thin lipid hydration method (Bangham *et al.*, 1965). In brief, 2.5 mol% of the PS stock solution (10 mM m-THPP or m-THPC in chloroform/methanol 9/1 v/v) was added to the POPC-DOTAP 75/25 solution prior to the formation of the thin lipid film. The film formed after solvent evaporation was hydrated with water and tip-sonicated to form SUVs. Subsequent centrifugations of the liposomal suspensions were performed to remove the titanium particles released by the probe during sonication and excess PSs.

IV.2.2.2. Preparation of β -Lap nanoparticles (β -Lap NPs)

β -Lap NPs were prepared by the nanoprecipitation method (Vauthier and Bouchemal, 2009), without using a surfactant. Briefly, 10% (w/w) of β -Lap was dissolved with PLA (50 mg) in acetone at the polymer concentration of 10 mg/mL. The obtained solution was added dropwise to ultrapure water under the same condition as described in Chapter II and the solvent was removed under reduced pressure. The final suspension was filtered through a 1 μ m pore-sized membrane (Glass acrodisc®, Waters) to remove β -Lap aggregates (Figure 47), and it was centrifuged 3 times at 3000g for 15 min at 15°C to separate unloaded β -Lap from NPs. The supernatant was discarded, and the pellet was diluted to the final concentration of 2 mg/mL. The suspension was concentrated for in vitro cytotoxicity studies.

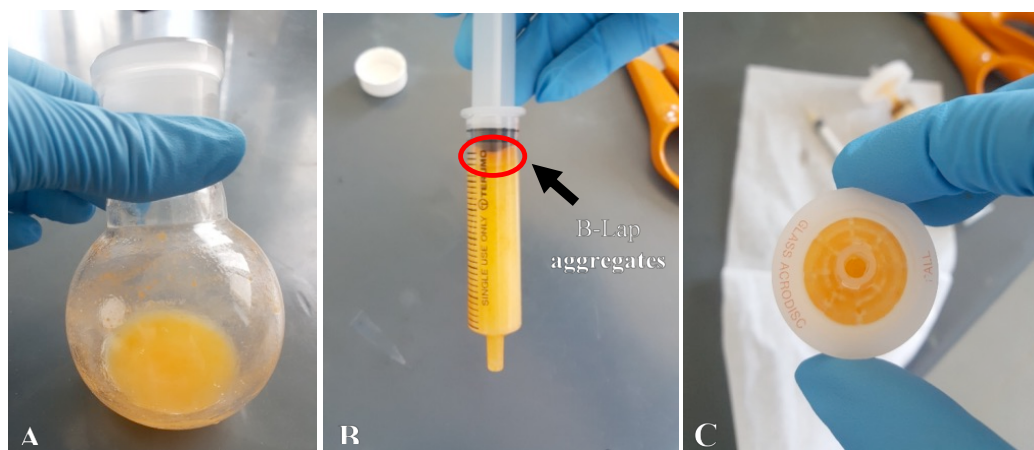


Figure 47: Removal of β -Lap aggregates following nanoprecipitation. The organic solvent was evaporated in a round bottom flask (A) followed by the filtration of the suspension (B and C).

IV.2.2.3. Preparation of β -Lap and PSs-loaded LNPs

PS or/and β -Lap-loaded LNPs were prepared by incorporating m-THPC or m-THPP in the lipid bilayer shell and β -Lap in the polymeric core. All LNPs were obtained by the same procedure as detailed in Chapter II. Briefly, PS-LNPs were prepared by mixing PS-loaded liposomes with unloaded PLA NPs, while β -Lap LNPs were formed by incubation of unloaded liposomes (POPC/DOTAP 75/25) with β -Lap NPs. PS and β -Lap were co-encapsulated in the LNPs by mixing PS-loaded liposomes with β -Lap NPs to obtain drug/PS molar ratio of 97/3 and 93/7 for LNPs containing m-THPP and m-THPC, respectively. All mixtures were sonicated for 6 min and incubated for 1h at room temperature (condition **iii**). Finally, the obtained LNPs were separated from excess vesicles by centrifugation and resuspended in pure water. They were concentrated for in vitro cyto/phototoxicity studies. Lipid concentration in both liposomes and LNPs formulations was determined by HPLC-ELSD as described in Chapter II.

IV.2.2.4. Particles characterization

IV.2.2.4.1. Size and zeta potential measurements

Particles size and size distribution were determined by dynamic light scattering (DLS) and zeta potential (ζ) by laser doppler electrophoresis using a zetasizer Nano ZS90 (Malvern). All formulations prepared in water were diluted in 1 mM NaCl solution while those prepared in buffer were diluted using the same buffer. Measurements were carried out in triplicate at 25°C.

IV.2.2.4.1. Evaluation of drug loading (DL) and encapsulation efficiency

Both PSs and β -Lap encapsulated in the formulations were quantified by measuring their absorption at 417 and 257 nm, respectively, using a CARY 100 Bio UV-visible spectrophotometer. All samples, prepared in triplicate, were dissolved in a mixture of water/methanol/THF (ratio 1:4:5). At the studied wavelengths, neither the polymer nor lipids interfered with drugs absorbance (Figure 48). Standard solutions were prepared at various concentrations using stock solutions of β -Lap in methanol (10 mM) and PS (m-THPC or m-THPP) in a chloroform:methanol (9:1 v/v) mixture (1 mM). Drug loading (DL) and encapsulation efficiency (EE) were calculated using equations (12) and (13):

$$DL (\%) = \frac{\text{Mass of drug in the nanosystem} \times 100}{\text{Mass of the nanosystem}} \quad (12)$$

$$EE (\%) = \frac{\text{Mass of drug in the nanosystem} \times 100}{\text{Total mass of drug added}} \quad (13)$$

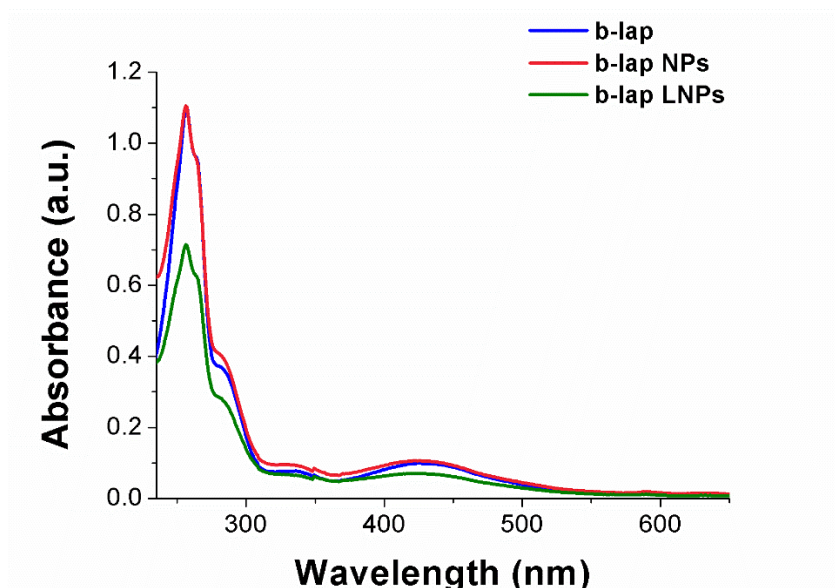


Figure 48: UV-visible spectrum of free β -Lap (blue line), β -Lap NPs (red line) and β -Lap LNPs (green line). Both lipids and PLA did not interfere with the absorbance spectrum of β -Lap.

IV.2.2.5. Cell culture

Human retinoblastoma Y79 cells (ATTC[®] HTB-18[™]), isolated from a child of two and a half years old by Reid *et al.* (1974), were grown using DMEM supplemented with 20 % v/v of FBS and 1 % of antibiotics at 37°C in a humidified incubator with 5% of CO₂. They were sub-cultured twice a week in a 75 cm² flask until reaching 80-95% confluency ($1-1.5 \times 10^6$ cells/mL). The harvested cells were centrifuged at 200 g for 5 min at 25°C and the medium renewed. They were seeded at a concentration of $0.25-0.5 \times 10^6$ cells/mL in a new flask. The cells were counted after being stained with trypan blue dye using a KOVA slide.

IV.2.2.6. Cytotoxicity and phototoxicity experiments

The cyto/phototoxicity activity of unloaded particles (NPs, liposomes and LNPs), free drugs (β -Lap, m-THPC and m-THPP), drug-loaded NPs and LNPs (β -Lap NPs, β -Lap LNPs, m-

THPC LNPs and m-THPP LNPs), and LNPs with co-encapsulated drugs (β -Lap/m-THPC LNPs and β -Lap/m-THPP LNPs) were evaluated in Y79 cells using the colorimetric MTT viability assay (Mosmann, 1983). Figure 49 illustrates the protocol of experiments.

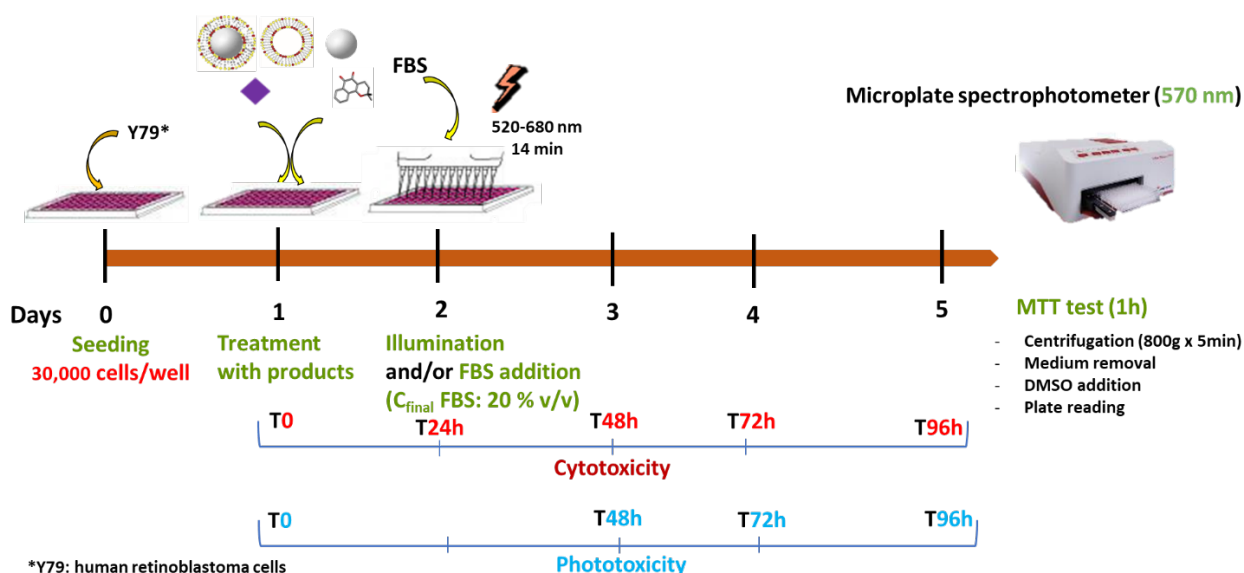


Figure 49: Summary of the procedure used for in vitro cytotoxicity and phototoxicity experiments.

Retinoblastoma cells once at confluency were harvested, centrifuged at 500 g for 5 min at 25°C and the pellet was resuspended in the culture medium supplemented with 10% v/v FBS and 1% antibiotics (ATB). The cells were seeded at a density of 30,000 cells per well (100 μ L) into 96-well plates and allowed to incubate for 24h at 37°C in the cell culture incubator (D0). Stock solutions of β -Lap, m-THPC and m-THPP prepared in DMSO (10 mM) and the tested formulations were diluted with DMEM containing 1% ATB at various concentrations. Twenty-four hours following seeding (D1), cells were treated by adding 100 μ L of the diluted solutions or suspensions at increasing concentrations (Treatment T0). The concentrations range assayed are summarized in Table 8. Control cells were treated by DMEM solutions containing the same volume of DMSO, DPBS or H₂O as the samples. Three wells were treated for each condition. After treatment, the plates were returned to the incubator. Incubations were carried out in the dark.

For cytotoxicity assays, FBS was supplemented to each well 24 hours after treatment (T24h) reaching a final concentration of 20 % v/v FBS and the plates were then incubated for two (T72h) and/or three more days (T96h). All presented results were obtained after T72h and/or T96h.

For phototoxicity assays, the plates were illuminated at T24h for 14 minutes, using a homemade lamp equipped with an orange filter ($\lambda \sim 520\text{--}680$ nm with a $\lambda_{\text{max}} = 590$ nm) at a fluence of 2 J/cm², before addition of FBS and incubation until the end of the experiment. The lamp is made of 4 Philips TL fluorescent tubes covered by a flat diffusing glass plate. Cell viability was evaluated by adding to the cells the MTT solution prepared in DPBS (at 5 mg/mL), and then incubating them for 1 hour at 37°C. During this period, living cells reduced the MTT into colored formazan crystals in their mitochondria. Then, the plates were centrifuged at 800 g for 5 minutes, the medium was cautiously removed with a manual multichannel pipette and 200 μ L of DMSO was added in each well. The formazan crystals were solubilized by stirring the plates on an orbital shaker and the absorbance was measured at 570 nm using a labsystem multiskan MS microplate to determine the half inhibitory concentration of the drugs (IC₅₀). The absorbance measured for the medium alone and with the products was subtracted from the resulting data and the cell survival rate (%) was expressed as a ratio between the absorbance of treated cells and that of cell controls.

The nature of interaction between β -Lap and each PS co-encapsulated in LNPs was evaluated using CompuSyn software (Version 1.0, CompuSyn nc., U.S., www.combosyn.com), based on the determination of the combination index (CI) theorem developed by Chou and Talalay (Chou and Talalay, 1984; Chou, 2006, 2010).

Products		Concentration ranges		
		β -Lap (μ M)	PS (μ M)	Suspension (μ g/mL)
Unloaded PLA NPs			—	100 - 1000
Unloaded POPC/DOTAP Liposomes	100/0	—	—	182 - 710
	75/25	—	—	162 - 810
	0/100	—	—	46-314
Unloaded LNP 75/25			—	100 - 1000
β -Lapachone		1 - 5	—	—
β -Lap NPs		0.5 - 10	—	5 - 105
β -Lap LNPs		0.5 - 15	—	4 - 120
<i>m</i> -THPC		—	0.04 - 0.3	—
<i>m</i> -THPC LNP		—	0.04 - 0.4	14 - 235
<i>m</i> -THPP		—	0.1 - 0.5	—
<i>m</i> -THPP LNP		—	0.002 - 1.3	25 - 1000
β -Lap/ <i>m</i> -THPC LNP		0.1 - 2.6	0.01 - 0.2	2 - 46
β -Lap/ <i>m</i> -THPP LNP		0.3 - 9.8	0.01 - 0.3	4 - 118

Table 8: Summary of the tested concentration ranges for *in vitro* cyto/phototoxicity experiments.

IV.3. Results and discussion

In chapter II, we have described the various preparation methods for LNPs and their characterization. The optimal formulation chosen for further experiments was prepared with PLA NPs and POPC/DOTAP 75/25 liposomes. The conditions used for their formation consisted in sonicating the mixture of particles and vesicles for 6 min in a water bath, and then incubating it at room temperature for one hour (condition **iii**).

The following step was to evaluate the potential of these LNPs as *m*-THPP or *m*-THPC nanocarriers. The two PSs were easily loaded in the lipid shell of LNPs as shown for *m*-THPP

in chapter II. Further, we evaluated the co-encapsulation capacity of these LNPs for the combinatory treatment of retinoblastoma by chemo/PDT. The cytotoxic drug, beta-lapachone, was encapsulated in the PLA nanoparticles, which were then covered by PS-loaded POPC-DOTAP bilayers.

We first analyzed the physico-chemical properties of the drug-loaded nanoparticles and PS/drug-loaded liponanoparticles, then their cytotoxicity and phototoxicity on retinoblastoma cells (Y79).

IV.3.1. Physico-chemical characterization of drug loaded particles

The main characteristics of β -Lap nanoparticles are summarized in Table 9. The incorporation of β -Lap in PLA nanoparticles did not alter their size, PDI nor ζ -potential. Our results agree with those of [Sharma *et al.* \(2014\)](#) who encapsulated the hydrophobic lorazepam in PLGA NPs by the nanoprecipitation process, and those of [Roussaki *et al.* \(2014\)](#) for aureusidin-loaded PLA NPs prepared by an emulsion-solvent evaporation method. In both cases, a surfactant was added.

All loaded LNPs showed an increase in their mean diameter as well as an inversion of the sign of their surface charge, which confirmed the formation of the lipid shell around the NPs core. They exhibited a narrow size distribution and a short-term stability as indicated by the value of their ζ -potential. A slight increase of their size was noticed as compared to unloaded LNP.

Formulation	HD (nm)	PDI	ζ -potential (mV)
Unloaded NPs	170 $\pm$ 3	0.08 $\pm$ 0.02	- 46 $\pm$ 1
β -Lap NPs	176 $\pm$ 9	0.06 $\pm$ 0.03	- 50 $\pm$ 2
Unloaded LNPs	193 $\pm$ 8	0.07 $\pm$ 0.03	+ 27 $\pm$ 6
β -Lap LNPs	221 $\pm$ 1	0.09 $\pm$ 0.03	+ 37 $\pm$ 1
<i>m</i> -THPC LNPs	229 $\pm$ 3	0.13 $\pm$ 0.02	+ 26 $\pm$ 1
<i>m</i> -THPP LNPs	206 $\pm$ 20	0.12 $\pm$ 0.04	+ 20 $\pm$ 4
β -Lap/ <i>m</i> -THPC LNPs	212 $\pm$ 0	0.10 $\pm$ 0.03	+38 $\pm$ 0
β -Lap/ <i>m</i> -THPP LNPs	211 $\pm$ 3	0.07 $\pm$ 0.04	+36 $\pm$ 1

Table 9: Size, PDI and zeta potential of unloaded and loaded NPs and LNPs.

IV.3.2. Encapsulation efficiency (EE) and drug/PS loading (DL) in LNPs

Encapsulation efficiency (EE) and drug loading (DL) of the PSs and β -Lap in the various studied systems are presented in Table 10.

Formulations		EE (%)	DL (%)
Drugs	Type of nanovectors		
β -Lapachone	PLA NPs	12.82	1.97
<i>m</i> -THPP	LNPs	3.92	0.17
<i>m</i> -THPC		4.01	0.18

Table 10: Summary of the encapsulation efficiency (EE) and drug loading (DL) for β -Lapachone-loaded PLA NPs and PS (*m*-THPP or *m*-THPC)-loaded LNPs.

β -Lapachone (10% w/w) was initially mixed with the polymer to form NPs by the nanoprecipitation method. The EE and DL were 12.82 and 1.97 % respectively, which seem relatively low percentages. However, Wang *et al.* (2017) designed core-shell PLGA-lipid hybrid nanoparticles in which docetaxel was loaded with 10% EE, a slightly lower value than for β -Lap. Also, Stavropoulos described PLGA lipid-polymer NPs with 1.7% (DL) camptothecin (CPT) (Stavropoulos, 2011). As NPs are spontaneously formed during the nanoprecipitation process by diffusion of the water-miscible organic phase into the aqueous one, it can be assumed that if β -Lap had limited affinity for PLA, it migrated in the solvent mixture and precipitated after solvent evaporation. Blanco *et al.* (2007) observed that β -Lap was released very fast from PLA-PEG micelles. This would account for a weak affinity of the drug for the polymer. Aggregation of β -Lap occurred during evaporation of the organic solvent (Figure 47). It is possible that the interaction between the drug and the organic solvent was the cause for its low entrapment in the nanoparticle (Budhian *et al.*, 2007).

A PS (2.5 mol%) was loaded in POPC/DOTAP 75/25 vesicles, which in turn were added in excess to the NPs to obtain PS LNPs or β -Lap-PS LNPs. The EE of the PS loaded in liposome bilayers was not determined, but it was expected to be ~85% from previous reported work (Massiot *et al.*, 2017). The EE and DL of PSs in LNPs were ~4 and 0.2% respectively. These values are very low, but may be explained by what follows.

LNPs were prepared by mixing the NPs with excess lipid vesicles, which contained the PS. Once LNPs were formed, they were separated from the remaining vesicles by centrifugation. Actually, we have shown in Chapter II that less than 10% of the total lipid vesicles used truly adsorbed onto the NPs. Thus, we believe that the low encapsulation efficiency (~4%) and drug loading (0.2%) of the PSs in LNPs is related to the preparation method of LNPs as almost 90% of the PS loaded liposomes initially mixed to the NPs were lost.

IV.3.3. Cytotoxicity of the studied formulations

We have evaluated the cytotoxicity of (i) the excipients used for the preparation of our formulations, (ii) the free drugs and (iii) drugs loaded or co-loaded in the nanocarriers, on Y79 cells after 72h or 96h of treatment by performing a MTT assay.

IV.3.3.1. Cytotoxicity of the excipients

IV.3.3.1.1. Cytotoxicity of unloaded vesicles

We have assessed the cytotoxic effect of unloaded POPC/DOTAP 100/0, 75/25 and 0/100 liposomes. The vesicles were maintained in contact with Y79 cells for 72h. As can be seen in Figure 50, neither the pure POPC liposomes nor those containing 25 mol% DOTAP showed a significant cytotoxic effect for concentrations up to $\sim 900 \mu\text{M}$ (Table 11). Only pure DOTAP liposomes were cytotoxic ($\text{IC}_{50} = 170 \mu\text{M}$), as expected from literature data (Knudsen *et al.*, 2015; Lappalainen *et al.*, 1994). An IC_{50} of $\sim 59 \mu\text{M}$ was reported by treating HEK 293T cells with DOTAP liposomes for 24h (Wan *et al.*, 2016). This IC_{50} value is 3-fold lower than ours. The difference with our result can be due to the sensitivity of the cell line to DOTAP and to the working density of seeded cells. Indeed, Wan *et al.* worked with lower cell density than us (20,000 vs 30,000 cells/well). Cells seeded at low density are more sensitive to toxins than those seeded at high density (Riss and Moravec, 2004). This is due to the higher proliferation rate of the former compared to the latter (Jagan *et al.*, 2012). Cells seeded at high density thus require higher concentration to achieve the same effect response.

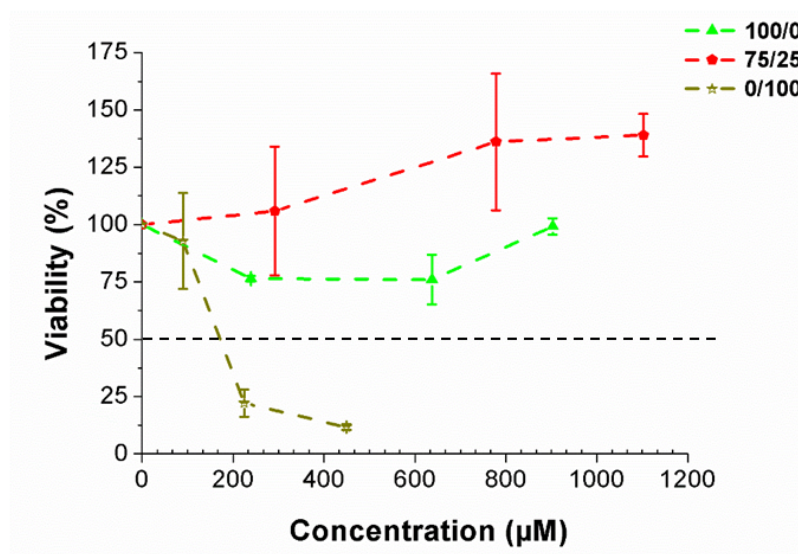


Figure 50: Viability of Y79 cells in contact with POPC/DOTAP 100/0, 75/25 and 0/100 vesicles for 72h.

IV.3.3.1.2. Cytotoxicity of unloaded NPs and LNPs

Figure 51 shows the viability plots for Y79 cells in contact with PLA NPs and LNP 75/25 for 72 h. As expected, no cytotoxic effect was observed for both nanocarriers at concentrations up to 1 mg/mL. The results obtained for LNPs 75/25 are coherent with those obtained for POPC/DOTAP 75/25 liposomes (Figure 50) as the maximum concentration of DOTAP in LNPs quantified by HPLC-ELSD was $\sim 26 \mu\text{M}$. Our results agree with previous works. [Pieper *et al.* \(2018\)](#) showed that incubating PLA NPs of $\sim 200 \text{ nm}$ in diameter up to $0.5 \mu\text{g/mL}$ with neuroblastoma cells for 120 h did not affect their viability. Also, a low cytotoxic effect (viability $> 70\%$) was reported by [Bose *et al.* \(2015\)](#) for LNPs composed of 24% DOTAP and PLGA.

From the results described above, we can conclude that our formulation was safe.

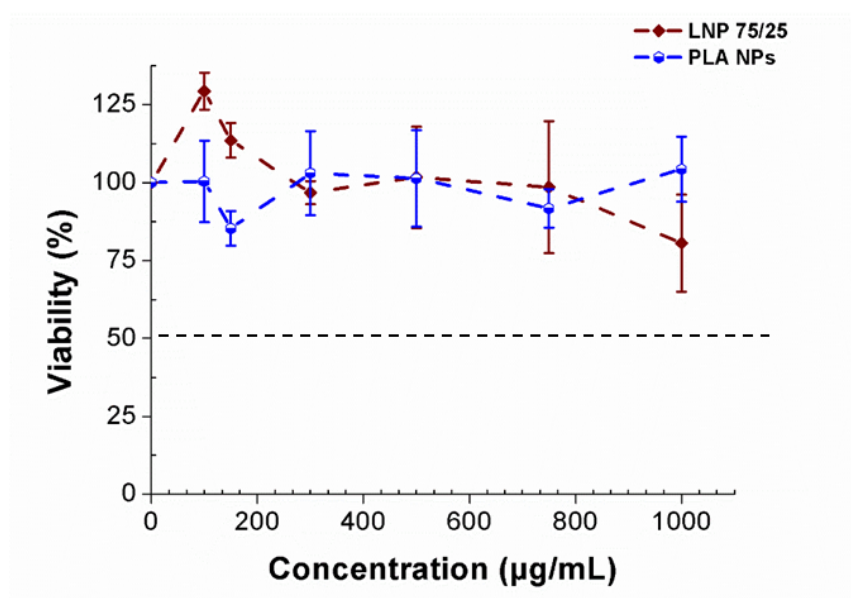


Figure 51: Viability of Y79 cells in contact with PLA NPs and LNPs 75/25 for 72h.

Products		IC ₅₀		
		(μ M)	(μ g/mL)	Final suspension (Drug+NPs or LNPs) (μ g/mL)
PLA NPs			> 1000	—
POPC/DOTAP Liposomes	100/0	> 900	> 680	—
	75/25	> 1100	> 810	—
	0/100	170	120	—
LNPs 75/25			> 1000	—
<i>m</i> -THPP		> 0.5	> 0.34	—
<i>m</i> -THPP LNPs		> 1.3	> 0.88	> 1000

Table 11: Cytotoxicity of excipients, m-THPP and m-THPP LNPs against Y79 cells after treatment for 72h. In the first two columns are presented the IC₅₀ values for drugs or excipients, while the mass concentration of LNPs or NPs containing encapsulated drugs is indicated in the last column (right).

IV.3.3.2. Cytotoxicity of free drugs

IV.3.3.2.1. *m*-THPP and *m*-THPC

The cytotoxic effects of *m*-THPP and *m*-THPC were evaluated after incubation with Y79 cells for 72h and 96h, respectively. The results are presented in Figure 52. As awaited, both photosensitizers showed no toxicity in the dark for concentrations up to 0.5 μ M (Table 11) and 0.3 μ M (Table 12), respectively. This is in agreement with previous results obtained by [Maillard et al. \(2007\)](#) showing that there was no cytotoxic effect on Y79 cells for *m*-THPP and *m*-THPC at concentrations up to 15 μ M and 8 μ M, respectively. Our tested concentrations were at least 25-fold lower than theirs.

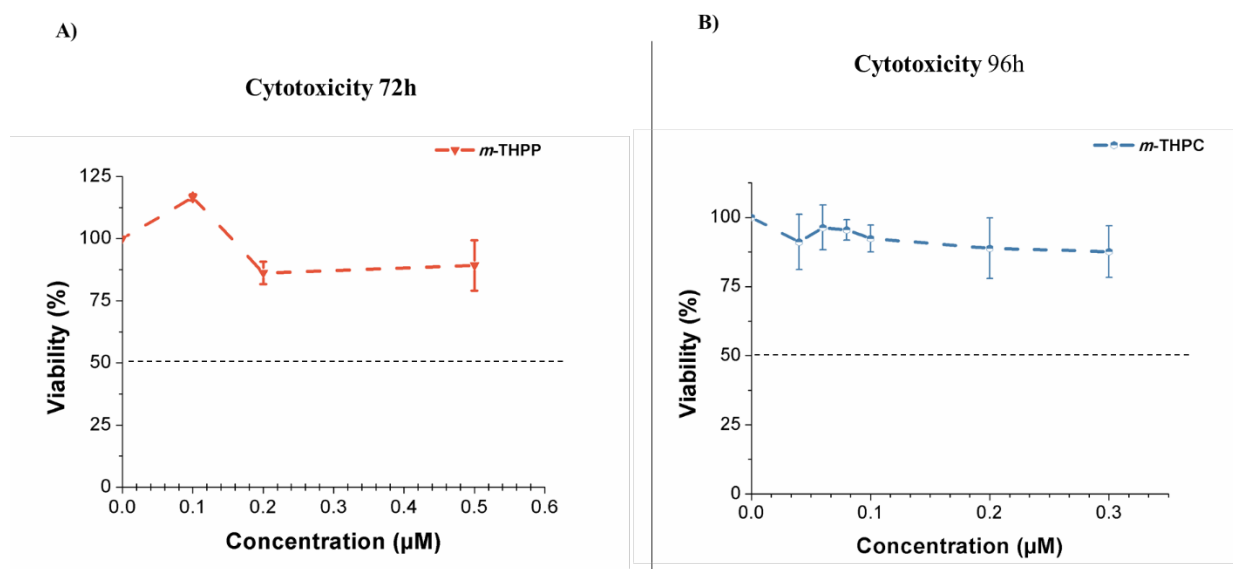


Figure 52: Viability of Y79 cells in contact with free *m*-THPP (A) and *m*-THPC (B) after 72h and 96h of treatment.

IV.3.3.2.2. Beta-lapachone

The cytotoxicity activity of β -Lap on Y79 cells after 96h of incubation is presented in Figure 53. Unsurprisingly, the increase in drug concentration from 1 to 5 μ M resulted in a significant reduction of the cell viability. β -Lap exhibited an IC_{50} of 1.6 μ M (Table 11), which is in the same order of magnitude than that reported by [Shah et al. \(2008\)](#) using Y79 cells at a density of 10,000 cells/well.

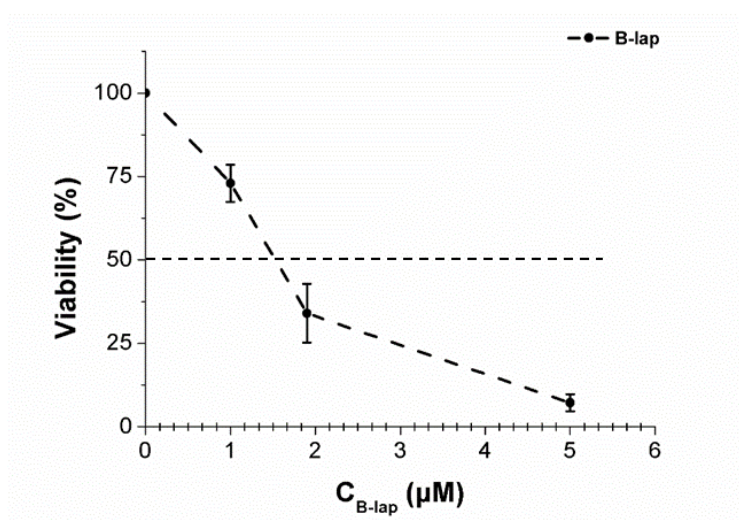


Figure 53: Viability of Y79 cells following contact with β -Lapachone for 96h.

IV.3.3.3. Cytotoxicity of beta-lapachone-loaded NPs and LNPs

The cytotoxicity of β -Lap– loaded PLA NPs and LNPs 75/25 was assessed after 96h incubation with Y79 cells. As shown in Figure 54, free and encapsulated β -Lap showed similar efficacy. No significant differences in IC_{50} values were observed (Table 12). Moreover, the effect of beta-lapachone on cell viability was not affected by the surface charge of the nanocarriers; PLA NPs were negatively charged and LNPs positively charged. The uptake of the latter was expected to be higher than that of the former, as the cell membrane is usually slightly negatively charged. [Moreno *et al.* \(2015\)](#) reported a moderate increase in nor- β -Lapachone toxicity when it was loaded in lecithin-chitosan nanoparticles, compared to the free drug. Conversely, [Costa *et al.* \(2016\)](#) reported a significant increase in the cytotoxic effect of β -Lapachone when it was loaded in PLGA microparticles. The effect of the encapsulation of the drug on its cytotoxicity is very dependent upon the system used. It is noteworthy that the maximum concentration of DOTAP in β -Lap-LNPs, as quantified by HPLC-ELSD, was only $\sim 1.5 \mu\text{M}$ (a very low concentration compared to DOTAP IC_{50}). Therefore, the cytotoxic effect observed with LNPs was only induced by the presence of β -Lapachone.

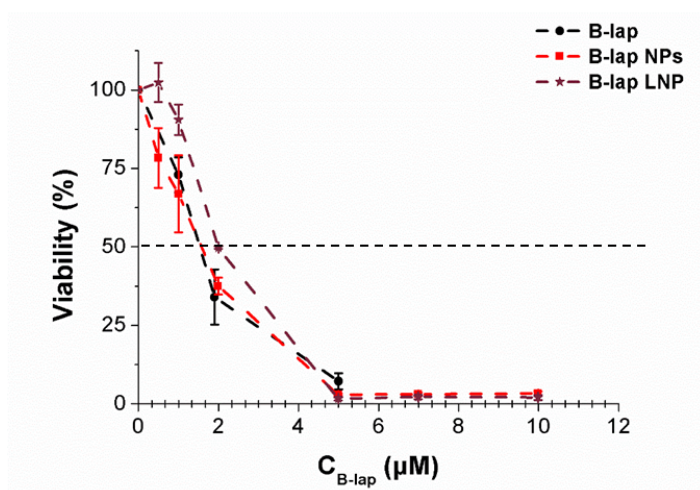


Figure 54: Viability of Y79 cells in contact with free β -Lap, β -Lap loaded NPs and β -Lap loaded LNPs for 96h.

Products		IC ₅₀		
		(μM)	($\mu\text{g/mL}$)	Final suspension (Drug + NPs or LNPs) ($\mu\text{g/mL}$)
β -Lapachone		1.6	—	—
β -Lap NPs		1.6	0.4	16
β -Lap LNPs		1.8	0.4	16
<i>m</i> -THPC		> 0.3	> 0.2	—
<i>m</i> -THPC LNPs		> 0.4	> 0.3	> 235
β -Lap/ <i>m</i> -THPC LNPs	β -Lap	1.3	0.31	21
	<i>m</i> -THPC	> 0.2	> 0.14	

β -Lap/ <i>m</i> -THPP LNPs	β -Lap	1.9	0.5	23
	<i>m</i> -THPP	> 0.3	> 0.2	

Table 12: Cytotoxicity of free drug and PSs, and drug and PS encapsulated in PLA NPs or LNPs after 96h incubation with Y79 cells. IC_{50} values of drugs are presented in the first two columns and the last column (right) indicates the mass concentration of the nanocarriers in which the drugs were entrapped.

IV.3.3.4. Cytotoxicity of photosensitizer-loaded LNPs

The cytotoxicity of *m*-THPP and *m*-THPC loaded in the lipid shell of LNPs after treatment for 72h and 96h, respectively, are presented in Figure 55. No cytotoxicity was observed for both PSs. The maximal concentrations of DOTAP in the tested suspensions were 15 μ M and 4 μ M for *m*-THPP and *m*-THPC LNPs, respectively. At these concentrations, at least 90% of the cells were still alive (Figure 51). These results are coherent with those previously obtained for free PSs (Figure 52).

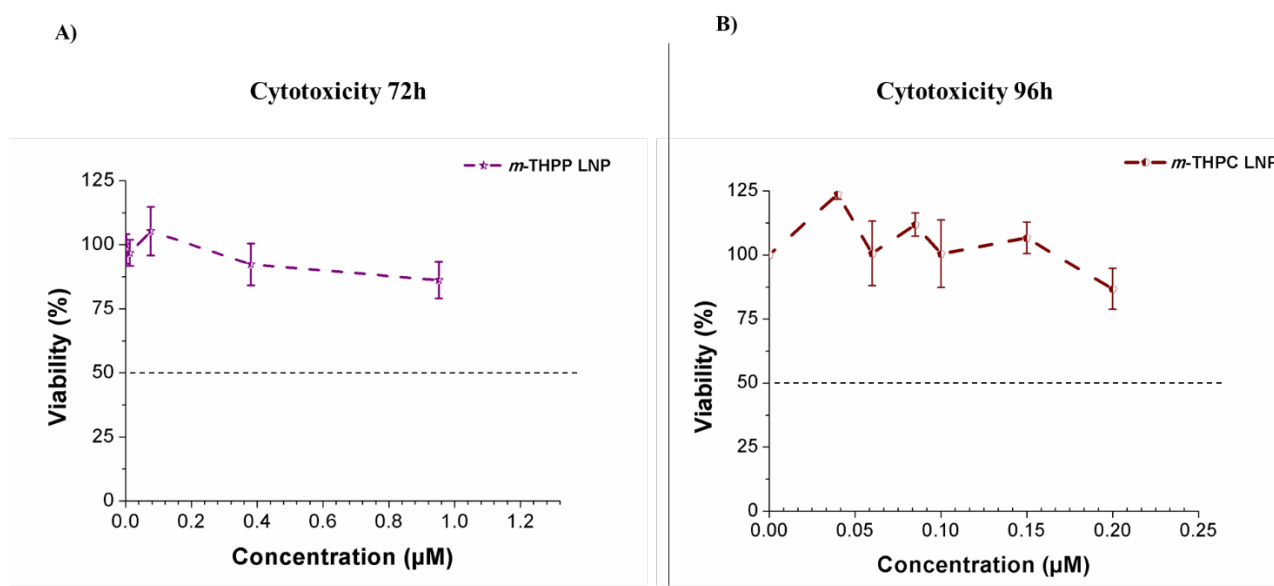


Figure 55: Cytotoxic effect of *m*-THPP LNPs (A) and *m*-THPC LNPs (B) on Y79 cells, after incubation for 72h and 96h, respectively.

IV.3.3.5. Cytotoxicity of beta-lapachone and photosensitizers co-encapsulated in LNPs

Figure 56 shows the viability results obtained for β -Lapachone co-encapsulated with m-THPP or m-THPC in LNPs. For both LNP systems, cell viability decreased in a dose-dependent manner. The IC_{50} was 1.9 μ M and 1.3 μ M for β -Lap/m-THPP LNPs and β -Lap/m-THPC LNPs, respectively (Table 12). These IC_{50} values are of the same order as those obtained for β -Lap LNPs. As we have demonstrated earlier that PSs loaded in LNPs were not cytotoxic in the dark, and since the concentration of DOTAP in the tested samples did not exceed 1 μ M, the cytotoxic effect observed was obviously induced by the presence of β -Lap in the formulation.

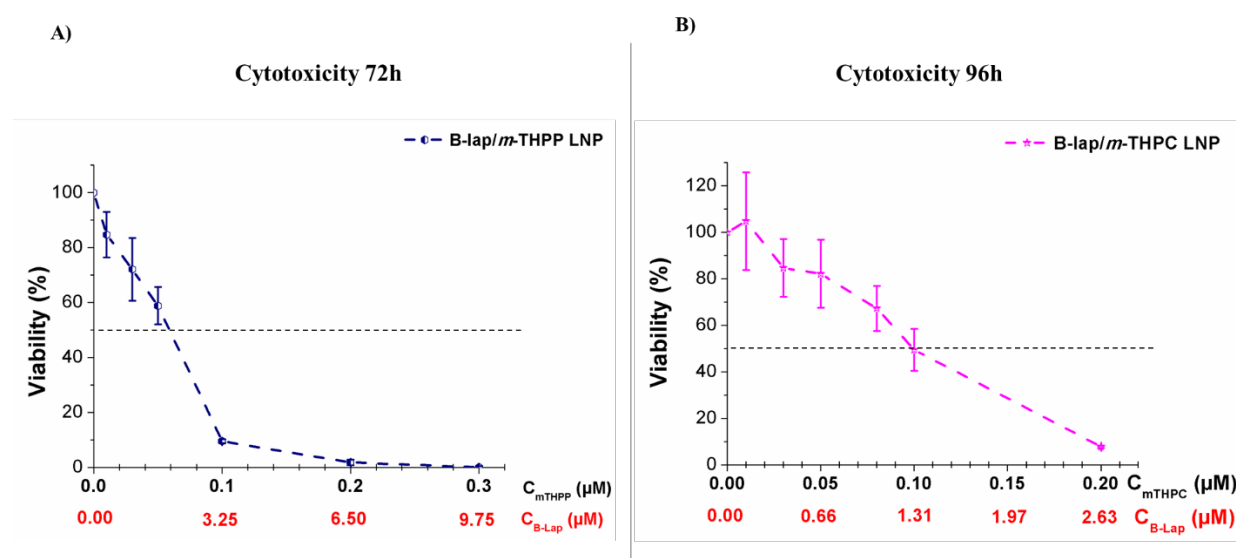


Figure 56: Cytotoxic effect of β -Lap co-encapsulated with m-THPP (A) or m-THPC (B) in LNPs after 96h incubation with Y79 cells. PS concentrations are indicated in black, and β -Lapachone ones in red.

To summarize the above results, we have assessed the cytotoxicity of our excipients which showed that unloaded LNPs 75/25 were not cytotoxic. β -Lap was cytotoxic and exhibited the same effect when free or encapsulated in NPs or LNPs. Free PSs and PS-loaded LNPs were not

cytotoxic in the dark. Finally, β -Lap/m-THPP LNPs and β -Lap/m-THPC LNPs showed similar cytotoxic effect thanks to the presence of β -Lap in the formulation.

IV.3.4 Phototoxicity of the studied formulations

The phototoxic effect of the excipients, free drugs, loaded and co-loaded drugs on Y79 cells was evaluated by MTT assays after 96h incubation. As a reminder, the cells were illuminated for 14 min, 24h following their treatment and were then incubated for 72h before the MTT test was performed.

IV.3.4.1. Phototoxicity of excipients

The aim of this evaluation was to assess whether the illumination impacted the cytotoxicity previously reported for the various systems. Figure 57A shows the results obtained for POPC/DOTAP liposomes. No significant phototoxic effect was observed for pure POPC vesicles at concentrations up to 900 μ M. Surprisingly, for POPC/DOTAP 75/25 liposomes, a significant decrease in cell viability was noticed, almost reaching 50% at the highest tested concentration (1000 μ g/mL). Pure DOTAP liposomes appeared toxic to the cells when they were illuminated, with an IC_{50} ~2-fold lower than when they were incubated with cells in the dark (Table 13). These results would suggest that the effect of DOTAP was potentiated by the illumination. [Luo *et al.* \(2017\)](#) demonstrated by liquid chromatography–mass spectrometry that both unsaturated tails of DOTAP can be photo-oxidized under light, generating peroxidized species. These species might be responsible for additional toxicity. Another explanation could be the difference in treatment duration in cytotoxicity and phototoxicity experiments. Indeed, cytotoxic experiments were performed for 72h, whereas the phototoxic ones lasted 96h.

Figure 57B shows the results for PLA NPs and LNP 75/25. PLA NPs did not exhibit any significant toxicity following illumination at concentrations up to 1000 $\mu\text{g/mL}$. Conversely, LNPs 75/25 showed similar phototoxicity profile as POPC/DOTAP liposomes 75/25.

By assessing the phototoxicity of the excipients, we showed evidence of a possible double toxic effect induced by both DOTAP and illumination. This effect was more pronounced at higher concentrations. As showed in Table 13, the concentration of the final suspensions, at which 50% of cell growth was inhibited, was lower than 100 $\mu\text{g/mL}$. Therefore, we are almost certain that at this concentration nearly 100% of the cells were viable.

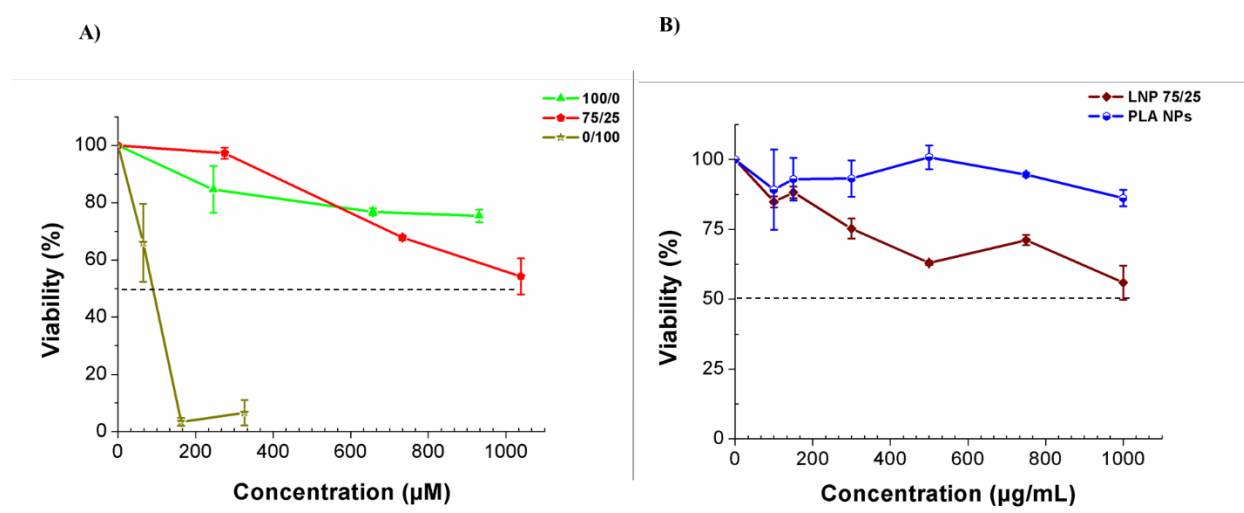


Figure 57: Phototoxic effect of A) POPC/DOTAP 100/0, 75/25 and 0/100 liposomes, and, B) PLA NPs and LNP 75/25 on Y79 cells after 96h of treatment.

Products		IC ₅₀		
		(μM)	($\mu\text{g/mL}$)	Final suspension (Drug+NPs or LNPs) ($\mu\text{g/mL}$)
PLA NPs			>1000	—
POPC/DOTAP Liposomes	100/0	>930	>700	—
	75/25	~1040	>764	—
	0/100	82	56	—
LNP 75/25			>1000	—
β -Lapachone		1.6	—	—
β -Lap LNPs		1.85	0.45	15
<i>m</i> -THPC		0.09	0.06	—

<i>m</i> -THPC LNPs		0.09	0.06	33
<i>m</i> -THPP		0.11	0.07	—
<i>m</i> -THPP LNPs		0.06	0.04	85
β -Lap/ <i>m</i> -THPC LNPs	β -Lap	0.8	0.2	12.5
	<i>m</i> -THPC	0.06	0.04	
β -Lap/ <i>m</i> -THPP LNPs	β -Lap	1.2	0.3	13
	<i>m</i> -THPP	0.04	0.02	

Table 13: Phototoxicity of excipients, free drug and PSs, and drug/PSs encapsulated in PLA NPs or LNPs on Y79 cells after 96h of incubation with Y79 cells. The first two columns show the IC₅₀ values of drugs or excipients. The last column (right) indicates the mass concentration of the nanocarriers containing the drugs.

IV.3.4.2. Phototoxicity of free β -lapachone and β -Lap-loaded LNPs

The phototoxicity of free β -Lapachone and β -lapachone encapsulated in LNPs was assessed to verify the effect of the illumination. As can be seen in Figure 58A, there was no effect of the light on the toxicity induced by β -Lapachone. The IC₅₀ remained the same with or without illumination (Table 12 and Table 13). Likewise, no additional effect of light was observed when β -Lap LNPs were illuminated in the presence of the cells (Figure 58B, Table 12 and Table 13). The concentration of DOTAP in the samples was lower than 4 μ M. The cytotoxic/phototoxic effect observed can thus only be attributed to the intrinsic cytotoxic activity of the drug.

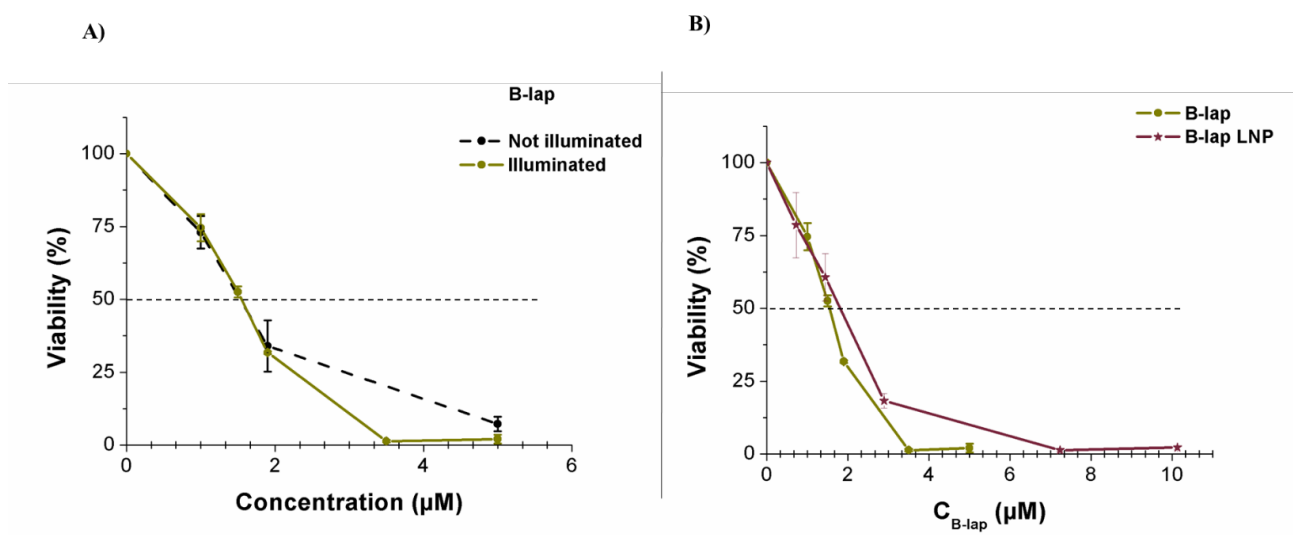


Figure 58: A) Cytotoxicity/phototoxicity of free β -Lap, and, B) phototoxicity of β -Lap loaded encapsulated in LNPs on Y79 cells after 96h of treatment of Y79 cells.

IV.3.4.3. Phototoxicity of free PS and PS-loaded LNPs

The phototoxicity of free m-THPP and m-THPP LNPs is presented in Figure 59A. Predictably, free m-THPP was phototoxic at low concentration with an IC_{50} of $\sim 0.1 \mu$ M (Table 13). Its incorporation in LNPs resulted in a slight decrease of the IC_{50} (Table 13). However, this effect is insignificant. The IC_{50} of free m-THPP obtained by [Maillard *et al.* \(2007\)](#) on Y79 cells was 0.5μ M. This value is 5-fold higher than the IC_{50} that we have obtained but considering that this difference is in the nanomolar range, it is not quite significant. Furthermore, it is noteworthy that they had 5×10^5 cells/well, while we used only 30,000 cells/well.

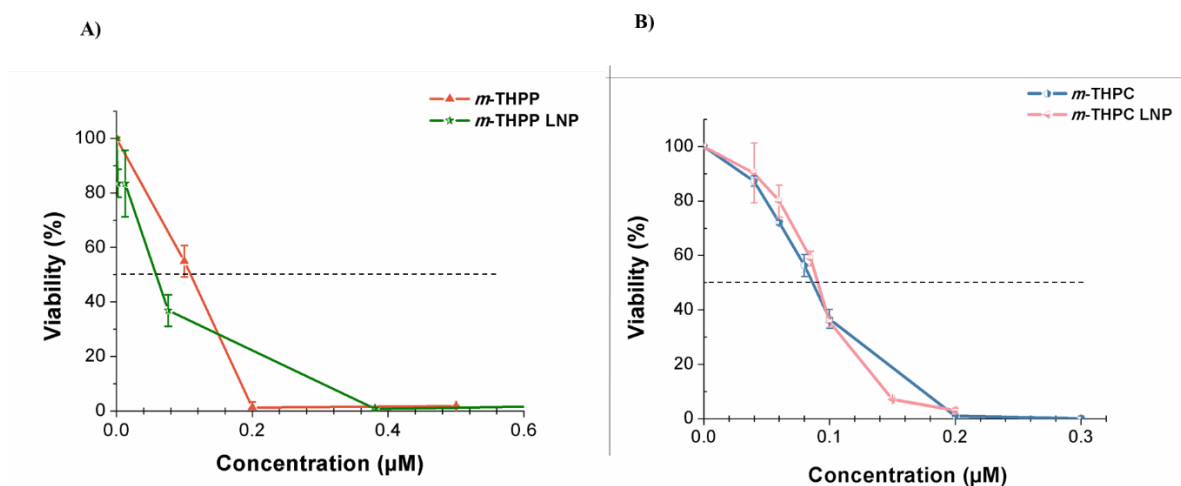


Figure 59: Phototoxicity of m-THPP and m-THPP LNPs (A), and m-THPC and m-THPC LNPs (B) on Y79 cells after 96h of treatment.

Figure 59B shows the phototoxicity of free m-THPC and m-THPC LNPs. As expected, m-THPC exhibited a phototoxic effect on Y79 at low doses. Its IC_{50} was 0.09 μ M which is significantly lower than that of previous reports (Gaio *et al.*, 2016; Maillard *et al.*, 2007). Whether free or incorporated in LNPs, m-THPC demonstrated the same phototoxic effect towards Y79 cells with 50% viability reduction at 0.09 μ M (Table 13).

IV.3.4.4. Phototoxicity of PS and β -Lap co-encapsulated in LNPs

We have co-encapsulated β -Lap and PSs in LNPs with the aim of combining PDT and chemotherapy. In the absence of light, only β -Lap was expected to exert its pharmacological effect, as demonstrated earlier, while in the presence of light, a conjugated effect of both β -Lap and the PSs could be expected.

Figure 60A shows the result obtained for β -Lap/m-THPP LNPs. Cell viability was reduced by the illumination of cells, as inferred from experiments in the dark. The IC_{50} of the LNPs was slightly lower than that of the same drug used separately (Table 13). Comparison of the phototoxicity curves of m-THPP LNPs and β -Lap/m-THPP LNPs confirmed that the latter was more effective than the former, thanks to the presence of β -Lap (Figure 60B). Figure 60C shows the result obtained for β -Lap/m-THPC LNPs. We can clearly observe a significant reduction of

the cell viability compared to that obtained with β -Lap/m-THPP LNPs suggesting that, as indicated in the literature, m-THPC is more effective than m-THPP. The comparison of the plots of m-THPC LNPs and β -Lap/m-THPC LNPs also show evidence of the combined effect of β -Lap and m-THPC on cell viability (Figure 60D).

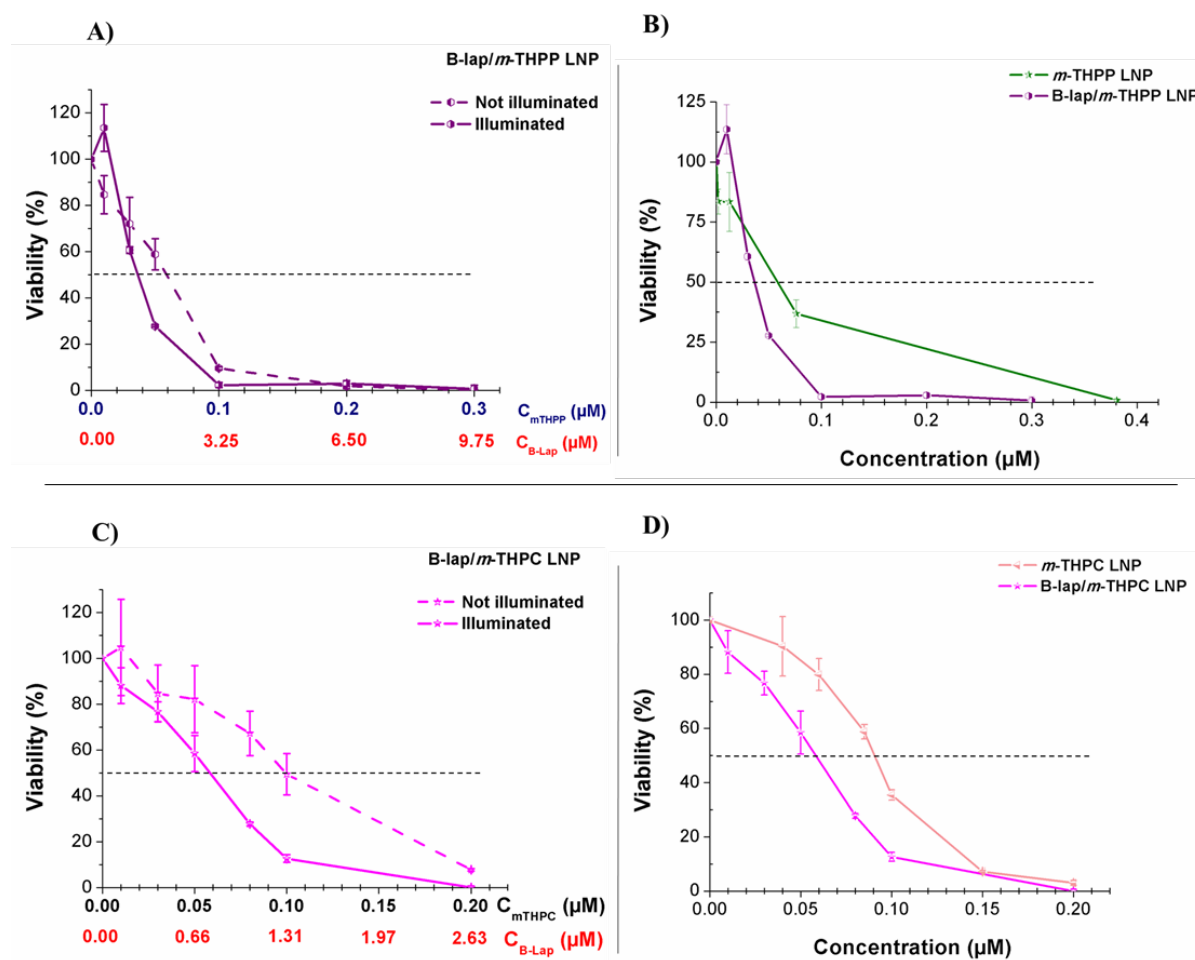


Figure 60: A) Cyto/phototoxicity of β -lap/m-THPP LNPs and B) Comparison of the phototoxicity of β -lap/m-THPP LNPs with that of m-THPP LNPs; C) Cyto/phototoxicity of β -lap/m-THPC LNPs and D) Comparison of the phototoxicity of β -lap/m-THPC LNPs with that of m-THPC LNPs; All these plots were obtained after 96h of incubation with Y79 cells.

To qualify the type of interactions between β -Lap and each PS leading to the combined effect observed in our experiments, we have applied the combination index (CI) theorem developed by Chou and Talalay, using the CompuSyn software (Version 1.0, CompuSyn nc., U.S., www.combosyn.com) (Chou and Talalay, 1984; Chou, 2006, 2010). This theorem allows

identifying the synergistic or antagonistic effect of two or more drugs co-encapsulated in a system, by calculating the combination index from equation (14).

$$CI = \frac{D_1}{ID_{X,1}} + \frac{D_2}{ID_{X,2}} \quad (14)$$

Where D_1 and D_2 are the concentrations of each drug (drug 1 and drug 2) in the combination that inhibit X% cells and $ID_{X,1}$ and $ID_{X,2}$, the concentrations of each drug used separately in the treatment that induces the same effect (X% cell inhibition).

When the $CI < 1$, the effect is considered synergistic, while it is antagonistic for $CI > 1$ (Bijnsdorp *et al.*, 2011). A $CI = 1$ indicates an additive effect.

In our phototoxicity studies, we have plotted the cell viability (%) as a function of drug concentration. For the calculation of the combination index, the values of growth-inhibitory effect of each drug and combination of both (Fraction affected, FA, equation 15) are required.

$$FA = 1 - \left(\frac{\%growth}{100} \right) \quad (15)$$

FA represents the fraction affected.

Figure 61A and Figure 61B show the FA plots for the LNPs and the pure drugs, respectively, and Figure 62, the CI-FA relationships.

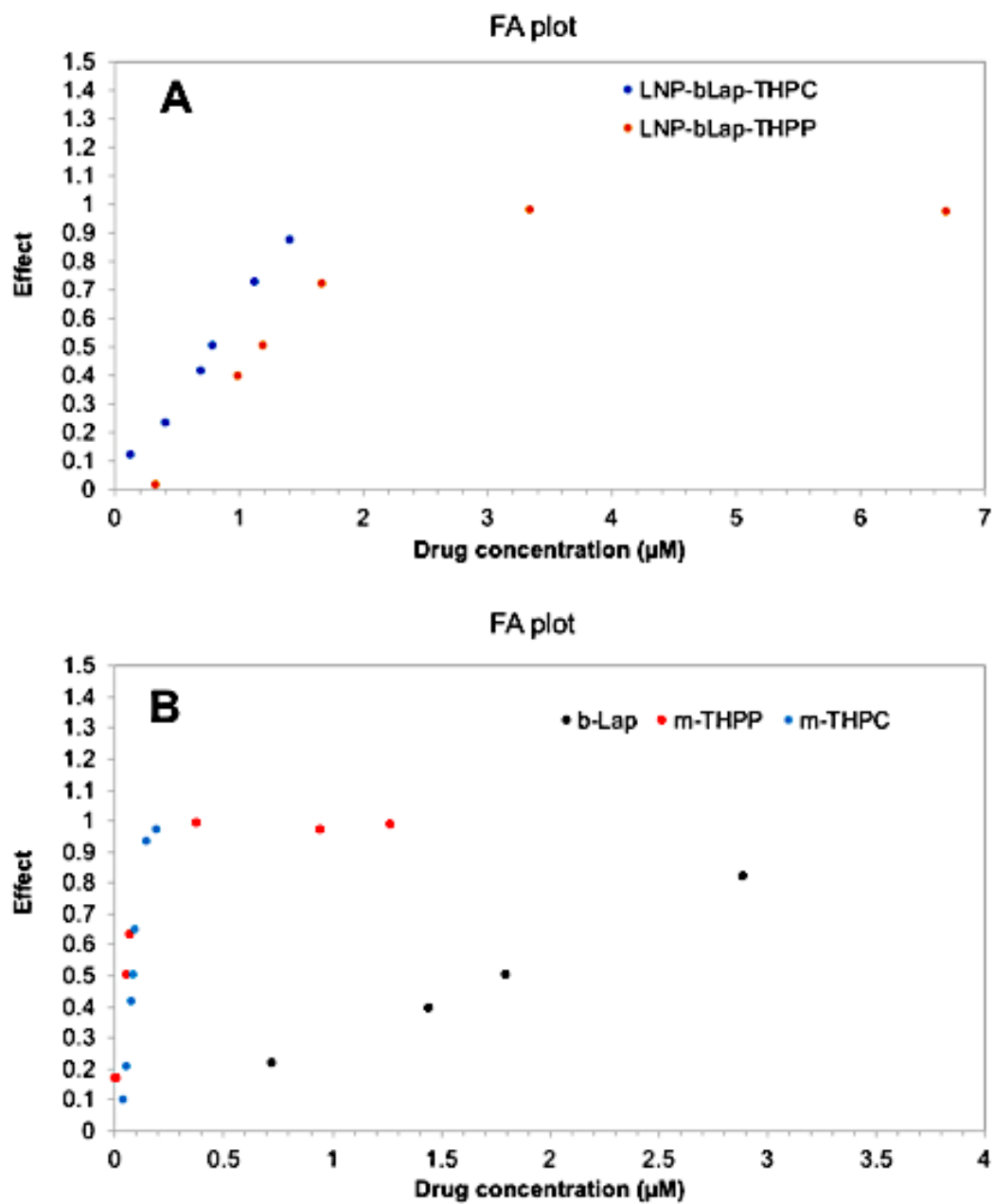


Figure 61: FA plots for (A) β -Lap/m-THPC and β -Lap/m-THPP LNPs, and (B) the pure drugs.

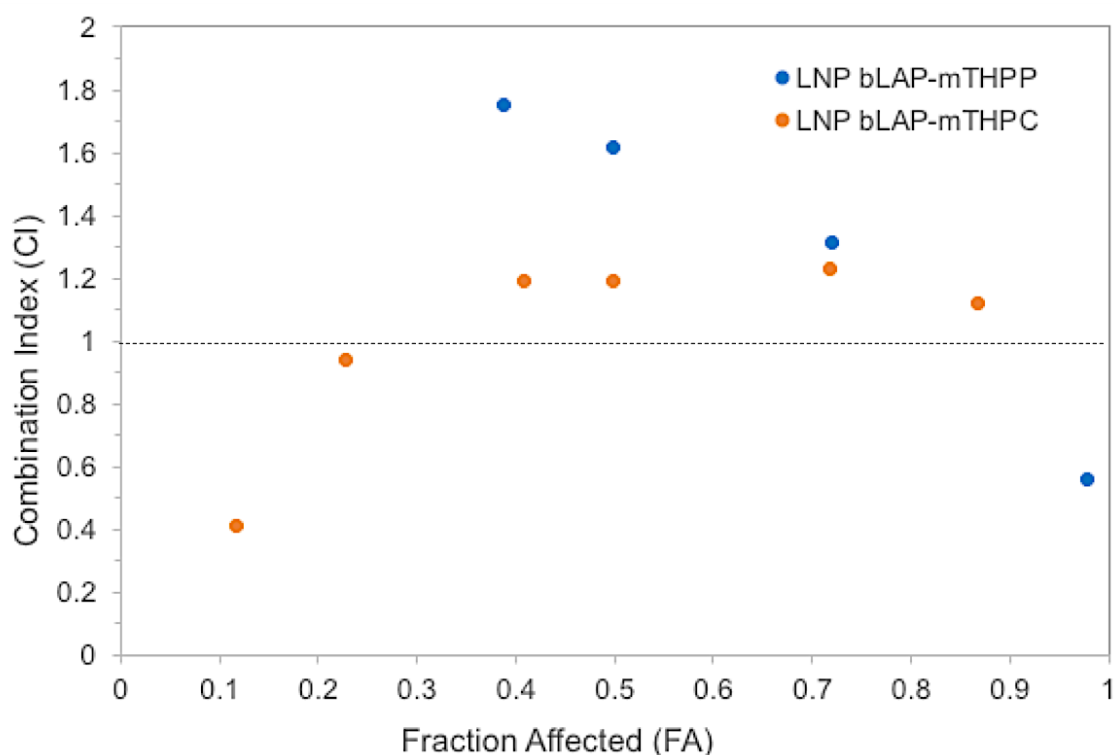


Figure 62: Combination index-fraction affected relationships for β -lap/m-THPC and β -lap/m-THPP LNPs, calculated using the CompuSyn software.

The CI values are different for the two studied systems. [Bijnsdorp *et al.* \(2011\)](#) have provided a simpler CI ranking than [Chou \(2006\)](#). In their system, CI below 0.8 indicates a synergy, $0.8 < CI < 1.2$ accounts for the additivity of effects and $CI > 1.2$, for drugs antagonism.

For β -lap/m-THPC LNPs (molar ratio = 93/7), at $FA > 0.2$, CI is comprised between 0.8 and 1.2. According to the ranking system defined above, effects of β -lapachone and m-THPC would be additive ([Bijnsdorp *et al.*, 2011](#)). For β -lap/m-THPP LNPs (molar ratio = 97/3), CI is above 1.3 for FA in the range [0.4-0.7]. There seems to be a real antagonism between the two drugs. Conversely at high FA, a synergic effect is observed.

The results of the simulation for the β -lap/m-THPP LNPs was unexpected because the viability plots in Figure 60B and Figure 60D show that the IC_{50} of both PS-LNPs are reduced in the presence of beta-lapachone, So, there is a gain for m-THPP or m-THPC to be associated with β -lap.

Some points must be considered when analyzing these results: (i) the number of FA values considered for β -lap/m-THPP LNPs was low (only 4 values); Also, (ii) most FA values considered for the simulation were below 0.5 for both LNP systems. According to [Bijnsdorp *et al.* \(2011\)](#) low FA values cannot be considered as significant as they correspond to a low level of growth inhibition. Unfortunately, at concentrations above the IC_{50} for β -lap/m-THPP LNPs, cell viability was strongly affected and most FA values above 0.5 were too close to 1 to be used in the simulation; (iii) The β -lap/PS molar ratio was not varied. The analysis of drug combination at a fixed constant ratio is interesting when the two drugs have similar cell viability-dose profiles. This is not the case for β -lapachone and the photosensitizers; (iv) In the literature, it is advised to combine drugs at a ratio corresponding to that between their IC_{50} . The IC_{50} for beta-lapachone is 20 to 30 times higher than that of the PSs. For m-THPP, the molar ratio of the two drugs should have been 30:1. It was 97:3. Likewise, for m-THPC, the molar ratio should have been 20:1 instead of 93:7. However, at high β -lap concentrations, it would be difficult to match the IC_{50} ratios. Indeed, high PSs concentrations could be difficult to incorporate in lipid vesicles, and even if it were possible, high PS concentrations could reduce PDT activity, by auto-quenching of the molecules.

In fact, a non-fixed ratio would have been more appropriate, as the PSs were more active (after illumination) and in a shorter time than beta-lapachone.

IV.4. Conclusion

We have co-encapsulated a cytotoxic agent, β -Lap and a PS in LNPs. Both substances did not induce significant changes in the physicochemical properties of the LNPs. Drug loading and encapsulation efficiency of β -Lap in PLA NPs and PSs in the LNPs were relatively low but acceptable. We have assessed the cytotoxic and phototoxic effect of our formulations on retinoblastoma cells. Our results show that the activity of β -Lap and the PS was preserved when loaded alone in the LNPs. The PSs were not cytotoxic in the dark, but they became phototoxic once illuminated. The light did not affect the activity of β -Lap. By co-encapsulating both molecules in LNPs - β -Lap in the core and a PS in the shell – we have demonstrated an improvement of their antitumoral effect as compared to single therapies.

General discussion

General discussion

There are many examples of liponanoparticles in the literature, either purely conceptual systems or systems actually prepared and experimentally tested ([Ashley *et al.*, 2011](#); [Hu *et al.*, 2015b](#); [Mornet *et al.*, 2005](#); [Thevenot *et al.*, 2008, 2007](#)). Among these are three types of systems: (i) lipid core systems surrounded by a polymer layer, (ii) polymer core systems stabilized by a monolayer of phospholipids, and (iii) inorganic or polymeric core systems coated with a phospholipid bilayer. These various systems, four in fact, if we distinguish the hybrid systems with inorganic core from those with a polymeric one, have different methods of preparation and applications.

It is at the end of the nineties that hybrid nanoparticules appeared as potential drug delivery systems. They were initially proposed to prevent the leakage of drugs from nanoparticles, improve the control of drug release, and they were more stable than liposomes and more biocompatible than nanoparticles. For example, it was suggested to coat metal nanoparticles for phototherapy with lipids so as to increase their biocompatibility.

Another reason justified the strategy of hybrid systems: the development of a new generation of nanocarriers allowing theranostic applications. Indeed, using LNPs it was possible to encapsulate in a same nanocarrier two or more compounds providing a therapeutic effect and imaging. To allow this co-encapsulation, it was necessary, in certain cases, to create hybrid systems, one compound being incorporated in the shell and the other in the core of the particle.

Combination of drugs usually aims to achieve a better efficiency of the treatment with one drug thanks to the action of the second drug. A synergy of effects is sought. For example, [Song *et al.* \(2017\)](#) co-encapsulated metformin (drug) and chlorin e6 (photosensitizer) in vesicles so that the former increased the tumor oxygenation necessary for the action of the latter.

Combination of an anti-cancer drug with a photosensitizer in mesoporous silica nanoparticules (MSN) was also studied ([Gary-Bobo *et al.*, 2012](#)). The authors observed an increase in cell death upon PDT treatment. However, the mechanism of enhancement of PDT efficiency by the two-treatment modality (chemotherapeutic and photodynamic) was not determined. These

MSNs, which were also functionalized with sugar moieties grafted to their surface, were found to be highly effective against RB cells. Co-encapsulation of drug and PS was also performed in LNPs for enhancement of the cytotoxic effect of PDT (Pramual *et al.*, 2017; Spring *et al.*, 2010).

In our work, we focused on hybrid nanoparticles with a polymer core and a lipid shell to obtain a biocompatible and rapidly biodegradable system.

Rationale of the design of a hybrid nanocarrier

The choice of the form and composition of our nanocarrier was dictated by the nature and mechanism of action of the photosensitizer and anti-cancer drug delivered by the intravitreal route. Several aspects were considered:

(i) the objective of the combination of the two substances was to potentiate their effects on retinoblastoma by applying a localized photodynamic treatment to the solid tumor and a chemotherapeutic treatment with a more prolonged and potentially active effect on the forming metastases. To target metastases and protect the healthy cells in the retina and the posterior area of the eye would require the presence of ligands specifically recognizing retinoblastoma cells in the tumor and in suspension in the vitreous liquid. Retinoblastoma cells are known to overexpress mannose receptors that have been recently identified (Gallud *et al.*, 2015). We originally envisaged using the dendrimeric and dimeric glycoconjugated derivatives developed by Philippe Maillard's team (Ballut *et al.*, 2012; Chen *et al.*, 2018; Makky *et al.*, 2012, 2011). This could not be done, for lack of product, because the synthesis is no longer carried out at the Institut Curie. If the PS is not modified with mannose moieties, it is non-specific and could be internalized by any cell present in the area. The interest of PDT is that only tumor cells are illuminated. So, the distribution of the PS is not important. A glycoconjugated PS would accumulate at the surface of the cells of interest. However, its effect might be controlled by availability/saturation of the receptor. For beta-lapachone, it is different. The drug is a powerful cytotoxic agent, with several possible mechanisms of action. So, if the drug is distributed in healthy as well as in RB cells, the damages could be non-negligible. On the other hand, beta-lapachone is the only one of the two compounds which could be active against forming metastases. So, the ideal treatment should combine the PS for PDT against the

tumor and beta-lapachone against the cells in suspension, and at the same time avoid internalization by healthy cells. For that, encapsulation in liponanoparticles would be a good strategy, provided that the liponanoparticle surface would be modified with saccharide moieties, for example. This was a first reason for choosing liponanoparticles for RB treatment.

(ii) The goal of vectorization was to couple two light-sensitive substances, which should be protected, solubilized (because hydrophobic) but also conveyed in the same place and, if possible, at the same time. Also, the two substances should be preferably administered intravitreally, in the same injection. Nanoparticles could allow this protocol and have demonstrated low toxicity when administered by this route, provided that their size is smaller than 200 μm (Jiang *et al.*, 2018; Merodio *et al.*, 2002; Prow *et al.*, 2008; C. Yang *et al.*, 2013).

(iii) Does it really make sense to transport the two cytotoxic agents at the same place in the same carrier for two different therapeutic protocols? As we have seen previously, photosensitizers are active at concentrations 20 to 30 times lower than beta-lapachone. At the level of the tumor, if the liponanoparticles are unmodified, the core and shell are internalized together in the cells, as demonstrated by the experiment with the two dyes in Chapter II. When we focus light on these cells, the photosensitizer is the most effective (Chapter IV). Is beta-lapachone of any use in these conditions? According to the literature, beta-lapachone could actually play an interesting role, if retinoblastoma cells overexpress the enzyme responsible for a large part of its activity, the NAD(P)H: quinone acceptor oxidoreductase NQO1. Indeed, beta-lapachone is activated when NQO1 is present in the cell cytosol. This enzyme catalyzes a so-called futile cycle reduction reaction that generates the superoxide ion $\text{O}_2^{\cdot-}$ (Figure 63).

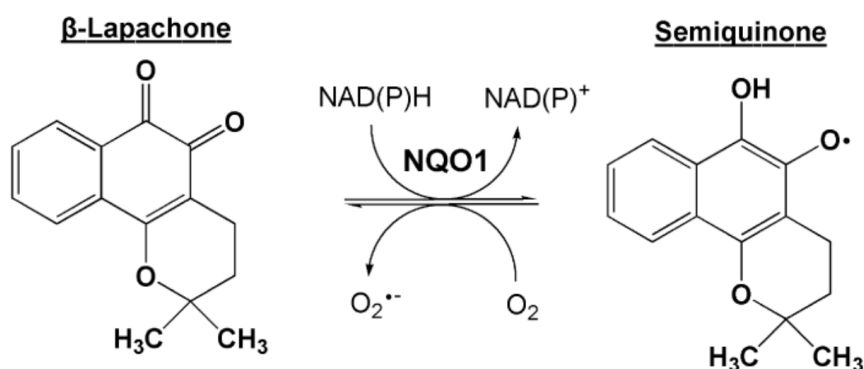


Figure 63: Futile oxidoreduction cycle of beta-lapachone catalyzed by NQO1 (from Kung, 2014)

We have verified that retinoblastoma cells express this enzyme, using the approach of Lamberti *et al.* (2013), and beta-lapachone is known to be cytotoxic to retinoblastoma cells (Figure 64). Shah *et al.* (2008) studied three cell lines (Y79, WERI-RB1 and RBM) and found IC_{50} of 1.9, 1.3 and 0.9 μM , respectively. Our own result (1.6 μM for Y79 cells) is in agreement with these data. In Y79 cells, Shah *et al.* observed a 1.7-fold increase in the frequency of apoptotic cells at 48h post-treatment, and a four-fold induction of caspase 3/7 activity at 72h post-treatment. This information is important because the caspase cascade directly results from intracellular ROS increase induced by the NQO1-catalyzed futile cycle (Kung *et al.*, 2014).

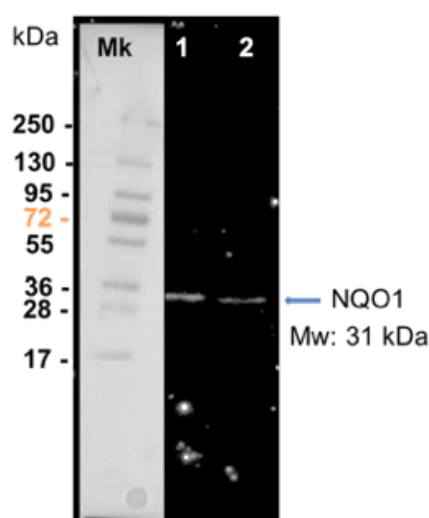


Figure 64: Western blot demonstrating the expression of NQO1 by Y79 cells.

It has been shown that beta-lapachone is a radiosensitizer, but also that conversely, its effect can be increased by ionizing radiation or by photodynamic therapy (Kung *et al.*, 2014; Park *et al.*, 2005). The superoxide ion produced through the futile cycle is the trigger for several mechanisms leading to apoptosis. It weakens the cells which can then be more receptive to the effect of singlet oxygen produced by PDT. Moreover, PDT is known to activate the expression of NQO1.

There is therefore potential synergy between the drug and a photosensitizer, provided that a sequential schedule is applied. The PDT must be carried out before bringing beta-lapachone into contact with the cells: indeed, it takes several hours to boost the expression of NQO1; if beta-lapachone is administered at the same time as the photosensitizer, the beta-lapachone should be released less rapidly than the PS and its activity should not be affected by the PDT treatment. Without having specifically studied the release of β -Lap, we observed that its effect was exerted over a long period of time, beyond the PDT treatment, when it was encapsulated in PLA nanoparticles; Moreover, we did not observe a difference in cytotoxicity according to whether β -lap was illuminated or not, or whether the drug was in nanoparticles or free.

(iv) Our two photosensitizers and beta-lapachone are hydrophobic, so a hydrophobic core was necessary for encapsulation and a polar surface was needed for electrostatic interaction with the liposomes. This is why we chose poly (D, L)-lactic acid. For rapid degradation in the body, PLGA would probably have been a better choice, but it contains hydrophilic glycolic acid units that could have hindered the encapsulation of the hydrophobic drug (Anderson and Shive, 1997). We have observed that even with the hydrophobic PLA, β -Lap was not easily encapsulated in a nanoparticle.

(v) The PS could have been co-encapsulated with beta-lapachone in the lipid bilayer (Song *et al.*, 2017). Recent work in our team (Wu, 2018) has shown that when beta-lapachone is incorporated into a liposome membrane, its encapsulation rate does not exceed 3 to 3.5 mol%. The drug is localized near the polar heads of phospholipids. It can be released quickly. However, as we mentioned above, it would be better if its release was more progressive. Thus, the drug should rather be encapsulated in nanoparticles. In addition, the space available for incorporation of β -lapachone and the PS into the phospholipid bilayer is limited. The encapsulation rate could be very low.

As previously mentioned, porphyrins and chlorins do not perform very well when encapsulated in the core of a nanoparticle, despite some promising studies (Chatterjee *et al.*, 2008; Konan-Kouakou *et al.*, 2005; Vargas *et al.*, 2008). When their concentration is high, the confinement favors quenching of the PS and when they are too diluted in the polymer mass, the singlet oxygen produced is inefficient because its lifetime and its diffusion length are limited. It tends to persist in the carrier. It would be more efficient if localized close to the surface of the drug carrier, in the lipid bilayer.

All these considerations led us to the design of a hybrid nanoparticle with a polymeric core and a phospholipid bilayer envelope. It was necessary for one to adhere to the other, and in order to facilitate this adhesion, the development of electrostatic interaction seemed appropriate. We therefore decided to encapsulate β -lap in PLA nanoparticles and to coat them with a bilayer formed of neutral and cationic phospholipids containing one of the two chosen photosensitizers.

Methods of preparation of the liponanoparticles.

Different methods of preparation have been experimented. After several trials with various POPC-DOTAP mixtures to modulate vesicle loading and achieve optimal NP surface covering, we selected the 75/25 mixture and chose to sonicate vesicles and nanoparticles for 6 minutes and then incubate them for one hour at room temperature. Although the colocalization of lipids and particles was demonstrated, not only with dyes but also with one of the photosensitizers, a homogeneous and continuous bilayer could not be clearly seen at the surface of the nanoparticles by cryoTEM experiments (Chapter II).

PLA being hydrophobic, a lipid monolayer instead of a bilayer could have been formed on its surface.

Further analysis of the liposome-polymer interaction by formation of a PLA layer on a solid support and addition of a vesicle suspension in QCM-D and AFM liquid chambers showed that the interaction between the two systems resulted in the formation of at least one bilayer and that the driving force of the fusion of liposomes on the PLA layer was mostly electrostatic.

However, the concentration of the vesicles and softness of the support could affect the process of bilayer formation and prevent full coverage of the polymer surface (Chapter III).

The experiments showed that under conditions where both systems carry opposite charges, the rupture of the vesicles is too fast to allow complete coating of the surface. The second and third stages of vesicle rupture mechanism (flattening and crowding of vesicles) do not occur. The liposomes break independently of each other and form patches of bilayers which cover at best 80% of the PLA-coated surface. According to our quantifications, the number of bilayers was 0.7, 1.1 and 1.4 bilayer for a proportion of DOTAP of 50, 25 or 10 mol%, respectively. The presence of two bilayers could correspond to flattened vesicles, emptied of their water. Literature suggests that a better result could be achieved by heating nanoparticles and liposomes during incubation and by playing on the salinity of the aqueous medium.

Stability of liponanoparticles

The formation of the LNPs being carried out in pure water, we wondered about the stability of the system in a biological medium, particularly in the cell culture medium. This is why we studied the morphology of LNPs in DPBS and in DMEM (Chapter II). In both cases, we observed a partial destabilization of the system, with detachment of the bilayer and sometimes a bilayer bridge between LNPs, favoring aggregation of these LNPs. It should be noted that due to the decrease in the zeta potential after interaction of DOTAP with PLA, the surface of the LNPs in the water was insufficiently charged to limit the effects of aggregation. To avoid this, it would be necessary to add PEG₂₀₀₀ chains on the surface, which could be easily achieved by inserting a little DSPE-PEG₂₀₀₀ in the liposome bilayer. In this condition, the adhesion of the bilayer to the polymer surface should be checked. Whatever the method of preparation adopted, we observed that the yield of LNP was low, as well as the rate of encapsulation of the two substances. In particular for beta-lapachone, it was about 60% of that of Xiao Wu in her lipid vesicles (Wu, 2018). How could we improve these yields?

Firstly, we could revise the vesicle-to-particle ratio (A_v/A_p). Decreasing this ratio could allow limiting PS loss. Secondly, waste of beta-lapachone could be avoided by decreasing the initial proportion (10%) of beta-lapachone used for nanoparticle preparation. Thirdly, during the nanoprecipitation process we observed rapid precipitation of the drug in excess. It is possible that diffusion of b-lap with acetone in water also occurred. There are several preparation

methods for LNPs as detailed in Chapter I. At first, we wanted to avoid using a non-miscible-to-water solvent. However, if another of the multi-step methods could prove effective for increasing the encapsulation rate, or at least increase the encapsulation efficiency, we might reconsider our strategy.

The one-step method could also be tried. In this case, the polymer could be neutral, with an anionic lipid monolayer adsorbed to its surface and a cationic bilayer on top of it.

Another encountered problem was the aggregation of the LNPs in DPBS and in the cell culture medium. This aggregation could be avoided by PEGylation. Finally, specific targeting of RB cells could be achieved by grafting of saccharide moieties to PEG ends.

Cytotoxicity and phototoxicity of the drug and PS-loaded liponanoparticles

Even if LNPs yield and encapsulation efficiency were low, we have been able to prepare liponanoparticles containing PS and beta-lapachone. We studied the effects of excipients, drug and photosensitizers, but also intermediate (β -lap NPs) and complete (PS-LNP and PS/ β -lap-LNP) systems on retinoblastoma cells. The number of experiments was particularly high because both the cytotoxicity and the phototoxicity of these systems had to be tested. The experiments showed the superiority of PDT treatment over chemotherapeutic treatment. The number of systems and controls made it possible to compare the effect of beta-lapachone on the phototoxicity of the two PSs studied. It was possible to highlight for each of them an effect of the reduction of the IC_{50} .

We have been able to show that the IC_{50} of LNPs was lower than those of β -lap and the PS used separately (more for m-THPC than for m-THPP). We tried to determine if there was a synergy of effects in the conditions where we combined the two molecules, by applying Chou's theorem (Chou, 2006). Unfortunately for various reasons that we have listed in Chapter IV, it was not possible to correctly characterize the combined effect of m-THPP and β -lap. The simulation showed an antagonism that did not seem realistic. For m-THPC and β -lap, on the other hand, a certain additivity of the effects could be highlighted. The conditions of the synergy study, however, appeared to be inappropriate: the combination of drugs should be tested by repeating the manipulations with more points at constant dose ratios, but also by testing different doses

of the two substances (non-constant dose ratio). Results could be compared to those obtained when one of the two substances administered is free.

Conclusion and perspectives

Conclusion and perspectives

Photodynamic therapy is a new and promising therapeutic approach for treating several types of cancers. It is considered, in particular, for the treatment of retinoblastoma (RB), a rare malignancy of the retina in childhood, thanks to its advantages over available conventional therapies.

The main objective of this work was to design a nanocarrier for the dual treatment of retinoblastoma by chemo/photodynamic therapy.

Our choice was oriented towards liponanoparticles which have a characteristic core-shell structure. We have successfully prepared biodegradable LNPs with PLA NPs and POPC/DOTAP vesicles. The ratio of the lipids and the mixing conditions of particles were varied, and the obtained suspensions were characterized by DLS, cryoTEM and confocal microscopy which showed that the optimal formulation was that obtained from the mixture of DOPC/DOTAP 75/25 vesicles and PLA NPs under 6 min of sonication and 1h of incubation. Unfortunately, these LNPs showed a tendency to aggregate in the cell culture medium. However, we have demonstrated that the destabilization was partial as we still observed the colocalization of lipid and NPs. We have also shown evidence of the adsorption of the LNPs onto retinoblastoma cells surface and their internalization within few hours. The same observation was made by encapsulating a photosensitizer in LNPs. The PS accumulated in the cells. We have then assessed the interaction of the liposomes and a PLA film leading to the formation of a lipid bilayer by performing AFM and QCM-D analysis. Our results showed that a rapid breakage of the cationic vesicles occurred once the vesicles are in contact with the PLA surface and the formation of a lipid bilayer onto the polymer surface.

We have successfully co-encapsulated a cytotoxic drug, β -lapachone in LNP's core, with two types of photosensitizers (m-THPP and m-THPC) incorporated in the lipid shell. Their drug loading and encapsulation efficiency were relatively low. This was due to the methods employed for their preparation. Finally, we have assessed the cyto/phototoxicity of each drug loaded alone in LNPs and that of co-loaded drugs. The aim of this combinatory therapy was to achieve a synergistic effect between both molecules. With this strategy PDT could help destroying the tumor while the cytotoxic agent would kill forming metastatic cells. We have

demonstrated an improved antitumor activity of the combined drugs loaded into LNPs compared to single PDT or chemotherapy.

We have shown that LNPs are suitable carriers for the dual treatment of retinoblastoma. Glycoconjugated photosensitizers could be incorporated in LNPs' shell in order to expose their sugar moieties towards the water medium for specific targeting of retinoblastoma cells. The surface of LNPs could be further functionalized with ligands to enhance their specific accumulation in the tumor.

Below are listed some investigations that could allow to improve our system and get a better understanding of the mechanisms involved in the effectiveness of the combined drugs.

- 1) It was reported that lipid-PEG conjugates can stabilize LNPs from aggregation in high ionic conditions. They could be added to our formulation for further study of the stability in the cell culture medium.
- 2) Other methods of preparation should be considered to improve the encapsulation yield of β -Lap in the polymeric nanoparticles. The emulsification-solvent evaporation method is an option although it implies the use of a non-water miscible solvent. Another solution might be to prepare loaded nanoparticles by a one-step modified nanoprecipitation method in which phospholipids would be used as stabilizer to form a barrier preventing the drug leakage during the nanoprecipitation process previously observed in our work. The lipid added should be cautiously chosen to allow the formation of a lipid bilayer on those NPs. One could also consider to change the polymer.
- 3) The proportion of lipid vesicles used to prepare LNPs was too high. Therefore, a significant amount of PSs were lost, which explains their low encapsulation yield in LNP. We have used an A_v/A_p ratio of 14. Lower ratios should be tried to determine the optimal conditions allowing good coverage of NPs by a lipid bilayer, for increasing the encapsulation efficiency and drug loading of the PSs.
- 4) In further experiments, it would also be useful to perform drug release experiments, in order to determine the kinetics of β -lap and photosensitizers release from the LNPs.
- 5) Following our confirmation of NQO1 expression in Y79 cells, it would be interesting to check the level of this expression. The share of NQO1 in the mechanism of action of

beta-lapachone could be deduced from cytotoxicity experiments following pre-treatment of cells by dicoumarol, a NQO1 inhibitor (Pink *et al.*, 2000; Planchon *et al.*, 2001).

- 6) Knowing this information, it would be easier to plan new experiments taking into account the properties of beta-lapachone for determining the synergy/additivity/antagonism of the drug and photosensitizers.
- 7) Finally, it is noteworthy that only one illumination protocol was followed (the protocol set at the Institut Curie). Another sequential treatment/illumination protocol could be used, taking into account the residence time of the LNPs in cells, drug and PS release, the time necessary for PDT-induced NQO1 expression (if relevant for Y79 cells) or for beta-lapachone-induced apoptosis mechanism.

References

References

- Ábrahám, Á., Katona, M., Kasza, G., Kiss, É., **2017**. Amphiphilic polymer layer – Model cell membrane interaction studied by QCM and AFM. *Eur. Polym. J.* 93, 212–221. doi: 10.1016/j.eurpolymj.2017.05.047
- Abrahamse, H., Hamblin, M.R., **2016**. New photosensitizers for photodynamic therapy. *Biochem. J.* 473, 347–64. doi: 10.1042/BJ20150942
- Ackroyd, R., Kelty, C., Brown, N., Reed, M., **2001**. The History of Photodetection and Photodynamic Therapy. *Photochem. Photobiol.* 74, 656–669. doi: 10.1562/0031-8655(2001)074<0656:THOPAP>2.0.CO;2
- Aerts, I., Leuraud, P., Blais, J., Pouliquen, A.L., Maillard, P., Houdayer, C., Couturier, J., Sastre-Garau, X., Grierson, D., Doz, F., Poupon, M.F., **2010**. In vivo efficacy of photodynamic therapy in three new xenograft models of human retinoblastoma. *Photodiagnosis Photodyn. Ther.* 7, 275–283. doi: 10.1016/j.pdpdt.2010.09.003
- Aerts, I., Lumbroso-Le Rouic, L., Gauthier-Villars, M., Brisse, H., Doz, F., Desjardins, L., **2006**. Retinoblastoma. *Orphanet J. Rare Dis.* 1, 1–11. doi: 10.1186/1750-1172-1-31
- Agostinis, P., Berg, K., Cengel, K.A., Foster, T.H., Girotti, A.W., Gollnick, S.O., Hahn, S.M., Hamblin, M.R., Juzeniene, A., Kessel, D., Korbelik, M., Moan, J., Mroz, P., Nowis, D., Piette, J., Wilson, B.C., Golab, J., **2011**. Photodynamic therapy of cancer: An update. *CA. Cancer J. Clin.* 61, 250–281. doi: 10.3322/caac.20114
- Ahmed, S., Madathingal, R.R., Wunder, S.L., Chen, Y., Bothun, G., **2011**. Hydration repulsion effects on the formation of supported lipid bilayers. *Soft Matter* 7, 1936-1947. doi: 10.1039/c0sm01045f
- Ahmed, S., Savarala, S., Chen, Y., Bothun, G., Wunder, S.L., **2012**. Formation of lipid sheaths around nanoparticle-supported lipid bilayers. *Small* 8, 1740–1751. doi: 10.1002/sml.201101833
- Ali, H., Van Lier, J.E., **1999**. Metal complexes as photo- and radiosensitizers. *Chem. Rev.* 99, 2379–2450. doi: 10.1021/cr980439y
- Allison, R.R., Downie, G.H., Cuenca, R., Hu, X.H., Childs, C.J.H., Sibata, C.H., **2004**. Photosensitizers in clinical PDT. *Photodiagnosis Photodyn. Ther.* 1, 27–42. doi: 10.1016/S1572-1000(04)00007-9

- Allison, R.R., Moghissi, K., **2013**. Photodynamic Therapy (PDT): PDT Mechanisms. *Clin. Endosc.* 46, 24–9. doi: 10.5946/ce.2013.46.1.24
- Almoustafa, H.A., Alshawsh, M.A., Chik, Z., **2017**. Technical aspects of preparing PEG-PLGA nanoparticles as carrier for chemotherapeutic agents by nanoprecipitation method. *Int. J. Pharm.* 533, 275–284. doi: 10.1016/j.ijpharm.2017.09.054
- Ambike, A., Rosilio, V., Stella, B., Lepe tre-Mouelhi, S., Couvreur, P., **2011**. Interaction of self-assembled squalenoyl gemcitabine nanoparticles with phospholipid-cholesterol monolayers mimicking a biomembrane. *Langmuir* 27, 4891–4899. doi: 10.1021/la200002d
- Anderson, J.M., Shive, M.S., **1997**. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv. Drug Deliv. Rev.* 28, 5–24. doi: 10.1016/S0169-409X(97)00048-3
- Anderson, T.H., Min, Y., Weirich, K.L., Zeng, H., Fygenson, D., Israelachvili, J.N., **2009**. Formation of supported bilayers on silica substrates. *Langmuir* 25, 6997–7005. doi: 10.1021/la900181c
- Andersson, J., K per, I., **2016**. Tethered and polymer supported bilayer lipid membranes: Structure and function. *Membranes (Basel)*. 6. doi: 10.3390/membranes6020030
- Andrejevic Blant, S., Ballini, J.-P., van den Bergh, H., Fontolliet, C., Wagn  res, G., Monnier, P., **2000**. Time-dependent Biodistribution of Tetra(m-hydroxyphenyl)chlorin and Benzoporphyrin Derivative Monoacid Ring A in the Hamster Model: Comparative Fluorescence Microscopy Study. *Photochem. Photobiol.* 71, 333. doi: 10.1562/0031-8655(2000)071<0333:TDBOTM>2.0.CO;2
- Ashcroft, N., Mermin, D., 1976. Solid state Physics. Holt-Saunders international eds, New York: Rinehart and Winston, 1976, 826p
- Ashley, C.E., Carnes, E.C., Phillips, G.K., Padilla, D., Durfee, P.N., Brown, P. a, Hanna, T.N., Liu, J., Phillips, B., Carter, M.B., Carroll, N.J., Jiang, X., Dunphy, D.R., Willman, C.L., Petsev, D.N., Evans, D.G., Parikh, A.N., Chackerian, B., Wharton, W., Peabody, D.S., Brinker, C.J., **2011**. The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. *Nat. Mater.* 10, 389–397. doi: 10.1038/nmat3042
- Asthana, S., Jaiswal, A.K., Gupta, P.K., Dube, A., Chourasia, M.K., **2015**. Th-1 biased immunomodulation and synergistic antileishmanial activity of stable cationic lipid-polymer hybrid nanoparticle: Biodistribution and toxicity assessment of encapsulated amphotericin B. *Eur. J. Pharm. Biopharm.* 89, 62–73. doi: 10.1016/j.ejpb.2014.11.019
- Avci, P., Gupta, A., Sadasivam, M., Vecchio, D., Pam, Z., Pam, N., Hamblin, M.R., **2013**. Low-level laser (light) therapy (LLLT) in skin: stimulating, healing, restoring, *in: Seminars in Cutaneous Medicine and Surgery*. p. 41. doi: 10.1016/j.biotechadv.2011.08.021.Secreted

- Bae, B.C., Na, K., **2012**. Development of polymeric cargo for delivery of photosensitizer in photodynamic therapy. *Int. J. Photoenergy* 2012, 1-14. doi: 10.1155/2012/431975
- Ballut, S., Makky, A., Chauvin, B., Michel, J.-P.P., Kasselouri, A., Maillard, P., Rosilio, V., **2012**. Tumor targeting in photodynamic therapy. from glycoconjugated photosensitizers to glycodendrimeric one. Concept, design and properties. *Org. Biomol. Chem.* 10, 4485–95. doi: 10.1039/c2ob25181g
- Bangham, A.D., Standish, M.M., Watkins, J.C., **1965**. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *J. Mol. Biol.* 13, 238–252. doi: 10.1016/S0022-2836(65)80093-6
- Bardelle, C., Furie, B.C., Furie, B.C., Gilbert, G.E., **1993**. Membrane binding kinetics of factor VIII indicate a complex binding process. *J. Biol. Chem.* 268, 8815–8824.
- Bathfield, M., Daviot, D., D'Agosto, F., Spitz, R., Ladavière, C., Charreyre, M.-T., Delair, T., **2008**. Synthesis of Lipid- α -End-Functionalized Chains by RAFT Polymerization . Stabilization of Lipid / Polymer Particle Assemblies Synthesis of Lipid-R-End-Functionalized Chains by RAFT Polymerization . Stabilization of Lipid / Polymer Particle Assemblies. *Macromolecules* 41, 8346–8353. doi: 10.1021/ma801567c
- Bayerl, T.M., Bloom, M., **1990**. Physical properties of single phospholipid bilayers adsorbed to micro glass beads. A new vesicular model system studied by ²H-nuclear magnetic resonance. *Biophys. J.* 58, 357–62. doi: 10.1016/S0006-3495(90)82382-1
- Benov, L., **2015**. Photodynamic therapy: Current status and future directions. *Med. Princ. Pract.* 24, 14–28. doi: 10.1159/000362416
- Berenbaum, M.C., Akande, S.L., Bonnett, R., Kaur, H., Ioannou, S., White, R.D., Winfield, U.J., **1986**. Meso-tetra(hydroxyphenyl)porphyrins, a new class of potent tumour photosensitisers with favourable selectivity. *Br. J. Cancer* 54, 717–725. doi: 10.1038/bjc.1986.232
- Berenbaum, M.C., Bonnett, R., Chevretton, E.B., Akande-Adebakin, S.L., Ruston, M., **1993**. Selectivity of meso-tetra(hydroxyphenyl)porphyrins and chlorins and of photofrin II in causing photodamage in tumour, skin, muscle and bladder. The concept of cost-benefit in analysing the results. *Lasers Med. Sci.* 8, 235–243. doi: 10.1007/BF02547845
- Bershteyna, A., Chaparroa, J., Yaua, R., Kimb, M., Reinherzb, E., Ferreira-Moitac, L., Irvine, D.J., **2009**. Polymer-supported lipid shells, onions, and flowers. *R. Soc. Chem.* 4, 1787–1791. doi: 10.1039/b804933e.Polymer-supported
- Betbeder, D., Sperandio, S., Latapie, J.P., De Nadai, J., Etienne, A., Zajac, J.M., Frances, B., **2000**. Biovector(TM) nanoparticles improve antinociceptive efficacy of nasal morphine. *Pharm. Res.* 17, 743–748. doi: 10.1023/A:1007594602449

- Bijnsdorp, I. V., Giovannetti, E., Peters, G.J., **2011**. Analysis of drug interactions. *Methods Mol. Biol.* 731, 421–34. doi: 10.1007/978-1-61779-080-5_34
- Blachon, F., Harb, F., Munteanu, B., Piednoir, A., Fulcrand, R., Charitat, T., Fragneto, G., Pierre-Louis, O., Tinland, B., Rieu, J.P., **2017**. Nanoroughness Strongly Impacts Lipid Mobility in Supported Membranes. *Langmuir* 33, 2444–2453. doi: 10.1021/acs.Langmuir.6b03276
- Blakeston, A.C., Alswieleh, A.M., Heath, G.R., Roth, J.S., Bao, P., Cheng, N., Armes, S.P., Leggett, G.J., Bushby, R.J., Evans, S.D., **2015**. New poly(amino acid methacrylate) brush supports the formation of well-defined lipid membranes. *Langmuir* 31, 3668–3677. doi: 10.1021/la504163s
- Blanco, E., Bey, E.A., Dong, Y., Weinberg, B.D., Sutton, D.M., Boothman, D.A., Gao, J., **2007**. Beta-lapachone-containing PEG-PLA polymer micelles as novel nanotherapeutics against NQO1-overexpressing tumor cells. *J. Control. Release* 122, 365–74. doi: 10.1016/j.jconrel.2007.04.014
- Bobo, D., Robinson, K.J., Islam, J., Thurecht, K.J., Corrie, S.R., **2016**. Nanoparticle-Based Medicines: A Review of FDA-Approved Materials and Clinical Trials to Date. *Pharm. Res.* 33, 2373–2387. doi: 10.1007/s11095-016-1958-5
- Bombelli, C., Bordi, F., Ferro, S., Giansanti, L., Jori, G., Mancini, G., Mazzuca, C., Monti, D., Ricchelli, F., Sennato, S., Venanzi, M., **2008**. New Cationic Liposomes as Vehicles of m-Tetrahydroxyphenylchlorin in Photodynamic Therapy of Infectious Diseases. *Mol. Pharm.* 5, 672–679. doi: 10.1021/mp800037d
- Bonnett, R., **1995**. Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy. *Chem. Soc. Rev.* 24, 19–33. doi: 10.1039/CS9952400019
- Bonnett, R., Charlesworth, P., Djelal, B.D., Foley, S., Mcgarvey, D.J., Truscott, T.G., **1999**. Photophysical properties of 5,10,15,20-tetrakis(m-hydroxyphenyl)- porphyrin (m-THPP), 5,10,15,20-tetrakis(m-hydroxyphenyl)chlorin (m-THPC) and 5,10,15,20-tetrakis(m-hydroxyphenyl)bacterio- chlorin (m-THPBC): a comparative study. *J. Chem. Soc. Perkin Trans. 2*, 325–328. doi: 10.1039/a805328f.
- Bonnett, R., White, R.D., Winfield, U.J., Berenbaum, M.C., **1989**. Hydroporphyrins of the meso-tetra(hydroxyphenyl)porphyrin series as tumour photosensitizers. *Biochem. J.* 261, 277–280. doi: 10.1042/bj2610277
- Bose, R.J., Arai, Y., Chan Ahn, J., Park, H., Lee, S.-H., **2015**. Influence of cationic lipid concentration on properties of lipid–polymer hybrid nanospheres for gene delivery. *Int. J. Nanomedicine* 10, 5367–5382. doi: 10.2147/IJN.S87120
- Bose, R.J., Lee, S.H., Park, H., **2016**. Lipid polymer hybrid nanospheres encapsulating antiproliferative agents for stent applications. *J. Ind. Eng. Chem.* 36, 284–292. doi:

10.1016/j.jiec.2016.02.015

- Bourré, L., Rousset, N., Thibaut, S., Eléouet, S., Lajat, Y., Patrice, T., **2002**. PDT effects of m-THPC and ALA, phototoxicity and apoptosis. *Apoptosis* 7, 221–30. doi: 10.1023/A:1015391415150
- Boury, F., Gulik, A., Dedieu, J.C., Proust, J.E., **1994**. First-Order Transition in a Polymer Monolayer: Structural Analysis by Transmission Electronic Microscopy and Atomic Force Microscopy. *Langmuir* 10, 1654–1656. doi: 10.1021/la00018a006
- Bozzini, G., Colin, P., Betrouni, N., Nevoux, P., Ouzzane, A., Puech, P., Villers, A., Mordon, S., **2012**. Photodynamic therapy in urology: What can we do now and where are we heading? *Photodiagnosis Photodyn. Ther.* 9, 261–273. doi: 10.1016/j.pdpdt.2012.01.005
- Brancaleon, L., Moseley, H., **2002**. Laser and Non-laser Light Sources for Photodynamic Therapy. *Lasers Med. Sci.* 17, 173–186. doi: 10.1007/s101030200027
- Brasseur, N., Brault, D., Couvreur, P., **1991**. Adsorption of hematoporphyrin onto polyalkylcyanoacrylate nanoparticles: carrier capacity and drug release. *Int. J. Pharm.* 70, 129–135. doi: 10.1016/0378-5173(91)90172-K
- Breskey, J.D., Lacey, S.E., Vesper, B.J., Paradise, W.A., Radosevich, J.A., Colvard, M.D., **2013**. Photodynamic Therapy: Occupational Hazards and Preventative Recommendations for Clinical Administration by Healthcare Providers. *Photomed. Laser Surg.* 31, 398–407. doi: 10.1089/pho.2013.3496
- Brevet, D., Gary-Bobo, M., Raehm, L., Richeter, S., Hocine, O., Amro, K., Loock, B., Couleaud, P., Frochot, C., Morère, A., Maillard, P., Garcia, M., Durand, J.O., **2009**. Mannose-targeted mesoporous silica nanoparticles for photodynamic therapy. *Chem. Commun.* 1475–1477. doi: 10.1039/b900427k
- Brian, A.A., McConnell, H.M., **1984**. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. U. S. A.* 81, 6159–6163.
- Bucak, S., Jones, D.A., Laibinis, P.E., Hatton, T.A., **2003**. Protein separations using colloidal magnetic nanoparticles. *Biotechnol. Prog.* 19, 477–484. doi: 10.1021/bp0200853.
- Budhian, A., Siegel, S.J., Winey, K.I., **2007**. Haloperidol-loaded PLGA nanoparticles: Systematic study of particle size and drug content. *Int. J. Pharm.* 336, 367–375. doi: 10.1016/j.ijpharm.2006.11.061
- Bulbake, U., Doppalapudi, S., Kommineni, N., Khan, W., **2017**. Liposomal formulations in clinical use: An updated review. *Pharmaceutics* 9, 1–33. doi: 10.3390/Pharmaceutics9020012
- Buranda, T., Huang, J., Ramarao, G. V., Ista, L.K., Larson, R.S., Ward, T.L., Sklar, L. a, Lopez,

- G.P., **2003**. Biomimetic Molecular Assemblies on Glass and Mesoporous Silica Microbeads for Biotechnology. *Langmuir* 19, 1654–1663. doi: 10.1021/la026405
- Buzzá, H.H., Silva, L.V., Moriyama, L.T., Bagnato, V.S., Kurachi, C., **2014**. Evaluation of vascular effect of Photodynamic Therapy in chorioallantoic membrane using different photosensitizers. *J. Photochem. Photobiol. B Biol.* 138, 1–7. doi: 10.1016/j.jphotobiol.2014.04.023
- Calixto, G., Bernegossi, J., de Freitas, L., Fontana, C., Chorilli, M., **2016**. Nanotechnology-Based Drug Delivery Systems for Photodynamic Therapy of Cancer: A Review. *Molecules* 21, 342. doi: 10.3390/molecules21030342
- Cao, S., Jiang, Y., Zhang, H., Kondza, N., Woodrow, K.A., **2018**. Core-shell nanoparticles for targeted and combination antiretroviral activity in gut-homing T cells. *Nanomedicine Nanotechnology, Biol. Med.* 14, 2143–2153. doi: 10.1016/j.nano.2018.06.005
- Carmona-Ribeiro, A.M., De Moraes Lessa, M., **1999**. Interactions between bilayer membranes and latex. *Colloids Surfaces A Physicochem. Eng. Asp.* 153, 355–361. doi: 10.1016/S0927-7757(98)00532-9
- Carmona-Ribeiro, A.M., Midmore, B.R., **1992**. Synthetic Bilayer Adsorption onto Polystyrene Microspheres. *Langmuir* 8, 801–806. doi: 10.1021/la00039a013
- Cauda, V., Engelke, H., Sauer, A., Arcizet, D., Brückle, C., Rüdler, J., Bein, T., **2010**. Colchicine-loaded lipid bilayer-coated 50 nm mesoporous nanoparticles efficiently induce microtubule depolymerization upon cell uptake. *Nano Lett.* 10, 2484–2492. doi: 10.1021/nl100991w
- Cavalcanti, I.M.F., Mendona, E.A.M., Lira, M.C.B., Honrato, S.B., Camara, C.A., Amorim, R.V.S., Filho, J.M., Rabello, M.M., Hernandez, M.Z., Ayala, A.P., Santos-Magalhães, N.S., **2011**. The encapsulation of β -lapachone in 2-hydroxypropyl- β -cyclodextrin inclusion complex into liposomes: A physicochemical evaluation and molecular modeling approach. *Eur. J. Pharm. Sci.* 44, 332–340. doi: 10.1016/j.ejps.2011.08.011
- Cerman, E., Çekiç, O., **2015**. Clinical use of photodynamic therapy in ocular tumors. *Surv. Ophthalmol.* 60, 557–574. doi: 10.1016/j.survophthal.2015.05.004
- Chan, J.M., Zhang, L., Yuet, K.P., Liao, G., Rhee, J.W., Langer, R., Farokhzad, O.C., **2009**. PLGA-lecithin-PEG core-shell nanoparticles for controlled drug delivery. *Biomaterials* 30, 1627–1634. doi: 10.1016/j.Biomaterials.2008.12.013
- Chatterjee, D.K., Fong, L.S., Zhang, Y., **2008**. Nanoparticles in photodynamic therapy: An emerging paradigm. *Adv. Drug Deliv. Rev.* 60, 1627–1637. doi: 10.1016/j.addr.2008.08.003
- Chen, B., Pogue, B.W., Hasan, T., **2005**. Liposomal delivery of photosensitising agents. *Expert*

- Opin. Drug Deliv.* 2, 477–487. doi: 10.1517/17425247.2.3.477
- Chen, S., Poyer, F., Garcia, G., Fiorini-Debuisschert, C., Rosilio, V., Maillard, P., **2018**. Amphiphilic Glycoconjugated Porphyrin Heterodimers as Two-Photon Excitable Photosensitizers: Design, Synthesis, Photophysical and Photobiological Studies. *ChemistrySelect* 3, 1887–1897. doi: 10.1002/slct.201703013
- Cheng, B., Qian, L., Qian, H.J., Lu, Z.Y., Cui, S., **2017**. Effects of stereo-regularity on the single-chain mechanics of polylactic acid and its implications on the physical properties of bulk materials. *Nanoscale* 9, 14312–14316. doi: 10.1039/c7nr06483g
- Cheow, W.S., Chang, M.W., Hadinoto, K., **2011**. The roles of lipid in anti-biofilm efficacy of lipid-polymer hybrid nanoparticles encapsulating antibiotics. *Colloids Surfaces A Physicochem. Eng. Asp.* 389, 158–165. doi: 10.1016/j.colsurfa.2011.08.035
- Cheow, W.S., Hadinoto, K., **2011**. Factors affecting drug encapsulation and stability of lipid-polymer hybrid nanoparticles. *Colloids Surfaces B Biointerfaces* 85, 214–220. doi: 10.1016/j.colsurfb.2011.02.033
- Cho, K., Wang, X., Nie, S., Chen, Z., Shin, D.M., **2008**. Therapeutic nanoparticles for drug delivery in cancer. *Clin. Cancer Res.* 14, 1310–1316. doi: 10.1158/1078-0432.CCR-07-1441
- Cho, N.J., Frank, C.W., **2010**. Fabrication of a planar zwitterionic lipid bilayer on titanium oxide. *Langmuir* 26, 15706–15710. doi: 10.1021/la101523f
- Cho, N.J., Hwang, L.Y., Solandt, J.J.R., Frank, C.W., **2013**. Comparison of extruded and sonicated vesicles for planar bilayer self-assembly. *Materials (Basel)*. 6, 3294–3308. doi: 10.3390/ma6083294
- Chorny, M., Fishbein, I., Danenberg, H.D., Golomb, G., **2002**. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: Effect of formulation variables on size, drug recovery and release kinetics. *J. Control. Release* 83, 389–400. doi: 10.1016/S0168-3659(02)00211-0
- Chou, T.-C., Talalay, P., **1984**. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22, 27–55. doi: 10.1016/0065-2571(84)90007-4
- Chou, T., **2006**. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58, 621–81. doi: 10.1124/pr.58.3.10
- Chou, T.C., **2010**. Drug combination studies and their synergy quantification using the chou-talalay method. *Cancer Res.* 70, 440–446. doi: 10.1158/0008-5472.CAN-09-1947

- Clarke, R.J., Apell, H.J., 1989. A stopped-flow kinetic study of the interaction of potential-sensitive oxonol dyes with lipid vesicles. *Biophys. Chem.* 34, 225–37.
- Colombo, S., Cun, D., Remaut, K., Bunker, M., Zhang, J., Martin-Bertelsen, B., Yagmur, A., Braeckmans, K., Nielsen, H.M., Foged, C., **2015**. Mechanistic profiling of the siRNA delivery dynamics of lipid-polymer hybrid nanoparticles. *J. Control. Release* 201, 22–31. doi: 10.1016/j.jconrel.2014.12.026
- Conway, J.W., Madwar, C., Edwardson, T.G., McLaughlin, C.K., Fahkoury, J., Lennox, R.B., Sleiman, H.F., Conway, J.W., Madwar, C., Edwardson, T.G., McLaughlin, C.K., Fahkoury, J., Lennox, R.B., Sleiman, H.F., **2014**. Dynamic Behavior of DNA Cages Anchored on Spherically Supported Lipid Bilayers. *J. Am. Chem. Soc.* 136, 1–15. doi: 10.1021/ja506095n
- Copp, J.A., Fang, R.H., Luk, B.T., Hu, C.-M.J., Gao, W., Zhang, K., Zhang, L., **2014**. Clearance of pathological antibodies using biomimetic nanoparticles. *Proc. Natl. Acad. Sci. U. S. A.* 111, 13481–6. doi: 10.1073/pnas.1412420111
- Costa, M.P., Feitosa, A.C.S.S., Oliveira, F.C.E.E., Cavalcanti, B.C., Da Silva, E.N., Dias, G.G., Sales, F.A.M.M., Sousa, B.L., Barroso-Neto, I.L., Pessoa, C., Caetano, E.W.S.S., Di Fiore, S., Fischer, R., Ladeira, L.O., Freire, V.N., **2016**. Controlled Release of Nor- β -lapachone by PLGA Microparticles: A Strategy for Improving Cytotoxicity against Prostate Cancer Cells. *Molecules* 21. doi: 10.3390/molecules21070873
- Couleaud, P., Morosini, V., Frochot, C., Richeter, S., Raehm, L., Durand, J.-O., **2010**. Silica-based nanoparticles for photodynamic therapy applications. *Nanoscale* 2, 1083. doi: 10.1039/c0nr00096e
- Cowell, J.K., **1991**. The genetics of retinoblastoma. *Br. J. Cancer* 63, 333–336. doi: 10.1038/bjc.1991.79
- da Cunha, M.M.L., Trepout, S., Messaoudi, C., Wu, T. Di, Ortega, R., Guerquin-Kern, J.L., Marco, S., **2016**. Overview of chemical imaging methods to address biological questions. *Micron* 84, 23–36. doi: 10.1016/j.micron.2016.02.005
- Da, M., Vicente, G.H., **1996**. Porphyrins and the photodynamic therapy of cancer, *Revista portuguesa de química*.
- Dąbrowski, J.M., Pucelik, B., Regiel-Futyra, A., Brindell, M., Mazuryk, O., Kyzioł, A., Stochel, G., Macyk, W., Arnaut, L.G., **2016**. Engineering of relevant photodynamic processes through structural modifications of metallotetrapyrrolic photosensitizers. *Coord. Chem. Rev.* 325, 67–101. doi: 10.1016/j.ccr.2016.06.007
- Daghildjian, K., **2013**. Etude des mécanismes d'interaction entre des porphyrines dendrimériques et des membranes de cellules tumorales : validation d'un modèle artificiel par une approche cellulaire. Université Paris sud. p. 185.

- Dai, T., Fuchs, B.B., Coleman, J.J., Prates, R.A., Astrakas, C., St. Denis, T.G., Ribeiro, M.S., Mylonakis, E., Hamblin, M.R., Tegós, G.P., **2012**. Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. *Front. Microbiol.* 3, 1–16. doi: 10.3389/fmicb.2012.00120
- Daniell, M.D., Hill, J.S., **1991**. A history of photodynamic therapy. *Aust. N. Z. J. Surg.* 61, 340–348. doi: 10.1111/j.1445-2197.1991.tb00230.x
- Das, M., Sahoo, S.K., **2012**. Folate decorated dual drug loaded nanoparticle: Role of curcumin in enhancing therapeutic potential of nutlin-3a by reversing multidrug resistance. *PLoS One* 7. doi: 10.1371/journal.pone.0032920
- Dave, V., Yadav, R.B., Kushwaha, K., Yadav, S., Sharma, S., Agrawal, U., **2017**. Lipid-polymer hybrid nanoparticles: Development & statistical optimization of norfloxacin for topical drug delivery system. *Bioact. Mater.* 2, 269–280. doi: 10.1016/j.bioactmat.2017.07.002
- De Cuyper, M., Joniau, M., **1991**. Mechanistic aspects of the adsorption of phospholipids onto lauric acid stabilized magnetite nanocolloids. *Langmuir* 7, 647–652. doi: 10.1021/la00052a010
- De Jong, W.H., Borm, P.J. a, **2008**. Drug delivery and nanoparticles: applications and hazards. *Int. J. Nanomedicine* 3, 133–149. doi: 10.2147/IJN.S596
- De Miguel, I., Imbertie, L., Rieumajou, V., Major, M., Kravtsoff, R., Betbeder, D., **2000**. Proofs of the structure of lipid coated nanoparticles (SMBV^(TM)) used as drug carriers. *Pharm. Res.* 17, 817–824. doi: 10.1023/A:1007504124603
- Deda, D.K., Uchoa, A.F., Caritá, E., Baptista, M.S., Toma, H.E., Araki, K., **2009**. A new micro/nanoencapsulated porphyrin formulation for PDT treatment. *Int. J. Pharm.* 376, 76–83. doi: 10.1016/j.ijpharm.2009.04.024
- Dehaini, D., Fang, R.H., Luk, B.T., Pang, Z., Hu, C.M.J., Kroll, A. V., Yu, C.L., Gao, W., Zhang, L., **2016**. Ultra-small lipid-polymer hybrid nanoparticles for tumor-penetrating drug delivery. *Nanoscale* 8, 14411–14419. doi: 10.1039/c6nr04091h
- Delhiwala, K.S., Vadakkal, I.P., Mulay, K., Khetan, V., Wick, M.R., **2016**. Retinoblastoma: An update. *Semin. Diagn. Pathol.* 33, 133–140. doi: 10.1053/j.semdp.2015.10.007
- Denis, T.G. St., Hamblin, M.R., Huang, Y.-Y., Hamblin, M.R., **2013**. Cyclic Tetrapyrroles in Photodynamic Therapy in The Chemistry of Porphyrins and Related Compounds in Medicine. *Handb. Porphyr. Sci.* 27, 255–301. doi: doi:10.1142/9789814407755_0016
- DeRosa, M.C., Crutchley, R.J., **2002**. Photosensitized singlet oxygen and its applications. *Coord. Chem. Rev.* 233–234, 351–371. doi: 10.1016/S0010-8545(02)00034-6

- Derycke, A.S.L., De Witte, P.A.M., **2004**. Liposomes for photodynamic therapy. *Adv. Drug Deliv. Rev.* 56, 17–30. doi: 10.1016/j.addr.2003.07.014
- DeSimone, J.M., **2016**. Co-opting Moore's law: Therapeutics, vaccines and interfacially active particles manufactured via PRINT®. *J. Control. Release* 240, 541–543. doi: 10.1016/j.jconrel.2016.07.019
- Diamanti, E., Gregurec, D., Rodríguez-Presa, M.J., Gervasi, C.A., Azzaroni, O., Moya, S.E., **2016**. High resistivity lipid bilayers assembled on polyelectrolyte multilayer cushions: An impedance study. *Langmuir* 32, 6263–6271. doi: 10.1021/acs.Langmuir.6b01191
- Diddens, H., Birngruber, R., Hasan, T., U, S.-E., Diddens, H., Birngruber, R., Hasan, T., Schmidt-Erfurth, U., Diddens, H., Birngruber, R., Hasan, T., **1997**. Photodynamic targeting of human retinoblastoma cells using covalent low-density lipoprotein conjugates. *Br. J. Cancer* 75, 54–61. doi: 10.1038/bjc.1997.9
- Dimaras, H., Corson, T.W., **2018**. Retinoblastoma, the visible CNS tumor: A review. *J. NeuroSci. Res.* 1–16. doi: 10.1002/jnr.24213
- Dimitrievski, K., Kasemo, B., **2008**. Simulations of lipid vesicle adsorption for different lipid mixtures. *Langmuir* 24, 4077–4091. doi: 10.1021/la703021u
- Ding, H., Mora, R., Gao, J., Sumer, B.D., **2011**. Characterization and optimization of mTHPP nanoparticles for photodynamic therapy of head and neck cancer. *Otolaryngol. - Head Neck Surg.* 145, 612–617. doi: 10.1177/0194599811412449
- Dolainsky, C., Köchy, T., Naumann, C., Brumm, T., Johnson, S.J., **1992**. Structure and Dynamics of Planar and Spherical Supported Phospholipid Bilayers, *in*: The Structure and Conformation of Amphiphilic Membranes. pp. 34–39. doi: 10.1007/978-3-642-84763-9_6.
- Dolmans, D.E.J.G.J., Fukumura, D., Jain, R.K., **2003**. Photodynamic therapy for cancer. *Nat. Rev. Cancer* 3, 380–7. doi: 10.1038/nrc1071
- Dombu, C.Y., Kroubi, M., Zibouche, R., Matran, R., Betbeder, D., **2010**. Characterization of endocytosis and exocytosis of cationic nanoparticles in airway epithelium cells. *Nanotechnology* 21. doi: 10.1088/0957-4484/21/35/355102
- Dougherty, T.J., **1987**. Photosensitizers: Therapy and Detection of Malignant Tumors. *Photochem. Photobiol.* 45, 879–889. doi: 10.1111/j.1751-1097.1987.tb07898.x
- Dougherty, T.J., **1983**. Hematoporphyrin As a Photosensitizer of Tumors. *Photochem. Photobiol.* 38, 377–379. doi: 10.1111/j.1751-1097.1983.tb02687.x
- Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbely, M., Moan, J., Peng, Q., **1993**. Photodynamic therapy. *Photochem. Photobiol.* 58, 895–900. doi:

10.1111/j.1751-1097.1993.tb04990.x

- Duan, R., Li, C., Wang, F., Yangi, J.C., **2017**. Polymer–lipid hybrid nanoparticles-based paclitaxel and etoposide combinations for the synergistic anticancer efficacy in osteosarcoma. *Colloids Surfaces B Biointerfaces* 159, 880–887. doi: 10.1016/j.colsurfb.2017.08.042
- Dunphy, E.B., 1964. The Story of Retinoblastoma. *Am. J. Ophthalmol.* 58, 539–552. doi: 10.1016/0002-9394(64)91368-6
- Dwivedi, P., Karumbaiah, K.M., Das, R., **2016**. Nano-size polymers via precipitation of polymer solutions, *in*: Nano-Size Polymers: Preparation, Properties, Applications. *Springer International Publishing*, Cham, pp. 251–282. doi: 10.1007/978-3-319-39715-3_9
- Dyer, M.A., **2016**. Lessons from Retinoblastoma: Implications for Cancer, Development, Evolution, and Regenerative Medicine. *Trends Mol. Med.* 22, 863–876. doi: 10.1016/j.molmed.2016.07.010
- Enlow, E.M., Luft, J.C., Napier, M.E., DeSimone, J.M., **2011**. Potent Engineered PLGA Nanoparticles by Virtue of Exceptionally High Chemotherapeutic Loadings. *Nano Lett.* 11, 808–813. doi: 10.1021/nl104117p
- Eschwège, V., Laude, I., Toti, F., Pasquali, J.L., Freyssinet, J.M., **1996**. Detection of bilayer phospholipid-binding antibodies using flow cytometry. *Clin. Exp. Immunol.* 103, 171–175. doi: 10.1046/j.1365-2249.1996.00908.x
- Eshghi, H., Sazgarnia, A., Rahimizadeh, M., Attaran, N., Bakavoli, M., Soudmand, S., **2013**. Protoporphyrin IX-gold nanoparticle conjugates as an efficient photosensitizer in cervical cancer therapy. *Photodiagnosis Photodyn. Ther.* 10, 304–312. doi: 10.1016/j.pdpdt.2013.02.003
- Fadel, M., Kassab, K., Abdel Fadeel, D., **2010**. Zinc phthalocyanine-loaded PLGA biodegradable nanoparticles for photodynamic therapy in tumor-bearing mice. *Lasers Med. Sci.* 25, 283–292. doi: 10.1007/s10103-009-0740-x
- Fales, A.M., Yuan, H., Vo-Dinh, T., **2011**. Silica-coated gold nanostars for combined surface-enhanced Raman scattering (SERS) detection and singlet-oxygen generation: A potential nanoplatform for *Theranostics*. *Langmuir* 27, 12186–12190. doi: 10.1021/la202602q
- Fang, R.H., Aryal, S., Hu, C.M.J., Zhang, L., **2010**. Quick synthesis of lipid-polymer hybrid nanoparticles with low polydispersity using a single-step sonication method. *Langmuir* 26, 16958–16962. doi: 10.1021/la103576a
- Fang, R.H., Hu, C.-M.J., Luk, B.T., Gao, W., Copp, J.A., Tai, Y., O'Connor, D.E., Zhang, L., **2014**. Cancer Cell Membrane-Coated Nanoparticles for Anticancer Vaccination and Drug

- Delivery. *Nano Lett.* 14, 2181–2188. doi: 10.1021/nl500618u
- Fattal, E., Tsapis, N., **2014**. *Nanomedicine* technology: Current achievements and new trends. *Clin. Transl. Imaging* 2, 77–87. doi: 10.1007/s40336-014-0053-3
- Ferhan, A.R., Jackman, J.A., Cho, N.J., **2017**. Investigating how vesicle size influences vesicle adsorption on titanium oxide: A competition between steric packing and shape deformation. *Phys. Chem. Chem. Phys.* 19, 2131–2139. doi: 10.1039/c6cp07930j
- Ferreira, G.C., Kadish, K.M., Smith, K.M., Guillard, R., **2013**. Handbook of Porphyrin Science (Volume 26), Handbook of Porphyrin Science. World Scientific Publishing Company. doi: 10.1142/8504-vol26
- Fingar, V.H., **1996**. Vascular effects of photodynamic therapy. *Chem. Chem. Phys.* 14, 323–8. doi: 10.1089/clm.1996.14.323
- Fingar, V.H., Kik, P.K., Haydon, P.S., Cerrito, P.B., Tseng, M., Abang, E., Wieman, T.J., **1999**. Analysis of acute vascular damage after photodynamic therapy using benzoporphyrin derivative (BPD). *Br J Cancer* 79, 1702–1708. doi: 10.1038/sj.bjc.6690271
- Francis, J.H., Habib, L.A., Abramson, D.H., **2017**. Vitreous Disease in Retinoblastoma: Clinical Findings and Treatment. *Adv. Ophthalmol. Optom.* 2, 177–195. doi: 10.1016/j.yaoo.2017.03.008
- Francis, J.H., Schaiquevich, P., Buitrago, E., Del Sole, M.J., Zapata, G., Croxatto, J.O., Marr, B.P., Brodie, S.E., Berra, A., Chantada, G.L., Abramson, D.H., **2014**. Local and systemic toxicity of intravitreal melphalan for vitreous seeding in retinoblastoma: A preclinical and clinical study. *Ophthalmology* 121, 1810–1817. doi: 10.1016/j.ophtha.2014.03.028
- Friedberg, J.S., Mick, R., Stevenson, J., Metz, J., Zhu, T., Buyske, J., Serman, D.H., Pass, H.I., Glatstein, E., Hahn, S.M., **2003**. A phase I study of Foscan-mediated photodynamic therapy and surgery in patients with mesothelioma. *Ann Thorac Surg* 75, 952–959. doi: 10.1016/S0003-4975(02)04474-0
- Fuhrmans, M., Müller, M., **2013**. Mechanisms of Vesicle Spreading on Surfaces: Coarse-Grained Simulations. *Langmuir* 29, 4335–4349. doi: 10.1021/la400119e
- Gaio, E., Scheglmann, D., Reddi, E., Moret, F., **2016**. Uptake and photo-toxicity of Foscan®, Foslip® and Fospeg® in multicellular tumor spheroids. *J. Photochem. Photobiol. B Biol.* 161, 244–252. doi: 10.1016/j.jphotobiol.2016.05.011
- Gál, D., **1992**. Effect of photosensitizers in chemical and biological processes: the MTO mechanism in photodynamic therapy. *Biochem. Biophys. Res. Commun.* 186, 1032–6.
- Gallud, A., Warther, D., Maynadier, M., Sefta, M., Poyer, F., Thomas, C.D., Rouxel, C., Mongin, O., Blanchard-Desce, M., Morère, A., Raehm, L., Maillard, P., Durand, J.O.,

- Garcia, M., Gary-Bobo, M., **2015**. Identification of MRC2 and CD209 receptors as targets for photodynamic therapy of retinoblastoma using mesoporous silica nanoparticles. *RSC Adv.* 5, 75167–75172. doi: 10.1039/C5RA14640B
- Gao, W., Fang, R.H., Thamphiwatana, S., Luk, B.T., Li, J., Angsantikul, P., Zhang, Q., Hu, C.M.J., Zhang, L., **2015**. Modulating antibacterial immunity via bacterial membrane-coated nanoparticles. *Nano Lett.* 15, 1403–1409. doi: 10.1021/nl504798g
- Garbo, G.M., **1996**. Purpurins and benzochlorins as sensitizers for photodynamic therapy. *J. Photochem. Photobiol. B Biol.* 34, 109–116. doi: 10.1016/1011-1344(96)07343-5
- García-Díaz, M., Foged, C., Nielsen, H.M., **2015**. Improved insulin loading in poly(lactic-co-glycolic) acid (PLGA) nanoparticles upon self-assembly with lipids. *Int. J. Pharm.* 482, 84–91. doi: 10.1016/j.ijpharm.2014.11.047
- Gary-Bobo, M., Mir, Y., Rouxel, C., Brevet, D., Hocine, O., Maynadier, M., Gallud, A., Da Silva, A., Mongin, O., Blanchard-Desce, M., Richeter, S., Loock, B., Maillard, P., Morère, A., Garcia, M., Raehm, L., Durand, J.O., **2012**. Multifunctionalized mesoporous silica nanoparticles for the in vitro treatment of retinoblastoma: Drug delivery, one and two-photon photodynamic therapy. *Int. J. Pharm.* 432, 99–104. doi: 10.1016/j.ijpharm.2012.04.056
- Gdowski, A., Johnson, K., Shah, S., Gryczynski, I., Vishwanatha, J., Ranjan, A., **2018**. Optimization and scale up of microfluidic nanolipomer production method for preclinical and potential clinical trials. *J. Nanobiotechnology* 16, 1–11. doi: 10.1186/s12951-018-0339-0
- Ge, Y., Li, S., Wang, S., Moore, R., **2014**. *Nanomedicine, Nanostructure Science and Technology*. Springer, New York. doi: 10.1007/978-1-4614-2140-5
- Gibot, L., Lemelle, A., Till, U., Moukarzel, B., Mingotaud, A.F., Pimienta, V., Saint-Aguet, P., Rols, M.P., Gaucher, M., Violleau, F., Chassenieux, C., Vicendo, P., **2014**. Polymeric micelles encapsulating photosensitizer: Structure/photodynamic therapy efficiency relation. *Biomacromolecules* 15, 1443–1455. doi: 10.1021/bm5000407
- Gijssens, A., Derycke, A., Missiaen, L., De Vos, D., Huwyler, J., Eberle, A., De Witte, P., **2002**. Targeting of the photocytotoxic compound A1PcS4 to Hela cells by transferrin conjugated PEG-liposomes. *Int. J. Cancer* 101, 78–85. doi: 10.1002/ijc.10548
- Gilbert, G.E., Drinkwater, D., Barter, S., Clouse, S.B., **1992**. Specificity of phosphatidylserine-containing membrane binding sites for factor VIII: Studies with model membranes supported by glass microspheres (lipospheres). *J. Biol. Chem.* 267, 15861–15868.
- Godar, D.E., **1999**. Light and death: Photons and apoptosis. *J. Investig. Dermatology Symp. Proc.* 4, 17–23. doi: 10.1038/sj.jidsp.5640175

- Gollnick, S.O., Vaughan, L., Henderson, B.W., **2002**. Generation of effective antitumor vaccines using photodynamic therapy. *Cancer Res.* 62, 1604–8.
- Gomes, A.T.P.C., Neves, M.G.P.M.S., Cavaleiro, J.A.S., **2018**. Cancer, photodynamic therapy and porphyrin-type derivatives. *An. Acad. Bras. Cienc.* 90, 993–1026. doi: 10.1590/0001-3765201820170811
- Govender, T., Stolnik, S., Garnett, M.C., Illum, L., Davis, S.S., **1999**. PLGA nanoparticles prepared by nanoprecipitation: Drug loading and release studies of a water soluble drug. *J. Control. Release* 57, 171–185. doi: 10.1016/S0168-3659(98)00116-3
- Griegel, S., Rajewsky, M.F., Ciesiolka, T., Gabius, H.J., 1989. Endogenous sugar receptor (lectin) profiles of human retinoblastoma and retinoblast cell lines analyzed by cytological markers, affinity chromatography and neoglycoprotein-targeted photolysis. *AntiCancer Res.* 9, 723–730.
- Grigoras, A.G., **2017**. Polymer-lipid hybrid systems used as carriers for insulin delivery. *Nanomedicine Nanotechnology, Biol. Med.* 13, 2425–2437. doi: 10.1016/j.nano.2017.08.005
- Gromelski, S., Saraiva, A.M., Krastev, R., Brezesinski, G., **2009**. The formation of lipid bilayers on surfaces. *Colloids Surfaces B Biointerfaces* 74, 477–483. doi: 10.1016/j.colsurfb.2009.08.006
- Grossniklaus, H.E., **2014**. Retinoblastoma. Fifty years of progress. the LXXI Edward Jackson memorial lecture. *Am. J. Ophthalmol.* 158, 875–891. doi: 10.1016/j.ajo.2014.07.025
- Guo, Y., Rogelj, S., Zhang, P., **2010**. Rose Bengal-decorated silica nanoparticles as photosensitizers for inactivation of gram-positive bacteria. *Nanotechnology* 21, 065102. doi: 10.1088/0957-4484/21/6/065102
- Gurtovenko, A.A., Patra, M., Karttunen, M., Vattulainen, I., **2004**. Cationic DMPC/DMTAP lipid bilayers: Molecular dynamics study. *Biophys. J.* 86, 3461–3472. doi: 10.1529/biophysj.103.038760
- Gutberlet, T., Steitz, R., Fragneto, G., Klösgen, B., **2004**. Phospholipid bilayer formation at a bare Si surface: A time-resolved neutron reflectivity study. *J. Phys. Condens. Matter* 16. doi: 10.1088/0953-8984/16/26/020
- Hadinoto, K., Sundaresan, A., Cheow, W.S., **2013**. Lipid-polymer hybrid nanoparticles as a new generation therapeutic delivery platform: A review. *Eur. J. Pharm. Biopharm* 85, 427–443. doi: 10.1016/j.ejpb.2013.07.002
- Hadjur, C., Wagnieres, G., Monnier, P., Bergh, H., **1997**. EPR and Spectrophotometric Studies of Free Radicals and Singlet Oxygen Generated by Irradiation of Benzoporphyrin Derivative Monoacid Ring A. *Photochem. Photobiol.* 65, 818–827. doi: 10.1111/j.1751-

1097.1997.tb01929.x

- Hamblin, M.R., Avci, P., **2015**. Applications of Nanoscience in Photomedicine. *Elsevier*. doi: 10.1016/C2013-0-23222-0
- Han, J., Park, W., Park, S.J., Na, K., **2016**. Photosensitizer-Conjugated Hyaluronic Acid-Shielded Polydopamine Nanoparticles for Targeted Photomediated Tumor Therapy. *ACS Appl. Mater. Interfaces* 8, 7739–7747. doi: 10.1021/acsami.6b01664
- Harush-Frenkel, O., Rozentur, E., Benita, S., Altschuler, Y., **2008**. Surface charge of nanoparticles determines their endocytic and transcytotic pathway in polarized MDCK cells. *Biomacromolecules* 9, 435–443. doi: 10.1021/bm700535p
- Hasan, W., Chu, K., Gullapalli, A., Dunn, S.S., Enlow, E.M., Luft, J.C., Tian, S., Napier, M.E., Pohlhaus, P.D., Rolland, J.P., DeSimone, J.M., **2012**. Delivery of Multiple siRNAs Using Lipid-Coated PLGA Nanoparticles for Treatment of Prostate Cancer. *Nano Lett.* 12, 287–292. doi: 10.1021/nl2035354
- He, C., Hu, Y., Yin, L., Tang, C., Yin, C., **2010**. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* 31, 3657–3666. doi: 10.1016/j.Biomaterials.2010.01.065
- Heerklotz, H., Seelig, J., **2002**. Application of pressure perturbation calorimetry to lipid bilayers. *Biophys. J.* 82, 1445–1452. doi: 10.1016/S0006-3495(02)75498-2
- Hertz, H., 1895. Ueber die Beruehrung elastischer Koerper (On Contact Between Elastic Bodies). *Gesammelte Werke (Collected Work)*. 1, 1895.
- Hianik, T., Haburcák, M., Lohner, K., Prenner, E., Paltauf, F., Hermetter, A., **1998**. Compressibility and density of lipid bilayers composed of polyunsaturated phospholipids and cholesterol. *Colloids Surfaces A Physicochem. Eng. Asp.* 139, 189–197. doi: 10.1016/S0927-7757(98)00280-5
- Hilkert, S.M., Farooq, A. V., Greenwald, M.J., **2017**. Retinoblastoma: A Global Perspective. *Curr. Ophthalmol. Rep.* 5, 119–127. doi: 10.1007/s40135-017-0138-z
- Hirsjärvi, S. **2008**. Preparation and characterization of poly (lactic acid) Nanoparticles for pharmaceutical use. PhD thesis, University of Helsinki, Faculty of Pharmacy, Division of Pharmaceutical Technology, Helsinki, Finland, 2008.
- Hitzman, C.J., Elmquist, W.F., Wattenberg, L.W., Wiedmann, T.S., **2006**. Development of a Respirable, Sustained Release Microcarrier for 5-Fluorouracil I: In Vitro Assessment of Liposomes, Microspheres, and Lipid Coated Nanoparticles. *J. Pharm. Sci.* 95, 1114–1126. doi: 10.1002/jps.20591
- Hofman, J.W., Carstens, M.G., Van Zeeland, F., Helwig, C., Flesch, F.M., Hennink, W.E., Van

- Nostrum, C.F., **2008**. Photocytotoxicity of mTHPC (temoporfin) loaded polymeric micelles mediated by lipase catalyzed degradation. *Pharm. Res.* 25, 2065–2073. doi: 10.1007/s11095-008-9590-7
- Holthuis, J.C.M., Levine, T.P., **2005**. Lipid traffic: Floppy drives and a superhighway. *Nat. Rev. Mol. Cell Biol.* 6, 209–220. doi: 10.1038/nrm1591
- Hu, C.-M.J., Fang, R.H., Wang, K.-C., Luk, B.T., Thamphiwatana, S., Dehaini, D., Nguyen, P., Angsantikul, P., Wen, C.H., Kroll, A. V., Carpenter, C., Ramesh, M., Qu, V., Patel, S.H., Zhu, J., Shi, W., Hofman, F.M., Chen, T.C., Gao, W., Zhang, K., Chien, S., Zhang, L., **2015a**. Nanoparticle biointerfacing by platelet membrane cloaking. *Nature* 526, 118–121. doi: 10.1038/Nature15373
- Hu, C.-M.J., Zhang, L., Aryal, S., Cheung, C., Fang, R.H., Zhang, L., **2011**. Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform. *Proc. Natl. Acad. Sci. USA* 108, 10980–10985. doi: 10.1073/pnas.1106634108
- Hu, M., Stanzione, F., Sum, A.K., Faller, R., Deserno, M., **2015b**. Design Principles for Nanoparticles Enveloped by a Polymer-Tethered Lipid Membrane. *ACS Nano* 9, 9942–9954. doi: 10.1021/acsnano.5b03439
- Hu, Y., Ehrich, M., Fuhrman, K., Zhang, C., **2014**. In vitro performance of lipid-PLGA hybrid nanoparticles as an antigen delivery system: lipid composition matters. *Nanoscale Res. Lett.* 9, 434. doi: 10.1186/1556-276X-9-434
- Hu, Y., Zhao, Z., Ehrich, M., Fuhrman, K., Zhang, C., **2015c**. In vitro controlled release of antigen in dendritic cells using pH-sensitive liposome-polymeric hybrid nanoparticles. *Polymer (Guildf)*. 80, 171–179. doi: 10.1016/j.polymer.2015.10.048
- Huang, Y.-Y., Sharma, S.K., Dai, T., Chung, H., Yaroslavsky, A., Garcia-Diaz, M., Chang, J., Chiang, L.Y., Hamblin, M.R., **2012a**. Can Nanotechnology potentiate photodynamic therapy? *Nanotechnol. Rev.* 1, 111–146. doi: 10.1515/ntrev-2011-0005
- Huang, Y.Y., Balasubramanian, T., Yang, E., Luo, D., Diers, J.R., Bocian, D.F., Lindsey, J.S., Holten, D., Hamblin, M.R., **2012b**. Stable Synthetic Bacteriochlorins for Photodynamic Therapy: Role of Dicyano Peripheral Groups, Central Metal Substitution (2H, Zn, Pd), and CremophorEL Delivery. *ChemMedChem* 7, 2155–2167. doi: 10.1002/cmdc.201200351
- Huang, W., Zhang, C., **2018**. Tuning the Size of Poly(lactic-co-glycolic Acid) (PLGA) Nanoparticles Fabricated by Nanoprecipitation. *Biotechnol. J.* 13, 1–8. doi: 10.1002/biot.201700203
- Huang, Z., **2005**. A Review of Progress in Clinical Photodynamic Therapy Zheng. *Technol Cancer Res Treat* 4, 283–293. doi: 10.1177/153303460500400308
- Isele, U., van Hoogevest, P., Leuenberger, H., Capraro, H.-G., Schieweck, K., **1994**.

- Development of CGP 55847, a liposomal Zn-phthalocyanine formulation using a controlled organic solvent dilution method. *Proc. SPIE* 2078, 397–403. doi: 10.1117/12.168680
- Jagan, S., Paganessi, L.A., Frank, R.R., Venugopal, P., Larson, M., Christopherson, K.W., **2012**. Bone marrow and peripheral blood AML cells are highly sensitive to CNDAC, the active form of sapacitabine. *Adv. Hematol.* 2012. doi: 10.1155/2012/727683
- Jäger, A., Jäger, E., Giacomelli, F.C., Nallet, F., Steinhart, M., Putaux, J.L., Konefał, R., Spěvák, J., Ulbrich, K., Štěpánek, P., **2018**. Structural changes on polymeric nanoparticles induced by hydrophobic drug entrapment. *Colloids Surfaces A Physicochem. Eng. Asp.* 538, 238–249. doi: 10.1016/j.colsurfa.2017.10.059
- Jensen, D.K., Jensen, L.B., Koocheki, S., Bengtson, L., Cun, D., Nielsen, H.M., Foged, C., **2012**. Design of an inhalable dry powder formulation of DOTAP-modified PLGA nanoparticles loaded with siRNA. *J. Control. Release* 157, 141–148. doi: 10.1016/j.jconrel.2011.08.011
- Jeong, H., Huh, M., Lee, S.J., Koo, H., Kwon, I.C., Jeong, S.Y., Kim, K., **2011**. Photosensitizer-conjugated human serum albumin nanoparticles for effective photodynamic therapy. *Theranostics* 1, 230–239. doi: 10.7150/thno/v01p0230
- Jeong, Y.I., Shim, Y.H., Kim, C., Lim, G.T., Choi, K.C., Yoon, C., **2005**. Effect of cryoprotectants on the reconstitution of surfactant-free nanoparticles of poly(DL-lactide-co-glycolide). *J. Microencapsul.* 22, 593–601. doi: 10.1080/02652040500162659
- Jiang, F., Lilge, L., Grenier, J., Li, Y., Wilson, M.D., Chopp, M., **1998**. Photodynamic therapy of U87 human glioma in nude rat using liposome-delivered photofrin. *Lasers Surg. Med.* 22, 74–80. doi: 10.1002/(SICI)1096-9101(1998)22:2<74::AID-LSM2>3.0.CO;2-T
- Jiang, S., Franco, Y.L., Zhou, Y., Chen, J., **2018**. Nanotechnology in retinal drug delivery. *Int. J. Ophthalmol.* doi: 10.18240/ijo.2018.06.23
- Jiang, X., Lo, P., Tsang, Y., Yeung, S., **2010**. Phthalocyanine-Polyamine Conjugates as pH-Controlled Photosensitizers for Photodynamic Therapy. *Chem. - A Eur. J. NA.* 16, 4777–4783. doi: 10.1002/chem.200903580
- Jin, C.S., Zheng, G., **2011**. Liposomal nanostructures for photosensitizer delivery. *Lasers Surg. Med.* 43, 734–748. doi: 10.1002/lsm.21101
- Jing, Y., Trefna, H., Persson, M., Kasemo, B., Svedhem, S., **2014**. Formation of supported lipid bilayers on silica: relation to lipid phase transition temperature and liposome size. *Soft Matter* 10, 187–95. doi: 10.1039/c3sm50947h
- Johnson, S.J., Bayerl, T.M., McDermott, D.C., Adam, G.W., Rennie, A.R., Thomas, R.K., Sackmann, E., **1991**. Structure of an adsorbed dimyristoylphosphatidylcholine bilayer

- measured with specular reflection of neutrons. *Biophys. J.* 59, 289–294. doi: 10.1016/S0006-3495(91)82222-6
- Jones, H.J., Vernon, D.I., Brown, S.B., **2003**. Photodynamic therapy effect of m-THPC (Foscan®) in vivo: Correlation with pharmacokinetics. *Br. J. Cancer* 89, 398–404. doi: 10.1038/sj.bjc.6601101
- Josefsen, L.B., Boyle, R.W., **2008**. Photodynamic therapy and the development of metal-based photosensitisers. *Met. Based. Drugs* 2008, 1–23. doi: 10.1155/2008/276109
- Junping, W., Takayama, K., Nagai, T., Maitani, Y., **2003**. Pharmacokinetics and antitumor effects of vincristine carried by microemulsions composed of PEG-lipid, oleic acid, vitamin E and cholesterol. *Int. J. Pharm.* 251, 13–21. doi: 10.1016/S0378-5173(02)00580-X
- Juzeniene, A., Peng, Q., Moan, J., **2007**. Milestones in the development of photodynamic therapy and fluorescence diagnosis. *Photochem. Photobiol. Sci.* 6, 1234–1245. doi: 10.1039/b705461k
- Kadam, R.S., Bourne, D.W.A., Kompella, U.B., **2012**. Nano-advantage in enhanced drug delivery with biodegradable nanoparticles: contribution of reduced clearance. *Drug Metab. Dispos.* 40, 1380–8. doi: 10.1124/dmd.112.044925
- Kalita, D., Shome, D., Jain, V.G., Chadha, K., Bellare, J.R., **2014**. In vivo intraocular distribution and safety of periocular nanoparticle carboplatin for treatment of advanced retinoblastoma in humans. *Am. J. Ophthalmol.* 157, 1109–1115.e2. doi: 10.1016/j.ajo.2014.01.027
- Kamaly, N., Xiao, Z., Valencia, P.M., Radovic-Moreno, A.F., Farokhzad, O.C., **2012**. Targeted polymeric therapeutic nanoparticles: design, development and clinical translation. *Chem. Soc. Rev.* 41, 2971. doi: 10.1039/c2cs15344k
- Kelty, C.J., Brown, N.J., Reed, M.W.R., Ackroyd, R., **2002**. The use of 5-aminolaevulinic acid as a photosensitizer in photodynamic therapy and photodiagnosis. *Photochem. Photobiol. Sci.* 1, 158–168. doi: 10.1039/b201027p
- Kennedy, J.C., Pottier, R.H., **1992**. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. *J Photochem Photobiol B* 14, 275–292.
- Kennedy, J.C., Pottier, R.H., Pross, D.C., **1990**. Photodynamic therapy with endogenous protoporphyrin. IX: Basic principles and present clinical experience. *J. Photochem. Photobiol. B Biol.* 6, 143–148. doi: 10.1016/1011-1344(90)85083-9
- Khan, S.A., Schneider, M., **2013**. Improvement of Nanoprecipitation Technique for Preparation of Gelatin Nanoparticles and Potential Macromolecular Drug Loading. *Macromol. BioSci.* 13, 455–463. doi: 10.1002/mabi.201200382

- Kharkwal, G.B., Sharma, S.K., Huang, Y.-Y., Dai, T., Hamblin, M.R., **2011**. Photodynamic therapy for infections: Clinical applications. *Lasers Surg. Med.* 43, 755–767. doi: 10.1002/lsm.21080
- Khdair, A., Gerard, B., Handa, H., Mao, G., Shekhar, M.P. V, Panyam, J., **2008**. Surfactant–Polymer Nanoparticles Enhance the Effectiveness of Anticancer Photodynamic Therapy. *Mol. Pharm.* 5, 795–807. doi: 10.1021/mp800026t
- Kim, K.H., Le, T.H., Oh, H.K., Heo, B., Moon, J., Shin, S., Jeong, S.H., **2017**. Protective microencapsulation of β -lapachone using porous glass membrane technique based on experimental optimisation. *J. Microencapsul.* 34, 545–559. doi: 10.1080/02652048.2017.1367850
- Kiss, É., Vargha, A., Vargha-Butler, E.I., **2004**. Interfacial behaviour of poly(lactic acid) and Pluronic6400 mixed monolayers at the air–water interface. *Phys. Chem. Chem. Phys.* 6, 1575–1579. doi: 10.1039/B312950K
- Klass, J.M., Lennox, R.B., Brown, G.R., Bourque, H., Pézolet, M., **2003**. Enantiomeric Polylactides at the Air–Water Interface: π – A Isotherms and PM-IRRAS Studies of Enantiomers and Their Blend. *Langmuir* 19, 333–340. doi: 10.1021/la020606w
- Knop, K., Mingotaud, A.-F., El-Akra, N., Violleau, F., Souchard, J.-P., **2009**. Monomeric pheophorbide(a)-containing poly(ethyleneglycol-b- ϵ -caprolactone) micelles for photodynamic therapy. *Photochem. Photobiol. Sci.* 8, 396. doi: 10.1039/b811248g
- Knudsen, K.B., Northeved, H., Pramod Kumar, E.K., Permin, A., Gjetting, T., Andresen, T.L., Larsen, S., Wegener, K.M., Lykkesfeldt, J., Jantzen, K., Loft, S., Møller, P., Roursgaard, M., **2015**. In vivo toxicity of cationic micelles and liposomes. *Nanomedicine Nanotechnology, Biol. Med.* 11, 467–477. doi: 10.1016/j.nano.2014.08.004
- Koenig, B.W., Krueger, S., Orts, W.J., Majkrzak, C.F., Berk, N.F., Silverton, J. V., Gawrisch, K., **1996**. Neutron Reflectivity and Atomic Force Microscopy Studies of a Lipid Bilayer in Water Adsorbed to the Surface of a Silicon Single Crystal. *Langmuir* 12, 1343–1350. doi: 10.1021/la950580r
- Konan-Kouakou, Y.N., Boch, R., Gurny, R., Allémann, E., **2005**. In vitro and in vivo activities of verteporfin-loaded nanoparticles. *J. Control. Release* 103, 83–91. doi: 10.1016/j.jconrel.2004.11.023
- Konan, Y.N., Cerny, R., Favet, J., Berton, M., Gurny, R., Allémann, E., **2003**. Preparation and characterization of sterile sub-200 nm meso-tetra(4-hydroxylphenyl)porphyrin-loaded nanoparticles for photodynamic therapy. *Eur. J. Pharm. Biopharm.* 55, 115–124. doi: 10.1016/S0939-6411(02)00128-5
- Kong, X., Lu, D., Wu, J., Liu, Z., **2016**. Spreading of a Unilamellar Liposome on Charged Substrates: A Coarse-Grained Molecular Simulation. *Langmuir* 32, 3785–3793. doi:

- 10.1021/acs.Langmuir.6b00043
- Koo, H., Moon, H., Han, H., Na, J.H., Huh, M.S., Park, J.H., Woo, S.J., Park, K.H., Chan Kwon, I., Kim, K., Kim, H., **2012**. The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection. *Biomaterials* 33, 3485–3493. doi: 10.1016/j.Biomaterials.2012.01.030
- Kostron, H., Hasan, T., **2016**. Photodynamic Medicine, Comprehensive Series in Photochemical & Photobiological Sciences. *Royal Society of Chemistry*, Cambridge. pp. 650. doi: 10.1039/9781782626824
- Krammer, B., Plaetzer, K., **2008**. ALA and its clinical impact, from bench to bedside. *Photochem. Photobiol. Sci.* 7, 283–289. doi: 10.1039/b712847a
- Krishnamurthy, S., Vaiyapuri, R., Zhang, L., Chan, J.M., **2015**. Lipid-coated polymeric nanoparticles for cancer drug delivery. *Sci. Sci.* 3, 923–36. doi: 10.1039/c4bm00427b
- Kriska, T., Jakus, J., Keszler, A., Vanyur, R., Németh, A., Gal, D., **1998**. Type III photosensitization: attempt for quantification and a way to new sensitizers. *Spie* 3563, 11–17. doi: 10.1117/12.339133
- Kung, H., **2014**. The chemotherapeutic effects of lapacho tree extract: β -lapachone. *Chemother. Open Access* 03, 26–29. doi: 10.4172/2167-7700.1000131
- Kung, H.N., Weng, T.Y., Liu, Y.L., Lu, K.S., Chau, Y.P., **2014**. Sulindac compounds facilitate the cytotoxicity of β -lapachone by up-regulation of NAD(P)H quinone oxidoreductase in human lung cancer cells. *PLoS One* 9. doi: 10.1371/journal.pone.0088122
- Lamberti, M.J., Rumie Vittar, N.B., De Carvalho Da Silva, F., Ferreira, V.F., Rivarola, V.A., **2013**. Synergistic enhancement of antitumor effect of β -Lapachone by photodynamic induction of quinone oxidoreductase (NQO1). *Phytomedicine* 20, 1007–1012. doi: 10.1016/j.phymed.2013.04.018
- Lanzkowsky, P., Lipton, J.M., Fish, J.D., **2016**. Manual of Pediatric Hematology and Oncology Manual of Pediatric Hematology and Oncology. *Elsevier*. pp. 788. doi: 10.1016/B978-0-1237-75154-6.00047-1
- Lapes, M., Petera, J., Jirsa, M., **1996**. Photodynamic therapy of cutaneous metastases of breast cancer after local application of meso-tetra-(para-sulphophenyl)-porphyrin (TPPS4). *J. Photochem. Photobiol. B.* 36, 205–7. doi: 10.1016/S1011-1344(96)07373-3
- Lapinski, M.M., Castro-Forero, A., Greiner, A.J., Ofoli, R.Y., Blanchard, G.J., **2007**. Comparison of liposomes formed by sonication and extrusion: Rotational and translational diffusion of an embedded chromophore. *Langmuir* 23, 11677–11683. doi: 10.1021/la7020963

- Lappalainen, K., Jääskeläinen, I., Syrjänen, K., Urtti, A., Syrjänen, S., **1994**. Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharm. Res.* 11, 1127–31. doi: 10.1023/A:1018932714745
- Lassalle, H.P., Dumas, D., Gräfe, S., D'Hallewin, M.A., Guillemin, F., Bezdetnaya, L., **2009**. Correlation between in vivo pharmacokinetics, intratumoral distribution and photodynamic efficiency of liposomal mTHPC. *J. Control. Release* 134, 118–124. doi: 10.1016/j.jconrel.2008.11.016
- Laville, I., Pigaglio, S., Blais, J.-C., Doz, F., Looock, B., Maillard, P., Grierson, D.S., Blais, J., **2006**. Photodynamic efficiency of diethylene glycol-linked glycoconjugated porphyrins in human retinoblastoma cells. *J. Med. Chem.* 49, 2558–67. doi: 10.1021/jm0580151
- Le Bihan, O., Bonnafous, P., Marak, L., Bickel, T., Trépout, S., Mornet, S., De Haas, F., Talbot, H., Taveau, J.C., Lambert, O., **2009**. Cryo-electron tomography of nanoparticle transmigration into liposome. *J. Struct. Biol.* 168, 419–425. doi: 10.1016/j.jsb.2009.07.006
- Lee, J. sook, Park, A.H., Lee, S.H., Lee, S.H., Kim, J.H., Yang, S.J., Yeom, Y. Il, Kwak, T.H., Lee, D., Lee, S.J., Lee, C.H., Kim, J.M., Kim, D., **2012**. Beta-Lapachone, a Modulator of NAD Metabolism, Prevents Health Declines in Aged Mice. *PLoS One* 7, e47122. doi: 10.1371/journal.pone.0047122
- Lee, Y.-E.K., Kopelman, R., **2011**. Polymeric Nanoparticles for Photodynamic Therapy, in: *Methods in Molecular Biology* (Clifton, N.J.). pp. 151–178. doi: 10.1007/978-1-61779-052-2_11
- Legrand, P., Lesieur, S., Bochot, A., Gref, R., Raatjes, W., Barratt, G., Vauthier, C., **2007**. Influence of polymer behaviour in organic solution on the production of polylactide nanoparticles by nanoprecipitation. *Int. J. Pharm.* 344, 33–43. doi: 10.1016/j.ijpharm.2007.05.054
- Lenne, P.F., Wawrezinieck, L., Conchonaud, F., Wurtz, O., Boned, A., Guo, X.J., Rigneault, H., He, H.T., Marguet, D., **2006**. Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J.* 25, 3245–3256. doi: 10.1038/sj.emboj.7601214
- Lesage, S., Hao Xu, Durham, L., **1993**. The occurrence and roles of porphyrins in the environment: possible implications for bioremediation. *Hydrol. Sci. Journal/Journal des Sci. Hydrol.* 38, 343–354. doi: 10.1080/02626669309492679
- Lesnefsky, E.J., Stoll, M.S.K., Minkler, P.E., Hoppel, C.L., **2000**. Separation and quantitation of phospholipids and lysophospholipids by high-performance liquid chromatography. *Anal. Biochem.* 285, 246–254. doi: 10.1006/abio.2000.4783
- Li, W., **2013**. Nanoparticles for Photodynamic Therapy, in: *Handbook of Biophotonics*. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 321–336. doi:

10.1002/9783527643981.bphot030

- Li, X., Anton, N., Arpagaus, C., Belleiteix, F., Vandamme, T.F., **2010**. Nanoparticles by spray drying using innovative new technology: The Büchi Nano Spray Dryer B-90. *J. Control. Release* 147, 304–310. doi: 10.1016/j.jconrel.2010.07.113
- Liang, R., Ma, L., Zhang, L., Li, C., Liu, W., Wei, M., Yan, D., Evans, D.G., Duan, X., **2014**. A monomeric photosensitizer for targeted cancer therapy. *Chem. Commun.* 50, 14983–14986. doi: 10.1039/c4cc07628a
- Lipson, R.L., Baldes, E.J., Olsen, A.M., **1961**. The use of a derivative of hematoporphyrin in tumor detection. *J. Natl. Cancer Inst.* 26, 1–11. doi: 10.1093/jnci/26.1.1
- Liu, J., Jiang, X., Ashley, C., Brinker, C.J., **2009a**. Electrostatically mediated liposome fusion and lipid exchange with a nanoparticle-supported bilayer for control of surface charge, drug containment, and delivery. *J. Am. Chem. Soc.* 131, 7567–9. doi: 10.1021/ja902039y
- Liu, J., Stace-Naughton, A., Jiang, X., Brinker, C.J., Juewen Liu, Alison Stace-Naughton, Xingmao Jiang, † and C. Jeffrey Brinker, **2009b**. Porous nanoparticle supported lipid bilayers (protocells) as delivery vehicles. *J. Am. Chem. Soc.* 131, 1354–5. doi: 10.1021/ja808018y
- Liu, L., Ye, Q., Lu, M., Lo, Y.C., Hsu, Y.H., Wei, M.C., Chen, Y.H., Lo, S.C., Wang, S.J., Bain, D.J., Ho, C., **2015**. A new approach to reduce toxicities and to improve bioavailabilities of platinum-containing anti-cancer nanodrugs. *Sci. Rep.* 5, 1–11. doi: 10.1038/srep10881
- Liu, Y., Li, K., Pan, J., Liu, B., Feng, S.S., **2010**. Folic acid conjugated nanoparticles of mixed lipid monolayer shell and biodegradable polymer core for targeted delivery of Docetaxel. *Biomaterials* 31, 330–338. doi: 10.1016/j.Biomaterials.2009.09.036
- Luo, D., Geng, J., Li, N., Carter, K.A., Shao, S., Atilla-Gokcumen, G.E., Lovell, J.F., **2017**. Vessel-Targeted Chemophototherapy with Cationic Porphyrin-Phospholipid Liposomes. *Mol. Cancer Ther.* 16, 2452–2461. doi: 10.1158/1535-7163.MCT-17-0276
- Lupu, M., Maillard, P., Mispelter, J., Poyer, F., Thomas, C.D., **2018**. A glycoporphyrin story: from chemistry to PDT treatment of cancer mouse models. *Photochem. Photobiol. Sci.* doi: 10.1039/C8PP00123E
- Lupu, M., Thomas, C.D., Poyer, F., Mispelter, J., Rosilio, V., Maillard, P., **2016**. Photobiology and Photochemistry Hand-in-Hand in Targeted Antitumoral Therapies, *in: Handbook of Porphyrin Science, Handbook of Porphyrin Science*. World Scientific Publishing Company, pp. 171–356. doi: 10.1142/9789813149595_0005
- Ma, P., Li, T., Xing, H., Wang, S., Sun, Y., Sheng, X., Wang, K., **2017**. Local anesthetic effects of bupivacaine loaded lipid-polymer hybrid nanoparticles: In vitro and in vivo evaluation.

- Biomed. Pharmacother. 89, 689–695. doi: 10.1016/j.biopha.2017.01.175
- Ma, X., Cheng, Z., Jin, Y., Liang, X., Yang, X., Dai, Z., Tian, J., **2014**. SM5-1-Conjugated PLA nanoparticles loaded with 5-fluorouracil for targeted hepatocellular carcinoma imaging and therapy. *Biomaterials* 35, 2878–2889. doi: 10.1016/j.Biomaterials.2013.12.045
- Ma, X., Huang, X., Moore, Z., Huang, G., Kilgore, J.A., Wang, Y., Hammer, S., Williams, N.S., Boothman, D.A., Gao, J., **2015**. Esterase-activatable β -lapachone prodrug micelles for NQO1-targeted lung cancer therapy. *J. Control. Release* 200, 201–211. doi: 10.1016/j.jconrel.2014.12.027
- Maaz, A., Abdelwahed, W., Tekko, I.A., Trefi, S., A. Maaz, W. Abdelwahed, I.A. Tekko, S. Tref, **2015**. Influence of nanoprecipitation method parameters on nanoparticles loaded with gatifloxacin for ocular drug delivery. *Int. J. Acad. Sci. Res.* 3, 1–12.
- Mackowiak, S.A., Schmidt, A., Weiss, V., Argyo, C., Von Schirnding, C., Bein, T., Bräuchle, C., **2013**. Targeted drug delivery in cancer cells with red-light photoactivated mesoporous silica nanoparticles. *Nano Lett.* 13, 2576–2583. doi: 10.1021/nl400681f
- Maeda, H., **2017**. Polymer therapeutics and the EPR effect. *J. Drug Target.* 25, 781–785. doi: 10.1080/1061186X.2017.1365878
- Maeda, H., Tsukigawa, K., Fang, J., **2016**. A Retrospective 30 Years After Discovery of the Enhanced Permeability and Retention Effect of Solid Tumors: Next-Generation Chemotherapeutics and Photodynamic Therapy—Problems, Solutions, and Prospects. *Microcirculation* 23, 173–182. doi: 10.1111/micc.12228
- Maillard, P., Looock, B., Grierson, D.S., Laville, I., Blais, J., Doz, F., Desjardins, L., Carrez, D., Guerquin-Kern, J.L., Croisy, A., **2007**. In vitro phototoxicity of glycoconjugated porphyrins and chlorins in colorectal adenocarcinoma (HT29) and retinoblastoma (Y79) cell lines. *Photodiagnosis Photodyn. Ther.* 4, 261–268. doi: 10.1016/j.pdpdt.2007.05.001
- Maira, M.-S., Pearson, M.A., Fabbro, D., García-Echeverría, C., **2007**. Cancer Biology, in: Comprehensive Medicinal Chemistry II. *Elsevier*, pp. 1–31. doi: 10.1016/B0-08-045044-X/00202-9
- Maisch, T., Santarelli, F., Schreml, S., Babilas, P., Szeimies, R.-M., **2009**. Fluorescence induction of protoporphyrin IX by a new 5-aminolevulinic acid nanoemulsion used for photodynamic therapy in a full-thickness ex vivo skin model. *Exp. Dermatol.* 19, e302–e305. doi: 10.1111/j.1600-0625.2009.01001.x
- Majewski, J., Wong, J.Y., Park, C.K., Seitz, M., Israelachvili, J.N., Smith, G.S., **1998**. Structural studies of polymer-cushioned lipid bilayers. *Biophys. J.* 75, 2363–2367. doi: 10.1016/S0006-3495(98)77680-5
- Major, M., Prieur, E., Tocanne, J.F., Betbeber, D., Sautereau, A.M., **1997**. Characterization and

- phase behaviour of phospholipids bilayers adsorbed on spherical polysaccharide nanoparticles. *Biochim. Biophys. Acta* 1327, 32–40.
- Makky, A., Daghildjian, K., Michel, J.P., Maillard, P., Rosilio, V., **2012**. Assessment of the relevance of supported planar bilayers for modeling specific interactions between glycodendrimeric porphyrins and retinoblastoma cells. *Biochim. Biophys. Acta - Biomembr.* 1818, 2831–2838. doi: 10.1016/j.bbamem.2012.07.003
- Makky, A., Michel, J.P., Maillard, P., Rosilio, V., **2011**. Biomimetic liposomes and planar supported bilayers for the assessment of glycodendrimeric porphyrins interaction with an immobilized lectin. *Biochim. Biophys. Acta - Biomembr.* 1808, 656–666. doi: 10.1016/j.bbamem.2010.11.028
- Mandal, B., Bhattacharjee, H., Mittal, N., Sah, H., Balabathula, P., Thoma, L.A., Wood, G.C., **2013**. Core-shell-type lipid-polymer hybrid nanoparticles as a drug delivery platform. *Nanomedicine Nanotechnology, Biol. Med.* 9, 474–491. doi: 10.1016/j.nano.2012.11.010
- Manjandavida, F.P., Shields, C.L., **2015**. The role of intravitreal chemotherapy for retinoblastoma. *Indian J. Ophthalmol.* 63, 141–5. doi: 10.4103/0301-4738.154390
- Marquardt, D., Geier, B., Pabst, G., **2015**. Asymmetric lipid membranes: Towards more realistic model systems. *Membranes (Basel)*. 5, 180–196. doi: 10.3390/membranes5020180
- Martens, T.F., Peynshaert, K., Nascimento, T.L., Fattal, E., Karlstetter, M., Langmann, T., Picaud, S., Demeester, J., De Smedt, S.C., Remaut, K., Braeckmans, K., **2017**. Effect of hyaluronic acid-binding to lipoplexes on intravitreal drug delivery for retinal gene therapy. *Eur. J. Pharm. Sci.* 103, 27–35. doi: 10.1016/j.ejps.2017.02.027
- Massiot, J., Makky, A., Di Meo, F., Chapron, D., Trouillas, P., Rosilio, V., **2017**. Impact of lipid composition and photosensitizer hydrophobicity on the efficiency of light-triggered liposomal release. *Phys. Chem. Chem. Phys.* 19, 11460–11473. doi: 10.1039/C7CP00983F
- Master, A.M., Livingston, M., Sen Gupta, A., **2013**. Photodynamic *Nanomedicine* in the treatment of solid tumors: Perspectives and challenges. *J. Control. Release* 168, 88–102. doi: 10.1016/j.jconrel.2013.02.020
- Master, A.M., Livingston, M., Oleinick, N.L., Sen Gupta, A., **2012a**. Optimization of a *Nanomedicine*-based silicon phthalocyanine 4 photodynamic therapy (Pc 4-PDT) strategy for targeted treatment of EGFR-overexpressing cancers. *Mol. Pharm.* 9, 2331–2338. doi: 10.1021/mp300256e
- Master, A.M., Qi, Y., Oleinick, N.L., Gupta, A. Sen, **2012b**. EGFR-mediated intracellular delivery of Pc 4 nanoformulation for targeted photodynamic therapy of cancer: in vitro studies. *Nanomedicine Nanotechnology, Biol. Med.* 8, 655–664. doi: 10.1016/j.nano.2011.09.012

- Mazzaglia, A., Micali, N., Scolaro, L.M., Sciortino, M.T., Sortino, S., Villari, V., **2010**. Design of photosensitizer/cyclodextrin nanoassemblies: spectroscopy, intracellular delivery and photodamage. *J. Porphyr. Phthalocyanines* 14, 661–677. doi: 10.1142/S1088424610002562
- McCarthy, J.R., Jaffer, F.A., Weissleder, R., **2006**. A macrophage-targeted theranostic nanoparticle for biomedical applications. *Small* 2, 983–987. doi: 10.1002/sml.200600139
- Menon, J.U., Kona, S., Wadajkar, A.S., Desai, F., Vadla, A., Nguyen, K.T., **2012**. Effects of surfactants on the properties of PLGA nanoparticles. *J. Biomed. Mater. Res. - Part A* 100 A, 1998–2005. doi: 10.1002/jbm.a.34040
- Merodio, M., Irache, J.M., Valamanesh, F., Mirshahi, M., **2002**. Ocular disposition and tolerance of ganciclovir-loaded albumin nanoparticles after intravitreal injection in rats. *Biomaterials* 23, 1587–1594. doi: 10.1016/S0142-9612(01)00284-8
- Messerschmidt, S.K.E.E., Musyanovych, A., Altvater, M., Scheurich, P., Pfizenmaier, K., Landfester, K., Kontermann, R.E., **2009**. Targeted lipid-coated nanoparticles: Delivery of tumor necrosis factor-functionalized particles to tumor cells. *J. Control. Release* 137, 69–77. doi: 10.1016/j.jconrel.2009.03.010
- Michel, J.P., Wang, Y.X., Kiesel, I., Gerelli, Y., Rosilio, V., **2017**. Disruption of Asymmetric Lipid Bilayer Models Mimicking the Outer Membrane of Gram-Negative Bacteria by an Active Plasticin. *Langmuir* 33, 11028–11039. doi: 10.1021/acs.Langmuir.7b02864
- Michel, R., Gradzielski, M., **2012**. Experimental Aspects of Colloidal Interactions in Mixed systems of liposome and inorganic nanoparticle and their applications. *Int. J. Mol. Sci.* 13, 11610–11642. doi: 10.3390/ijms130911610
- Michels, S., Schmidt-erfurth, U., **2001**. Photodynamic therapy with verteporfin : A new treatment in *Ophthalmology* Photodynamic therapy with verteporfin : A new treatment in *Ophthalmology. Semin. Ophthalmol.* 16, 201–206. doi: 10.1076/soph.16.4.201.10298
- Miladi, K., Sfar, S., Fessi, H., Elaissari, A., **2016**. Nanoprecipitation Process : From Particle Preparation to In Vivo Applications, *in*: Vauthier, C., Ponchel, G. (Eds.), *Polymer Nanoparticles for Nanomedicines. Springer International Publishing, Cham*, pp. 17–53. doi: 10.1007/978-3-319-41421-8_2
- Mingeot-Leclercq, M.P., Deleu, M., Brasseur, R., Dufrêne, Y.F., **2008**. Atomic force microscopy of supported lipid bilayers. *Nat. Protoc.* 3, 1654–1659. doi: 10.1038/nprot.2008.149
- Mitra, S., Foster, T.H., **2005**. Photophysical Parameters, Photosensitizer Retention and Tissue Optical Properties Completely Account for the Higher Photodynamic Efficacy of meso-Tetra-Hydroxyphenyl-Chlorin vs Photofrin[®]. *Photochem. Photobiol.* 81, 849. doi: 10.1562/2005-02-22-RA-447R.1

- Moan, J., Berg, K., **1991**. The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem. Photobiol.* 53, 549–53. doi: 10.1111/j.1751-1097.1991.tb03669.x
- Monge-Fuentes, V., Muehlmann, L.A., de Azevedo, R.B., **2014**. Perspectives on the application of *Nanotechnology* in photodynamic therapy for the treatment of melanoma. *Nano Rev.* 5, 24381. doi: 10.3402/nano.v5.24381
- Montforts, F., **1995**. Selective synthesis and photophysical properties of tailor-made chlorins for photodynamic therapy. *Proc. SPIE* 2325, 29–39. doi: 10.1117/12.199155
- Moor, A.C.E., **2000**. Signaling pathways in cell death and survival after photodynamic therapy. *J. Photochem. Photobiol. B Biol.* 57, 1–13. doi: 10.1016/S1011-1344(00)00065-8
- Moore, C.M., Pendse, D., Emberton, M., **2009**. Photodynamic therapy for prostate cancer—a review of current status and future promise. *Nat. Clin. Pract. Urol.* 6, 18–30. doi: 10.1038/ncpuro1274
- Moreno, E., Schwartz, J., Larrea, E., Conde, I., Font, M., Sanmartín, C., Irache, J.M., Espuelas, S., **2015**. Assessment of β -lapachone loaded in lecithin-chitosan nanoparticles for the topical treatment of cutaneous leishmaniasis in L. major infected BALB/c mice. *Nanomedicine Nanotechnology, Biol. Med.* 11, 2003–2012. doi: 10.1016/j.nano.2015.07.011
- Morgan, A.R., Garbo, G.M., Keck, R.W., Eriksen, L.D., Selman, S.H., **1990**. Metalloporphyrins and light: effect on transplantable rat bladder tumors and murine skin. *Photochem. Photobiol.* 51, 589–592. doi: 10.1111/j.1751-1097.1990.tb01970.x
- Mornet, S., Lambert, O., Duguet, E., Brisson, A., **2005**. The formation of supported lipid bilayers on silica nanoparticles revealed by cryoelectron microscopy. *Nano Lett.* 5, 281–285. doi: 10.1021/nl048153y
- Moser, E., Eggebrecht, A.T., Patterson, M.S., Lovell, J.F., Huang, H., Song, W., Rieffel, J., **2015**. Emerging applications of porphyrins in photomedicine. *Front. Phys.* 3, 1–15. doi: 10.3389/fphy.2015.00023
- Mosmann, T., **1983**. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63. doi: 10.1016/0022-1759(83)90303-4
- Moura, P., Carmona-ribeiro, A.M., **2005**. Biomimetic Particles : Optimization of Phospholipid Bilayer Coverage on Silica and Colloid Stabilization. *Langmuir* 21, 10160–10164. doi: 10.1021/la0504614
- Moura, S.P., Carmona-Ribeiro, A.M., **2003**. Cationic Bilayer Fragments on Silica at Low Ionic Strength: Competitive Adsorption and Colloid Stability. *Langmuir* 19, 6664–6667. doi:

10.1021/la034334o

- Muehlmann, L.A., Joanitti, G.A., Silva, J.R., Longo, J.P.F., Azevedo, R.B., **2011**. Liposomal photosensitizers: Potential platforms for anticancer photodynamic therapy. *Brazilian J. Med. Biol. Res.* 44, 729–737. doi: 10.1590/S0100-879X2011007500091
- Munier, F.L., Gaillard, M.C., Balmer, A., Beck-Popovic, M., **2013**. Intravitreal chemotherapy for vitreous seeding in retinoblastoma: Recent advances and perspectives. *Saudi J. Ophthalmol.* 27, 147–150. doi: 10.1016/j.sjopt.2013.06.003
- Murphree, A.L., Cote, M., Gomer, C.J., **1987**. The evolution of photodynamic therapy techniques in the treatment of intraocular tumors. *Photochem. Photobiol.* 46, 919–23. doi: 10.1111/j.1751-1097.1987.tb04869.x
- Nagle, J.F., Tristram-Nagle, S., Nagle John, F., Stephanie, T.N., **2009**. Structure of lipid bilayers. *Biochim. Biophys. Acta - Rev. Biomembr.* 1469, 159–195. doi: 10.1016/S0304-4157(00)00016-2
- Nakano, M., Fukuda, M., Kudo, T., Matsuzaki, N., Azuma, T., Sekine, K., Endo, H., Handa, T., **2009**. Flip-flop of phospholipids in vesicles: kinetic analysis with time-resolved small-angle neutron scattering. *J. Phys. Chem. B* 113, 6745–8. doi: 10.1021/jp900913w
- Nasongkla, N., Wiedmann, A.F., Bruening, A., Beman, M., Ray, D., Bornmann, W.G., Boothman, D. a, Gao, J., **2003**. Enhancement of solubility and bioavailability of beta-lapachone using cyclodextrin inclusion complexes. *Pharm. Res.* 20, 1626–1633. doi: 10.1023/a:1026143519395
- Ni, S., Lee, W., Li, B., Esker, A., **2006**. Thermodynamics of the liquid expanded to condensed phase transition of poly (L-lactic acid) in *Langmuir* monolayers. *Langmuir* 22, 3672–7. doi: 10.1021/la060084a
- Nichols, J.W., Bae, Y.H., **2013**. *Nanotechnology for Cancer Treatment: Possibilities and Limitations*, in: *Cancer Targeted Drug Delivery*. Springer New York, New York, pp. 37–56. doi: 10.1007/978-1-4614-7876-8_2
- Nirasay, S., Badia, A., Leclair, G., Claverie, J.P., Marcotte, I., **2012**. Polydopamine-supported lipid bilayers. *Materials (Basel)*. 5, 2621–2636. doi: 10.3390/ma5122621
- Nokes, B., Apel, M., Jones, C., Brown, G., Lang, J.E., **2013**. Aminolevulinic acid (ALA): Photodynamic detection and potential therapeutic applications. *J. Surg. Res.* 181, 262–271. doi: 10.1016/j.jss.2013.02.002
- Nordlund, G., Lönneborg, R., Brzezinski, P., Rosa, L., Brzezinski, P., **2009**. Formation of supported lipid bilayers on silica particles studied using flow cytometry. *Langmuir* 25, 4601–4606. doi: 10.1021/la8036296

- Nyokong, T., Ahsen, V., **2012**. Photosensitizers in Medicine, Environment, and Security. *Springer Netherlands*, Dordrecht. pp. 644. doi: 10.1007/978-90-481-3872-2
- O'Connor, A.E., Gallagher, W.M., Byrne, A.T., **2009**. Porphyrin and nonporphyrin photosensitizers in oncology: Preclinical and clinical advances in photodynamic therapy. *Photochem. Photobiol.* 85, 1053–1074. doi: 10.1111/j.1751-1097.2009.00585.x
- Obringer, A.R., Rote, N.S., Walter, A., **1995**. Antiphospholipid antibody binding to bilayer-coated glass microspheres. *J. Immunol. Methods* 185, 81–93. doi: 10.1016/0022-1759(95)00106-K
- Ochsner, M., **1997**. Photophysical and photobiological processes in the photodynamic therapy of tumours. *J. Photochem. Photobiol. B Biol.* doi: 10.1016/S1011-1344(96)07428-3
- Ohnishi, Y., Yamana, Y., Minei, M., **1986**. Photoradiation therapy using argon laser and a hematoporphyrin derivative for retinoblastoma--a preliminary report. *Jpn. J. Ophthalmol.* 30, 409–19. doi: 10.1109/IJCNN.2013.6706854
- Ohsawa, K., Hayashi, Y., Hasunuma, R., Yamabe, K., **2009**. Roughness increase on surface and interface of SiO₂ grown on atomically flat Si (111) terrace. *J. Phys. Conf. Ser.* 191. doi: 10.1088/1742-6596/191/1/012031
- Oleinick, N.L., Evans, H.H., **1998**. The Photobiology of Photodynamic Therapy: Cellular Targets and Mechanisms. *Radiat. Res.* 150, S146. doi: 10.2307/3579816
- Opitz, I., Krueger, T., Pan, Y., Altermatt, H.J., Wagnières, G., Ris, H.B., **2006**. Preclinical comparison of mTHPC and verteporfin for intracavitary photodynamic therapy of malignant pleural mesothelioma. *Eur. Surg. Res.* 38, 333–339. doi: 10.1159/000094028
- Ormond, A.B., Freeman, H.S., **2013**. Dye sensitizers for photodynamic therapy. *Materials (Basel)*. 6, 817–840. doi: 10.3390/ma6030817
- Palange, A.L., Di Mascolo, D., Carallo, C., Gnasso, A., Decuzzi, P., **2014**. Lipid-polymer nanoparticles encapsulating curcumin for modulating the vascular deposition of breast cancer cells. *Nanomedicine Nanotechnology, Biol. Med.* 10, e991–e1002. doi: 10.1016/j.nano.2014.02.004
- Pandey, S.K., Patel, D.K., Thakur, R., Mishra, D.P., Maiti, P., Haldar, C., **2015**. Anti-cancer evaluation of quercetin embedded PLA nanoparticles synthesized by emulsified nanoprecipitation. *Int. J. Biol. Macromol.* 75, 521–529. doi: 10.1016/j.ijbiomac.2015.02.011
- Park, S.H., Oh, S.G., Mun, J.Y., Han, S.S., **2005**. Effects of silver nanoparticles on the fluidity of bilayer in phospholipid liposome. *Colloids Surfaces B Biointerfaces* 44, 117–122. doi: 10.1016/j.colsurfb.2005.06.002

- Paszko, E., Ehrhardt, C., Senge, M.O., Kelleher, D.P., Reynolds, J. V., **2011**. Nanodrug applications in photodynamic therapy. *Photodiagnosis Photodyn. Ther.* 8, 14–29. doi: 10.1016/j.pdpdt.2010.12.001
- Pavot, V., Rochereau, N., Primard, C., Genin, C., Perouzel, E., Lioux, T., Paul, S., Verrier, B., **2013**. Encapsulation of Nod1 and Nod2 receptor ligands into poly(lactic acid) nanoparticles potentiates their immune properties. *J. Control. Release* 167, 60–67. doi: 10.1016/j.jconrel.2013.01.015
- Pelletier, I., Pézolet, M., **2004**. Compression-induced stereocomplexation of polylactides at the air/water interface. *Macromolecules* 37, 4967–4973. doi: 10.1021/ma035949v
- Peng, Q., Moan, J., Ma, L., Nesland, J.M., **1995**. Uptake , Localization , and Photodynamic Effect of meso -Tetra (hydroxyphenyl) porphine and Its Corresponding Chlorin in Normal and Tumor Tissues of Mice Bearing Mammary Carcinoma. *Cancer Res.* 55, 2620–2626.
- Pfeiffer, C., Rehbock, C., Hühn, D., Carrillo-Carrion, C., De Aberasturi, D.J., Merk, V., Barcikowski, S., Parak, W.J., **2014**. Interaction of colloidal nanoparticles with their local environment: The (ionic) nanoenvironment around nanoparticles is different from bulk and determines the physico-chemical properties of the nanoparticles. *J. R. Soc. Interface* 11. doi: 10.1098/rsif.2013.0931
- Pieper, S., Onafuye, H., Mulac, D., Cinatl, J., Wass, M.N., Michaelis, M., Langer, K., **2018**. Incorporation of doxorubicin in different polymer nanoparticles and their anti-cancer activity. *bioRxiv* 0044, 403923. doi: 10.1101/403923
- Pierre, M.B.R., Tedesco, A.C., Marchetti, J.M., Bentley, M.V.L.B., **2001**. Stratum corneum lipids liposomes for the topical delivery of 5-aminolevulinic acid in photodynamic therapy of skin cancer: Preparation and in vitro permeation study. *BMC Dermatol.* 1, 5. doi: 10.1186/1471-5945-1-5
- Pink, J.J., Planchon, S.M., Tagliarino, C., Varnes, M.E., Siegel, D., Boothman, D.A., **2000**. NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. *J. Biol. Chem.* 275, 5416–24.
- Plaetzer, K., Krammer, B., Berlanda, J., Berr, F., Kiesslich, T., **2009**. Photophysics and photochemistry of photodynamic therapy: Fundamental aspects. *Lasers Med. Sci.* 24, 259–268. doi: 10.1007/s10103-008-0539-1
- Planchon, S.M., Pink, J.J., Tagliarino, C., Bornmann, W.G., Varnes, M.E., Boothman, D.A., **2001**. β -Lapachone-induced apoptosis in human prostate cancer cells: Involvement of NQO1/xip3. *Exp. Cell Res.* 267, 95–106. doi: 10.1006/excr.2001.5234
- Pogue, B.W., Redmond, R.W., Trivedi, N., Hasan, T., **1998**. Photophysical Properties of Tin Ethyl Etiopurpurin I (SnET2) and Tin Octaethylbenzochlorin (SnOEBC) in Solution and

- Bound to Albumin. *Photochem. Photobiol.* 68, 809–815. doi: 10.1111/j.1751-1097.1998.tb05288.x
- Pramual, S., Lirdprapamongkol, K., Svasti, J., Bergkvist, M., Jouan-Hureaux, V., Arnoux, P., Frochot, C., Barberi-Heyob, M., Niamsiri, N., **2017**. Polymer-lipid-PEG hybrid nanoparticles as photosensitizer carrier for photodynamic therapy. *J. Photochem. Photobiol. B Biol.* 173, 12–22. doi: 10.1016/j.jphotobiol.2017.05.028
- Primo, F.L., Bentley, M.V.L.B., Tedesco, A.C., **2008**. Photophysical studies and in vitro skin permeation/retention of Foscan/nanoemulsion (NE) applicable to photodynamic therapy skin cancer treatment. *J. NanoSci. Nanotechnol.* 8, 340–7. doi: 10.1111/j.1600-0625.2009.01001.x
- Prow, T.W., Bhutto, I., Kim, S.Y., Grebe, R., Merges, C., McLeod, D.S., Uno, K., Mennon, M., Rodriguez, L., Leong, K., Luty, G.A., **2008**. Ocular nanoparticle toxicity and transfection of the retina and retinal pigment epithelium. *Nanomedicine* 4, 340–9. doi: 10.1016/j.nano.2008.06.003
- Puri, A., **2014**. Phototriggerable liposomes: Current research and future perspectives. *Pharmaceutics* 6, 1–25. doi: 10.3390/Pharmaceutics6010001
- Raemdonck, K., Braeckmans, K., Demeester, J., De Smedt, S.C., **2014**. Merging the best of both worlds: hybrid lipid-enveloped matrix nanocomposites in drug delivery. *Chem. Soc. Rev.* 43, 444–72. doi: 10.1039/c3cs60299k
- Ramírez-Ortiz, M.A., Lansingh, V.C., Eckert, K.A., Haik, B.G., Phillips, B.X., Bosch-Canto, V., González-Pérez, G., Villavicencio-Torres, A., Etulain-González, A., **2017**. Systematic review of the current status of programs and general knowledge of diagnosis and management of retinoblastoma. *Bol. Med. Hosp. Infant. Mex.* 74, 41–54. doi: 10.1016/j.bmhmx. 08.002
- Rao, L., Bu, L.-L., Meng, Q.-F., Cai, B., Deng, W.-W., Li, A., Li, K., Guo, S.-S., Zhang, W.-F., Liu, W., Sun, Z.-J., Zhao, X.-Z., **2017**. Antitumor Platelet-Mimicking Magnetic Nanoparticles. *Adv. Funct. Mater.* 1604774. doi: 10.1002/adfm.201604774
- Rapupano, R., Carmona-Ribeiro, A.M., **2000**. Supported Bilayers On Silica. *J. Colloid Interface Sci.* 307, 299–307. doi: 10.1006/jcis.2000.6824
- Reid, T.W., Albert, D.M., Rabson, A.S., Russell, P., Craft, J., Chu, E.W., Tralka, T.S., Wilcox, J.L., **1974**. Characteristics of an established cell line of retinoblastoma. *J. Natl. Cancer Inst.* 53, 347–360. doi: 10.1093/jnci/53.2.347
- Reimhult, E., Höök, F., Kasemo, B., **2003**. Intact vesicle adsorption and supported biomembrane formation from vesicles in solution: Influence of surface chemistry, vesicle size, temperature, and osmotic pressure. *Langmuir* 19, 1681–1691. doi: 10.1021/la0263920

- Reimhult, E., Kasemo, B., Höök, F., **2009**. Rupture pathway of phosphatidylcholine liposomes on silicon dioxide. *Int. J. Mol. Sci.* 10, 1683–1696. doi: 10.3390/ijms10041683
- Rejman, J., Oberle, V., Zuhorn, I.S., Hoekstra, D., **2004**. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* 377, 159–169. doi: 10.1042/bj20031253
- Richter, A.M., Cerruti-sola, S., **1990**. Biodistribution of tritiated benzoporphyrin derivative (3H-BPD-MA), a new potent photosensitizer, in normal and tumor-bearing mice. *J. Photochem. Photobiol. B.* 5, 231–44. doi: 10.1016/1011-1344(90)80008-L
- Richter, R.P., Bérat, R., Brisson, A.R., **2006**. Formation of solid-supported lipid bilayers: An integrated view. *Langmuir* 22, 3497–3505. doi: 10.1021/la052687c
- Richter, R.P., Brisson, A.R., **2005**. Following the formation of supported lipid bilayers on Mica: A study combining AFM, QCM-D, and ellipsometry. *Biophys. J.* 88, 3422–3433. doi: 10.1529/biophysj.104.053728
- Riley, T., Govender, T., Stolnik, S., Xiong, C., Garnett, M., Illum, L., Davis, S., **1999**. Colloidal stability and drug incorporation aspects of micellar-like PLA–PEG nanoparticles. *Colloids Surfaces B Biointerfaces* 16, 147–159. doi: 10.1016/S0927-7765(99)00066-1
- Riss, T.L., Moravec, R.A., **2004**. Use of Multiple Assay Endpoints to Investigate the Effects of Incubation Time, Dose of Toxin, and planting Density in Cell-Based Cytotoxicity Assays. *Assay Drug Dev. Technol.* 2, 51–62. doi: 10.1089/154065804322966315
- Roblero-Bartolon, G.V., Ramon-Gallegos, E., **2015**. Use of nanoparticles (NP) in photodynamic therapy (PDT) against cancer. *Gac. Med. Mex.* 151, 85–98.
- Roces, C.B., Kastner, E., Stone, P., Lowry, D., Perrie, Y., **2016**. Rapid quantification and validation of lipid concentrations within liposomes. *Pharmaceutics* 8, 1–11. doi: 10.3390/Pharmaceutics8030029
- Rodrigues, M.M.A., Simioni, A.R., Primo, F.L., Siqueira-Moura, M.P., Morais, P.C., Tedesco, A.C., **2009**. Preparation, characterization and in vitro cytotoxicity of BSA-based nanospheres containing nanosized magnetic particles and/or photosensitizer. *J. Magn. Magn. Mater.* 321, 1600–1603. doi: 10.1016/j.jmmm.2009.02.093
- Rodriguez-Galindo, C., Orbach, D.B., VanderVeen, D., **2015**. Retinoblastoma. *Pediatr. Clin. North Am.* 62, 201–223. doi: 10.1016/j.pcl.2014.09.014
- Rodriguez, E., Baas, P., Friedberg, J.S., **2004**. Innovative therapies: Photodynamic therapy. *Thorac. Surg. Clin.* 14, 557–566. doi: 10.1016/j.thorsurg.2004.06.004
- Rogers, L., Majer, F., Sergeeva, N.N., Paszko, E., Gilmer, J.F., Senge, M.O., **2013**. Synthesis and biological evaluation of Foscan® bile acid conjugates to target esophageal cancer

- cells. *Bioorganic Med. Chem. Lett.* 23, 2495–2499. doi: 10.1016/j.bmcl.2013.03.040
- Roussaki, M., Gaitanarou, A., Diamanti, P.C., Vouyiouka, S., Papaspyrides, C., Kefalas, P., Detsi, A., **2014**. Encapsulation of the natural antioxidant aureusidin in biodegradable PLA nanoparticles. *Polym. Degrad. Stab.* 108, 182–187. doi: 10.1016/j.polymdegradstab.2014.08.004
- Ruiz-Galindo, E., Arenas-Huertero, F., Ramón-Gallegos, E., **2007**. Expression of genes involved in heme biosynthesis in the human retinoblastoma cell lines WERI-Rb-1 and Y79: implications for photodynamic therapy. *J. Exp. Clin. Cancer Res.* 26, 195–200.
- Saavedra, R., Rocha, L.B., Dabrowski, J.M., Arnaut, L.G., **2014**. Modulation of biodistribution, pharmacokinetics, and photosensitivity with the delivery vehicle of a bacteriochlorin photosensitizer for photodynamic therapy. *ChemMedChem* 9, 390–398. doi: 10.1002/cmdc.201300449
- Salvador-Morales, C., Zhang, L., Langer, R., Farokhzad, O.C., **2009**. Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups. *Biomaterials* 30, 2231–2240. doi: 10.1016/j.Biomaterials.2009.01.005
- Samaroo, D., Perez, E., Aggarwal, A., Wills, A., O’connor, N., **2014**. Strategies for delivering porphyrinoid-based photosensitizers in therapeutic applications. *Ther. Deliv.* 5, 859–872. doi: 10.4155/tde.14.46
- Sato, S., Gondo, D., Wada, T., Kanehashi, S., Nagai, K., **2013**. Effects of various liquid organic solvents on solvent-induced crystallization of amorphous poly(lactic acid) film. *J. Appl. Polym. Sci.* 129, 1607–1617. doi: 10.1002/app.38833
- Savarala, S., Ahmed, S., Ilies, M.A., Wunder, S.L., **2010**. Formation and colloidal stability of dmpe supported lipid bilayers on SiO₂ nanobeads. *Langmuir* 26, 12081–12088. doi: 10.1021/la101304v
- Savarala, S., Monson, F., Ilies, M.A., Wunder, S.L., **2011**. Supported Lipid Bilayer NanoSystems: Stabilization by Undulatory-Protrusion Forces and Destabilization by Lipid Bridging. *Langmuir* 27, 5850–5861. doi: 10.1021/la200636k
- Schmidt-Erfurth, U., Bauman, W., Gragoudas, E., Flotte, T.J., Michaud, N.A., Birngruber, R., Hasan, T., **1994**. Photodynamic therapy of experimental choroidal melanoma using lipoprotein-delivered benzoporphyrin. *Ophthalmology* 101, 89–99. doi: 10.1016/S0161-6420(13)31242-1
- Senge, M.O., Brandt, J.C., **2011**. Temoporfin (Foscan, 5,10,15,20-Tetra(m-hydroxyphenyl)chlorin) - A second-generation photosensitizer. *Photochem. Photobiol.* 87, 1240–1296. doi: 10.1111/j.1751-1097.2011.00986.x
- Sengel-Turk, C.T., Gumustas, M., Uslu, B., Ozkan, S.A., **2018**. A novel approach for drug

- targeting: core-shell type lipid-polymer hybrid nanocarriers, *in*: Design of Nanostructures for *Theranostics* Applications. *Elsevier*, pp. 69–107. doi: 10.1016/B978-0-12-813669-0.00003-8
- Sengel-Turk, C.T., Hascicek, C., **2017**. Design of lipid-polymer hybrid nanoparticles for therapy of BPH: Part I. Formulation optimization using a design of experiment approach. *J. Drug Deliv. Sci. Technol.* 39, 16–27. doi: 10.1016/j.jddst.2017.02.012
- Sengupta, S., Eavarone, D., Capila, I., Zhao, G., Watson, N., Kiziltepe, T., Sasisekharan, R., **2005**. Temporal targeting of tumour cells and neovasculature with a *Nanoscale* delivery system. *Nature* 436, 568–572. doi: 10.1038/Nature03794
- Sève, A., Couleaud, P., Lux, F., Tillement, O., Arnoux, P., André, J.-C., Frochot, C., **2012**. Long-distance energy transfer photosensitizers arising in hybrid nanoparticles leading to fluorescence emission and singlet oxygen luminescence quenching. *Photochem. Photobiol. Sci.* 11, 803–811. doi: 10.1039/c2pp05324a
- Shah, H.R., Conway, R.M., Van Quill, K.R., Madigan, M.C., Howard, S. a, Qi, J., Weinberg, V., O'Brien, J.M., **2008**. Beta-lapachone inhibits proliferation and induces apoptosis in retinoblastoma cell lines. *Eye (Lond)*. 22, 454–60. doi: 10.1038/sj.eye.6702764
- Shao, J., Wen, C., Xuan, M., Zhang, H., Frueh, J., Wan, M., Gao, L., He, Q., **2017**. Polyelectrolyte multilayer-cushioned fluid lipid bilayers: A parachute model. *Phys. Chem. Chem. Phys.* 19, 2008–2016. doi: 10.1039/c6cp06787e
- Sharma, D., Maheshwari, D., Philip, G., Rana, R., Bhatia, S., Singh, M., Gabrani, R., Sharma, S.K., Ali, J., Sharma, R.K., Dang, S., **2014**. Formulation and optimization of polymeric nanoparticles for intranasal delivery of lorazepam using Box-Behnken design: In vitro and in vivo evaluation. *Biomed Res. Int.* 2014, 156010. doi: 10.1155/2014/156010
- Sharman, W.M., Allen, C.M., Van Lier, J.E., **1999**. Photodynamic therapeutics: Basic principles and clinical applications. *Drug Discov. Today* 4, 507–517. doi: 10.1016/S1359-6446(99)01412-9
- Shi, J., Xiao, Z., Votruba, A.R., Vilos, C., Farokhzad, O.C., **2011**. Differentially charged hollow core/shell lipid-polymer-lipid hybrid nanoparticles for small interfering rna delivery. *Angew. Chemie - Int. Ed.* 50, 7027–7031. doi: 10.1002/anie.201101554
- Shields, C.L., Fulco, E.M., Arias, J.D., Alarcon, C., Pellegrini, M., Rishi, P., Kaliki, S., Bianciotto, C.G., Shields, J.A., **2013**. Retinoblastoma frontiers with intravenous, intra-arterial, periocular, and intravitreal chemotherapy. *Eye* 27, 253–264. doi: 10.1038/eye.2012.175
- Shields, C.L., Manjandavida, F.P., Arepalli, S., Kaliki, S., Lally, S.E., Shields, J.A., **2014**. Intravitreal melphalan for persistent or recurrent retinoblastoma vitreous seeds: Preliminary results. *JAMA Ophthalmol.* 132, 319–325. doi:

10.1001/jamaophthalmol.2013.7666

- Silva, J.N., Filipe, P., Morlière, P., Mazière, J.C., Freitas, J.P., Gomes, M.M., Santus, R., **2008**. Photodynamic therapy: Dermatology and *Ophthalmology* as main fields of current applications in clinic. *Biomed. Mater. Eng.* 18, 319–327. doi: 10.3233/BME-2008-0546
- Simon, V., Devaux, C., Darmon, A., Donnet, T., Thiénot, E., Germain, M., Honnorat, J., Duval, A., Pottier, A., Borghi, E., Levy, L., Marill, J., **2010**. Pp IX silica nanoparticles demonstrate differential interactions with in vitro tumor cell lines and in vivo mouse models of human cancers. *Photochem. Photobiol.* 86, 213–222. doi: 10.1111/j.1751-1097.2009.00620.x
- Soares, M. V., Lanzarini, C.M., Oliveira, D.S., Ramos, P.R.S., Santos, E.P., Ricci, E., **2010**. Improving the phototoxicity of the zinc phthalocyanine by encapsulation in nanoparticles: Preparation, characterization and phototherapy studies. *Lat. Am. J. Pharm.* 29, 5–12.
- Sobot, D., Mura, S., Couvreur, P., **2016**. How can *Nanomedicines* overcome cellular-based anticancer drug resistance? *J. Mater. Chem. B* 4, 5078–5100. doi: 10.1039/c6tb00900j
- Song, L., Li, H., Sunar, U., Chen, J., Corbin, I., Yodh, A.G., Zheng, G., **2007**. Naphthalocyanine-reconstituted LDL nanoparticles for in vivo cancer imaging and treatment. *Int. J. Nanomedicine* 2, 767–774.
- Song, X., Feng, L., Liang, C., Gao, M., Song, G., Liu, Z., **2017**. Liposomes co-loaded with metformin and chlorin e6 modulate tumor hypoxia during enhanced photodynamic therapy. *Nano Res.* 10, 1200–1212. doi: 10.1007/s12274-016-1274-8
- Spikes, J.D., **1985**. The Historical Development of Ideas on Applications of Photosensitized Reactions in the Health Sciences. *Prim. Photo-Processes Biol. Med.* 209–227. doi: 10.1007/978-1-4684-1224-6
- Spikes, J.D., Bommer, J.C., **1993**. Photosensitizing properties of mono-L-aspartyl chlorin e6 (NPe6): a candidate sensitizer for the photodynamic therapy of tumors. *J. Photochem. Photobiol. B.* 17, 135–43. doi: 10.1016/1011-1344(93)80006-U
- Spring, B., Mai, Z., Rai, P., Chang, S., Hasan, T., **2010**. Theranostic nanocells for simultaneous imaging and photodynamic therapy of pancreatic cancer, *in*: Kessel, D.H. (Ed.). p. 755104. doi: 10.1117/12.843725
- Stallivieri, A., Colombeau, L., Jetpisbayeva, G., Moussaron, A., Myrzakhmetov, B., Arnoux, P., Acherar, S., Vanderesse, R., Frochot, C., **2017**. Folic acid conjugates with photosensitizers for cancer targeting in photodynamic therapy: Synthesis and photophysical properties. *Bioorganic Med. Chem.* 25, 1–10. doi: 10.1016/j.bmc.2016.10.004
- Stavropoulos, K., **2011**. Synthesis and Characterization of Lipid-Polymer Hybrid Nanoparticles

- for Combinatorial Drug Delivery. Faculty of the University of Kansas.
- Stephan, H., Boeleni, R., Eggert, A., Bornfeld, N., Schueler, A., **2008**. Photodynamic therapy in retinoblastoma: Effects of verteporfin on retinoblastoma cell lines. *Investig. Ophthalmol. Vis. Sci.* 49, 3158–3163. doi: 10.1167/iovs.07-1016
- Sternberg, E.D., Dolphin, D., Brückner, C., **1998**. Porphyrin-based photosensitizers for use in photodynamic therapy. *Tetrahedron* 54, 4151–4202. doi: 10.1016/S0040-4020(98)00015-5
- Strickley, R.G., **2004**. Solubilizing excipients in oral and injectable formulations. *Pharm. Res.* 21, 201–30. doi: 10.1023/B:PHAM.0000016235.32639.23
- Su, X., Fricke, J., Kavanagh, D.G., Irvine, D.J., **2011**. In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles. *Mol. Pharm.* 8, 774–787. doi: 10.1021/mp100390w
- Sun, Y., Chen, Z., Yang, X., Huang, P., Zhou, X., Du, X., **2009**. Magnetic chitosan nanoparticles as a drug delivery system for targeting photodynamic therapy. *Nanotechnology* 20, 135102. doi: 10.1088/0957-4484/20/13/135102
- Sundh, M., Svedhem, S., Sutherland, D.S., **2010**. Influence of phase separating lipids on supported lipid bilayer formation at SiO₂ surfaces. *Phys. Chem. Chem. Phys.* 12, 453–460. doi: 10.1039/b912598a
- Sutton, D., Wang, S., Nasongkla, N., Gao, J., Dormidontova, E.E., **2007**. Doxorubicin and -Lapachone Release and Interaction with Micellar Core Materials: Experiment and Modeling. *Exp. Biol. Med.* 232, 1090–1099. doi: 10.3181/0702-RM-31
- Swamy, N., Purohit, A., Fernandez-Gacio, A., Jones, G.B., Ray, R., **2006**. Nuclear estrogen receptor targeted photodynamic therapy: Selective uptake and killing of MCF-7 breast cancer cells by a C17 α -alkynylestradiol-porphyrin conjugate. *J. Cell. Biochem.* 99, 966–977. doi: 10.1002/jcb.20955
- Szurko, A., Rams, M., Sochanik, A., Sieroń-Stołtny, K., Koziół, A.M., Montforts, F.P., Wrzałik, R., Ratuszna, A., **2009**. Spectroscopic and biological studies of a novel synthetic chlorin derivative with prospects for use in PDT. *Bioorganic Med. Chem.* 17, 8197–8205. doi: 10.1016/j.bmc.2009.10.029
- Tahara, Y., Fujiyoshi, Y., **1994**. A new method to measure bilayer thickness: Cryo-electron microscopy of frozen hydrated liposomes and image simulation. *Micron* 25, 141–149. doi: 10.1016/0968-4328(94)90039-6
- Tahir, N., Madni, A., Balasubramanian, V., Rehman, M., Correia, A., Kashif, P.M., Mäkilä, E., Salonen, J., Santos, H.A., **2017**. Development and optimization of methotrexate-loaded lipid-polymer hybrid nanoparticles for controlled drug delivery applications. *Int. J. Pharm.*

- 533, 156–168. doi: 10.1016/j.ijpharm.2017.09.061
- Takeuchi, Y., Ichikawa, K., Yonezawa, S., Kurohane, K., Koishi, T., Nango, M., Namba, Y., Oku, N., **2004**. Intracellular target for photosensitization in cancer antiangiogenic photodynamic therapy mediated by polycation liposome. *J. Control. Release* 97, 231–240. doi: 10.1016/j.jconrel.2004.03.030
- Tan, S., Li, X., Guo, Y., Zhang, Z., **2013**. Lipid-enveloped hybrid nanoparticles for drug delivery. *Nanoscale* 5, 860–72. doi: 10.1039/c2nr32880a
- Tarhini, M., Benlyamani, I., Hamdani, S., Agusti, G., Fessi, H., Greige-Gerges, H., Bentaher, A., Elaissari, A., **2018**. Protein-based nanoparticle preparation via nanoprecipitation method. *Materials (Basel)*. 11, 1–18. doi: 10.3390/ma11030394
- Taylor, D.M., **2000**. Developments in the theoretical modelling and experimental measurement of the surface potential of condensed monolayers. *Adv. Colloid Interface Sci.* 87, 183–203. doi: 10.1016/S0001-8686(99)00044-5
- Teixo, R., Laranjo, M., Abrantes, A.M., Brites, G., Serra, A., Proença, R., Botelho, M.F., **2015**. Retinoblastoma: might photodynamic therapy be an option? *Cancer Metastasis Rev.* 34, 563–573. doi: 10.1007/s10555-014-9544-y
- Thanki, K., Zeng, X., Justesen, S., Tejlmann, S., Falkenberg, E., Van Driessche, E., Mørck Nielsen, H., Franzyk, H., Foged, C., **2017**. Engineering of small interfering RNA-loaded lipidoid-poly(DL-lactic-co-glycolic acid) hybrid nanoparticles for highly efficient and safe gene silencing: A quality by design-based approach. *Eur. J. Pharm. Biopharm.* 120, 22–33. doi: 10.1016/j.ejpb.2017.07.014
- Thevenot, J., Troutier, A., David, L., Delair, T., Ladavière, C., **2007**. Steric Stabilization of Lipid/Polymer Particle Assemblies by Poly(ethylene glycol)-Lipids. *Biomacromolecules* 8, 3651–3660. doi: 10.1021/bm700753q
- Thevenot, J., Troutier, A., Putaux, J., Delair, T., Ladavière, C., **2008**. Effect of the polymer Nature on the structural organization of lipid/polymer particle assemblies. *J. Phys. Chem. B* 112, 13812–22. doi: 10.1021/jp805865r
- Triesscheijn, M., Baas, P., Schellens, J.H.M., Stewart, F.A., **2006**. Photodynamic Therapy in Oncology. *Oncologist* 11, 1034–1044. doi: 10.1634/theoncologist.11-9-1034
- Troutier-Thuilliez, A.-L., Thevenot, J., Delair, T., Ladavière, C., **2009**. Adsorption of plasmid DNA onto lipid/polymer particle assemblies. *Soft Matter* 5, 4739. doi: 10.1039/b911260j
- Troutier, A.-L., Véron, L., Delair, T., Pichot, C., Ladavière, C., **2005a**. New insights into self-organization of a model lipid mixture and quantification of its adsorption on spherical polymer particles. *Langmuir* 21, 9901–10. doi: 10.1021/la050796l

- Troutier, A., Delair, T., Pichot, C., Ladavière, C., **2005b**. Physicochemical and interfacial investigation of lipid/polymer particle assemblies. *Langmuir* 21, 1305–13. doi: 10.1021/la047659t
- Troutier, A.L., Ladavière, C., **2007**. An overview of lipid membrane supported by colloidal particles. *Adv. Colloid Interface Sci.* 133, 1–21. doi: 10.1016/j.cis.2007.02.003
- Tsuji, H., Ikada, Y., **1997**. Blends of crystalline and amorphous poly(lactide). *J. Appl. Polym. Sci.* 63, 855–863. doi: papers://590F92D9-0B76-4B88-8729-9AF064BE5AC8/Paper/p4495
- Valencia, P.M., Basto, P.A., Zhang, L., Rhee, M., Langer, R., Farokhzad, O.C., Karnik, R., **2010**. Single-Step Assembly of Homogenous Lipid–Polymeric and Lipid–Quantum Dot Nanoparticles Enabled by Microfluidic Rapid Mixing. *ACS Nano* 4, 1671–1679. doi: 10.1021/nn901433u
- Van Dongen, G.A.M.S., Visser, G.W.M., Vrouenraets, M.B., **2004**. Photosensitizer-antibody conjugates for detection and therapy of cancer. *Adv. Drug Deliv. Rev.* 56, 31–52. doi: 10.1016/j.addr.2003.09.003
- Van Nostrum, C.F., **2004**. Polymeric micelles to deliver photosensitizers for photodynamic therapy. *Adv. Drug Deliv. Rev.* 56, 9–16. doi: 10.1016/j.addr.2003.07.013
- Vargas, A., Eid, M., Fanchaouy, M., Gurny, R., Delie, F., **2008**. In vivo photodynamic activity of photosensitizer-loaded nanoparticles: Formulation properties, administration parameters and biological issues involved in PDT outcome. *Eur. J. Pharm. Biopharm.* 69, 43–53. doi: 10.1016/j.ejpb.2007.09.021
- Vasilca, V., Sadeghpour, A., Rawson, S., Hawke, L.E., Baldwin, S.A., Wilkinson, T., Bannister, D., Postis, V.L.G., Rappolt, M., Muench, S.P., Jeuken, L.J.C., **2018**. Spherical-supported membranes as platforms for screening against membrane protein targets. *Anal. Biochem.* 549, 58–65. doi: 10.1016/j.ab.2018.03.006
- Vauthier, C., Bouchemal, K., **2009**. Methods for the Preparation and Manufacture of Polymeric Nanoparticles. *Pharm. Res.* 26, 1025–1058. doi: 10.1007/s11095-008-9800-3
- Vermette, P., **2009**. Liposome Characterization by Quartz Crystal Microbalance Measurements and Atomic Force Microscopy, *in: Methods in Enzymology. Elsevier Inc.*, pp. 43–73. doi: 10.1016/S0076-6879(09)65003-5
- Vidóczy, T., Elzemzam, S., Gál, D., **1992**. Physico-chemical modeling of the role of free radicals in photo-dynamic therapy. I. Utilization of quantum yield data of singlet oxygen formation for the study of the interaction between excited photosensitizer and stable free radicals. *Biochem. Biophys. Res. Commun.* 189, 1548–52.
- Von Hoegen, P., **2001**. Synthetic biomimetic supra molecular Biovector (SMBV) particles for

- nasal vaccine delivery. *Adv. Drug Deliv. Rev.* 51, 113–125. doi: 10.1016/S0169-409X(01)00175-2
- Wagner, M.L., Tamm, L.K., **2000**. Tethered polymer-supported planar lipid bilayers for reconstitution of integral membrane proteins: silane-polyethyleneglycol-lipid as a cushion and covalent linker. *Biophys. J.* 79, 1400–14. doi: 10.1016/S0006-3495(00)76392-2
- Walther, J., Schastak, S., Dukic-Stefanovic, S., Wiedemann, P., Neuhaus, J., Claudepierre, T., **2014**. Efficient photodynamic therapy on human retinoblastoma cell lines. *PLoS One* 9, 1–12. doi: 10.1371/journal.pone.0087453
- Wan, N., Jia, Y.-Y., Hou, Y.-L., Ma, X.-X., He, Y.-S., Li, C., Zhou, S.-Y., Zhang, B.-L., **2016**. Preparation, Physicochemical Properties, and Transfection Activities of Tartaric Acid-Based Cationic Lipids as Effective Nonviral Gene Delivery Vectors. *Biol. Pharm. Bull.* 39, 1112–20. doi: 10.1248/bpb.b16-00007
- Wang, F., Blanco, E., Ai, H., Boothman, D.A., Gao, J., **2006**. Modulating b-Lapachone Release from Polymer Millirods through Cyclodextrin Complexation. *J. Pharm. Sci.* 95, 2309–19. doi: 10.1002/jps.20721
- Wang, Q., Alshaker, H., Böhler, T., Srivats, S., Chao, Y., Cooper, C., Pchejetski, D., **2017**. Core shell lipid-polymer hybrid nanoparticles with combined docetaxel and molecular targeted therapy for the treatment of metastatic prostate cancer. *Sci. Rep.* 7, 1–8. doi: 10.1038/s41598-017-06142-x
- Wang, Z.-J., He, Y.-Y., Huang, C.-G., Huang, J.-S., Huang, Y.-C., An, J.-Y., Gu, Y., Jiang, L.-J., **1999**. Pharmacokinetics, tissue distribution and photodynamic therapy efficacy of liposomal-delivered hypocrellin A, a potential photosensitizer for tumor therapy. *Photochem Photobiol* 70, 773–780. doi: 10.1111/j.1751-1097.1999.tb08282.x
- White, R.J., Zhang, B., Daniel, S., Tang, J.M., Ervin, E.N., Cremer, P.S., White, H.S., **2006**. Ionic conductivity of the aqueous layer separating a lipid bilayer membrane and a glass Support. *Glass* 22, 10777–10783. doi: 10.1021/la061457a
- Wikene, K.O., Bruzell, E., Tønnesen, H.H., **2015**. Improved antibacterial phototoxicity of a neutral porphyrin in natural deep eutectic solvents. *J. Photochem. Photobiol. B Biol.* 148, 188–196. doi: 10.1016/j.jphotobiol.2015.04.022
- Wlodek, M., Szuwarzynski, M., Kolasinska-Sojka, M., **2015**. Effect of Supporting Polyelectrolyte Multilayers and Deposition Conditions on the Formation of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine Lipid Bilayers. *Langmuir* 31, 10484–10492. doi: 10.1021/acs.Langmuir.5b02560
- Wong, H.L., Rauth, A.M., Bendayan, R., Manias, J.L., Ramaswamy, M., Liu, Z., Erhan, S.Z., Wu, X.Y., **2006**. A new polymer-lipid hybrid nanoparticle system increases cytotoxicity

- of doxorubicin against multidrug-resistant human breast cancer cells. *Pharm. Res.* 23, 1574–1585. doi: 10.1007/s11095-006-0282-x
- Woodbury, D.J., Richardson, E.S., Grigg, A.W., Welling, R.D., Knudson, B.H., **2006**. Reducing liposome size with ultrasound: Bimodal size distributions. *J. Liposome Res.* 16, 57–80. doi: 10.1080/08982100500528842
- Wrobel, I., Collins, D., **1995**. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *BBA - Biomembr.* 1235, 296–304. doi: 10.1016/0005-2736(95)80017-A
- Wu, X., **2018**. Approche physico-chimique de la formulation de bêta-lapachone complexée ou non à des cyclodextrines, dans des préparations liposomales. Paris-Saclay University.
- Xiao, L., Wang, B., Yang, G., Gauthier, M., **2006**. Poly(Lactic Acid)-Based *Biomaterials* : Synthesis , Modification and Applications. *Biomed. Sci. Eng. Technol.* 247–282. doi: 10.5772/23927
- Yan, G.P., Zong, R.F., Li, L., Fu, T., Liu, F., Yu, X.H., **2010**. Anticancer drug-loaded nanospheres based on biodegradable amphiphilic ϵ -Caprolactone and carbonate copolymers. *Pharm. Res.* 27, 2743–2752. doi: 10.1007/s11095-010-0275-7
- Yang, C., Jiang, L., Bu, S., Zhang, L., Xie, X., Zeng, Q., Zhu, D., Zheng, Y., **2013**. Intravitreal administration of dexamethasone-loaded PLGA-TPGS nanoparticles for the treatment of posterior segment diseases. *J. Biomed. Nanotechnol.* 9, 1617–1623. doi: 10.1166/jbn.2013.1646
- Yang, X., Palasuberniam, P., Kraus, D., Chen, B., **2015**. Aminolevulinic acid-based tumor detection and therapy: Molecular mechanisms and strategies for enhancement. *Int. J. Mol. Sci.* 16, 25865–25880. doi: 10.3390/ijms161025865
- Yang, X.Z., Dou, S., Wang, Y.C., Long, H.Y., Xiong, M.H., Mao, C.Q., Yao, Y.D., Wang, J., **2012**. Single-step assembly of cationic lipid-polymer hybrid nanoparticles for systemic delivery of siRNA. *ACS Nano* 6, 4955–4965. doi: 10.1021/nn300500u
- Yang, Y., Song, W., Wang, A., Zhu, P., Fei, J., Li, J., **2010**. Lipid coated mesoporous silica nanoparticles as photosensitive drug carriers. *Phys. Chem. Chem. Phys.* 12, 4418. doi: 10.1039/b924370d
- Yang, Z., Luo, X., Zhang, X., Liu, J., Jiang, Q., **2013**. Targeted delivery of 10-hydroxycamptothecin to human breast cancers by cyclic RGD-modified lipid-polymer hybrid nanoparticles. *Biomed. Mater.* 8. doi: 10.1088/1748-6041/8/2/025012
- Yoo, J.O., Ha, K.S., **2012**. New Insights into the Mechanisms for Photodynamic Therapy-Induced Cancer Cell Death, 1st ed, International Review of Cell and Molecular Biology. Elsevier Inc. doi: 10.1016/B978-0-12-394306-4.00010-1

- Yoon, I., Li, J.Z., Shim, Y.K., **2013**. Advance in photosensitizers and light delivery for photodynamic therapy. *Clin. Endosc.* 46, 7–23. doi: 10.5946/ce.2013.46.1.7
- Zane, C., Panfilis, G. De, Calzavara-Pinton, P., **2001**. Chapter 7 Photosensitizers—systemic sensitization, *in: Photodynamic Therapy and Fluorescence Diagnosis in Dermatology. Elsevier*, pp. 101–114. doi: 10.1016/S1568-461X(01)80111-3
- Záruba, K., Králová, J., Øezanka, P., Pouèková, P., Veverková, L., Král, V., **2010**. Modified porphyrin-brucine conjugated to gold nanoparticles and their application in photodynamic therapy. *Org. Biomol. Chem.* 8, 3202–3206. doi: 10.1039/c002823a
- Zeisser-Labouèbe, M., Lange, N., Gurny, R., Delie, F., **2006**. Hypericin-loaded nanoparticles for the photodynamic treatment of ovarian cancer. *Int. J. Pharm.* 326, 174–181. doi: 10.1016/j.ijpharm.2006.07.012
- Zeisser-Labouèbe, M., Mattiuzzo, M., Lange, N., Gurny, R., Delie, F., **2009**. Quenching-induced deactivation of photosensitizer by nanoencapsulation to improve phototherapy of cancer. *J. Drug Target.* 17, 619–626. doi: 10.1080/10611860903118930
- Zhai, Y., Su, J., Ran, W., Zhang, P., Yin, Q., Zhang, Z., Yu, H., Li, Y., **2017**. Preparation and application of cell membrane-camouflaged nanoparticles for cancer therapy. *Theranostics* 7, 2575–2592. doi: 10.7150/thno.20118
- Zhang, J., Hu, J., Chan, H.F., Skibba, M., Liang, G., Chen, M., **2016**. iRGD decorated lipid-polymer hybrid nanoparticles for targeted co-delivery of doxorubicin and sorafenib to enhance anti-hepatocellular carcinoma efficacy. *Nanomedicine Nanotechnology, Biol. Med.* 12, 1303–1311. doi: 10.1016/j.nano.2016.01.017
- Zhang, J., Jiang, C., Figueiró Longo, J.P., Azevedo, R.B., Zhang, H., Muehlmann, L.A., **2018**. An updated overview on the development of new photosensitizers for anticancer photodynamic therapy. *Acta Pharm. Sin.* B 8, 137–146. doi: 10.1016/j.apsb.2017.09.003
- Zhang, L., Chan, J.M., Gu, F.X., Rhee, J., Wang, A.Z., Radovic-Moreno, A.F., Alexis, F., Langer, R., Farokhzad, O.C., **2008**. Self-Assembled Lipid–Polymer Hybrid Nanoparticles: A Robust Drug Delivery Platform. *ACS Nano* 2, 1696–1702. doi: 10.1021/nn800275r
- Zhang, L., Chen, Z., Yang, K., Liu, C., Gao, J., Qian, F., **2015**. β -Lapachone and Paclitaxel Combination Micelles with Improved Drug Encapsulation and Therapeutic Synergy as Novel Nanotherapeutics for NQO1-Targeted Cancer Therapy. *Mol. Pharm.* 12, 3999–4010. doi: 10.1021/acs.molpharmaceut.5b00448
- Zhang, L., Feng, Q., Wang, J., Zhang, S., Ding, B., Wei, Y., Dong, M., Ryu, J., Yoon, T., Shi, X., Sun, J., Jiang, X., **2015**. Microfluidic Synthesis of Hybrid Nanoparticles with Controlled Lipid Layers: Understanding Flexibility-Regulated Cell-Nanoparticle Interaction. *ACS Nano* 9, 9912–21. doi: 10.1021/acsnano.5b05792

- Zhang, L., Sun, H., Chen, Z., Liu, Z., Huang, N., Qian, F., **2017**. Intermolecular Interactions between Coencapsulated Drugs Inhibit Drug Crystallization and Enhance Colloidal Stability of Polymeric Micelles. *Mol. Pharm.* 14, 3568–3576. doi: 10.1021/acs.molpharmaceut.7b00591
- Zhang, L., Zhang, L., **2010**. Lipid–Polymer Hybrid Nanoparticles: Synthesis, Characterization and Applications. *Nano Life* 01, 163–173. doi: 10.1142/S179398441000016X
- Zhao, P., Wang, H., Yu, M., Liao, Z., Wang, X., Zhang, F., Ji, W., Wu, B., Han, J., Zhang, H., Wang, H., Chang, J., Niu, R., **2012**. Paclitaxel loaded folic acid targeted nanoparticles of mixed lipid-shell and polymer-core: In vitro and in vivo evaluation. *Eur. J. Pharm. Biopharm.* 81, 248–256. doi: 10.1016/j.ejpb.2012.03.004
- Zheng, G., Graham, A., Shibata, M., Missert, J.R., Oseroff, A.R., Dougherty, T.J., Pandey, R.K., **2001**. Synthesis of β -galactose-conjugated chlorins derived by enyne metathesis as galectin-specific photosensitizers for photodynamic therapy. *J. Org. Chem.* 66, 8709–8716. doi: 10.1021/jo0105080
- Zhong, Z., Ji, Q., Zhang, J.A., **2010**. Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection. *J. Pharm. Biomed. Anal.* 51, 947–951. doi: 10.1016/j.jpba.2009.10.001
- Zhu, T.C., Finlay, J.C., **2008**. The role of photodynamic therapy (PDT) physics. *Med. Phys.* 35, 3127–3136. doi: 10.1118/1.2937440
- Zuluaga, M.-F., Mailhos, C., Robinson, G., Shima, D.T., Gurny, R., Lange, N., **2007**. Synergies of VEGF inhibition and photodynamic therapy in the treatment of age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* 48, 1767–1772. doi: 10.1167/iovs.06-1224

Titre : Formulation de liponanoparticules pour le traitement du rétinoblastome par bithérapie chimio/photodynamique

Mots clés : Liponanoparticules, thérapie photodynamique, photosensibilisateur, chimiothérapie, rétinoblastome

Résumé : Le rétinoblastome est une tumeur maligne de la rétine qui touche essentiellement les nourrissons et jeunes enfants. Sa prise en charge est associée à la survenue d'effets secondaires sévères, certains traitements induisant le développement de tumeurs secondaires. Dans ce contexte, la thérapie photodynamique (PDT) apparaît comme une alternative prometteuse, car elle est non mutagène et génère des effets secondaires moins importants. Elle consiste à injecter un agent photosensibilisateur (PS) - une porphyrine par exemple - puis à illuminer la zone tumorale avec un laser. L'efficacité de la PDT nécessite l'accumulation de PS dans la tumeur. Cependant, la plupart des porphyrines sont hydrophobes et s'agrègent en milieu aqueux. Leur incorporation dans un nano-vecteur peut améliorer leur distribution au cytoplasme. Malheureusement, lorsqu'elles sont encapsulées

dans le cœur des nanoparticules, les molécules de PS perdent leur phototoxicité en raison de leur auto-extinction. Dans ce travail, nous avons conçu des lipo-nanoparticules biodégradables (LNP) constituées d'une nanoparticule (NP) de poly (D,L)-lactide (PLA) recouverte d'une bicouche de phospholipides (POPC-DOTAP). Un principe actif anticancéreux, la bêta-lapachone et un agent photosensibilisateur ont ensuite été co-encapsulés dans notre système en vue de favoriser un effet synergique sur le rétinoblastome. Nous avons démontré la formation effective des LNPs et leur internalisation dans les cellules de rétinoblastome en quelques heures. Enfin, nous avons démontré une amélioration de l'activité antitumorale en combinant les deux traitements dans notre système par rapport au traitement simple par PDT ou chimiothérapie.

Title: Formulation of liponanoparticles for dual chemo/photodynamic therapy of retinoblastoma

Keywords: Liponanoparticles, photodynamic therapy, photosensitizer, chemotherapy, retinoblastoma

Abstract: Retinoblastoma is a malignant tumor of the retina in infants. Conventional therapies are associated to severe side effects and some of them induce secondary tumors. Therefore, photodynamic therapy (PDT) appears as a promising alternative as it is non-mutagenic and generates minimal side effects. It consists in injection of a photosensitizer (PS) like a porphyrin, and then illumination of the tumor area with a laser. The effectiveness of PDT requires the accumulation of the PS in the tumor. However, most porphyrins are hydrophobic and aggregate in aqueous medium. Their incorporation into a nanocarrier may improve their delivery to the cytoplasm. Unfortunately, when incorporated into a nanoparticle core, PS molecules lose their

phototoxicity due to self-quenching. In this work, we have designed biodegradable liponanoparticles (LNPs) consisting of a poly(D,L)-lactide (PLA) nanoparticle (NP) coated with a phospholipid (POPC/DOTAP) bilayer. An anticancer drug, beta-lapachone (β -Lap), and a photosensitizer were then co-encapsulated in these LNPs for achieving synergistic effect on retinoblastoma. We have first demonstrated the effective formation of the LNPs and their internalization in retinoblastoma cells within few hours. Then we studied the cyto/phototoxicity of the system. The hybrid nanoparticles showed an improved antitumor activity when the PS and β -Lap were combined, compared to the single treatment by PDT or chemotherapy.

