International Journal of Pharmaceutics 420 (2011) 319–332

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Nuclear delivery of a therapeutic peptide by long circulating pH-sensitive liposomes: Benefits over classical vesicles

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ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form 16 August 2011
Accepted 17 August 2011
Available online 25 August 2011

Keywords:
Peptide
pH-sensitive liposomes
Drug delivery
PEG
Cellular uptake

ABSTRACT

The purpose of this study is to propose a suitable vector combining increased circulation lifetime and intracellular delivery capacities for a therapeutic peptide. Long circulating classical liposomes [SPC:CHOL:PEG-750-DSPE (47:47:6 molar% ratio)] or pH-sensitive stealth liposomes [DOPE:CHEMS:CHOL:PEG_{750}-DSPE (43:21:30:6 molar% ratio)] were used to deliver a therapeutic peptide to its nuclear site of action. The benefit of using stealth pH-sensitive liposomes was investigated and formulations were compared to classical liposomes in terms of size, shape, charge, encapsulation efficiency, stability and, most importantly, in terms of cellular uptake. Confocal microscopy and flow cytometry were used to evaluate the intracellular fate of liposomes themselves and of their hydrophilic encapsulated material. Cellular uptake of peptide-loaded liposomes was also investigated in three cell lines: Hs578t human epithelial cells from breast carcinoma, MDA-MB-231 human breast carcinoma cells and WI-26 human diploid lung fibroblast cells. The difference between formulations in terms of peptide delivery from the endosome to the cytoplasm and even to the nucleus was investigated as a function of time. Characterization studies showed that both formulations possess acceptable size, shape and encapsulation efficiency but cellular uptake studies showed the important benefit of the pH-sensitive formulation over the classical one, in spite of liposome PEGylation. Indeed, stealth pH-sensitive liposomes were able to deliver hydrophilic materials strongly to the cytoplasm. Most importantly, when encapsulated in pH-sensitive stealth liposomes, the peptide was able to reach the nucleus of tumorigenic and non tumorigenic breast cancer cells.

1. Introduction

Print3G is a putative antagonist of an oncoprotein involved in breast cancer growth and invasion. Until now, only a few biologically active peptides have been successfully used in clinical medicine. Indeed, administration of peptidic drugs presents several disadvantages, related to their rapid elimination from the blood circulation by the lymphatic system, their enzymatic degradation, their uptake by the reticulo-endothelial system (RES) and their non-selective accumulation (Banga and Chien, 1988; Zhou and Li Wan Po, 1991; Torchilin, 2006; Katanasaka et al., 2008).

The necessity for intravenous administration of Print3G has led to the development of PEGylated liposomes as drug carriers. Recently, several studies successfully explored the path of liposomes for the administration of several peptides (Katanasaka et al., 2008; Hanato et al., 2009; Petrikovics et al., 2009). Liposomes, spherical structures composed of one or several phospholipid bilayers, possess many attractive characteristics for stabilizing peptidic drugs and for improving their pharmacological properties. Being biocompatible and biodegradable, liposomes cause no or very mild antigenic, pyrogenic, allergic or toxic reactions. They can entrap hydrophilic drugs within their aqueous compartment, lipophilic compounds in their membrane or amphipathic drugs. First generation liposomes have been shown to be easily eliminated from the bloodstream and to accumulate in the Kupfer cells in the liver and in spleen macrophages (Klibanov et al., 2003). Therefore, the strategy has been to graft polymers onto nanoparticles and particularly onto liposomal phospholipids with polyethylene glycol (PEG). PEGs are synthetic, inert and biocompatible polymers, allowing the formation of a protective layer on the particle surface and providing protection against opsonization and capture by the RES (Torchilin, 2009). The increase in circulation lifetime by PEGs was demonstrated by Dos Santos et al., who showed an increase in vascular remanence for the surface-grafted PEG liposomes, preventing

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doi:10.1016/j.ijpharm.2011.08.034

aggregation and building a steric barrier around the liposome, in comparison with non-grafted vectors (Dos Santos et al., 2007). These two properties, namely the increase in circulation time and the decrease in capture by the RES, explain the Enhanced Permeability and Retention (EPR) effect, applicable to almost all rapidly growing solid tumors (Maeda et al., 2008). The enhanced extravasations of macromolecules from blood vessels to tumor and their retention within these tissues, due to the anatomical and permeability particularities of tumors, represent a phenomenon not observed in normal tissue, leading to a passive targeting towards affected tissues.

Print3G is a hydrophilic peptide composed of 25 natural amino acids (M.W. = 3000, p*I* = 11.1), freely soluble in water, as such it is encapsulated in the inner aqueous cavity of liposomes. The first formulation of PEGylated liposomes developed as Print3G vector (Ducat et al., 2010) was made of soybean phosphatidylcholine (SPC), methoxypolyethyleneglycol grafted onto distearoylphosphoethanolamine (PEG_{750}-DSPE) and cholesterol (CHOL), added because of their stabilizing properties (Kirby et al., 1980a,b; Dos Santos et al., 2007). In order to improve the introduction of encapsulated material into the cytoplasm, a second formulation was developed, replacing SPC with a combination of dioleylphosphoethanolamine (DOPE) and cholesterylhemisuccinate (CHEMS) (Simoes et al., 2004). DOPE exhibits a conical shape because of its small and minimally hydrated headgroup, compared to its lipophilic tail. DOPE is associated with a compound used as a stabilizer of the liposomal membrane: CHEMS. This molecule possesses an inverted cone shape at physiological pH, but, under acidic conditions, the carboxylic acid group becomes protonated and loses this particular shape. During endocytosis, the pH decreases within the endosome, resulting in a destabilization of the liposomal membrane transiting from a lamellar to a hexagonal phase. Some publications have described the mechanism of action of pH-sensitive liposomes containing a derivative of PE associated with a compound containing an acidic group as stabilizer (Straubinger et al., 1985; Skalko et al., 1998; Peschka-Suss and Schubert, 2003; Huth et al., 2006). PEG and cholesterol added into formulations decrease the membrane fluidity of fluid liposomes and can hamper their penetration capacity/potency and their pH-sensitivity (Bellavance et al., 2010; Liu and Huang, 1989). Cholesterol indeed is able to increase the fluidity of solid membranes. The use of PEG_{750}, characterized by a lower molecular weight than PEG often used to obtain long circulating liposomes, could allow finding a compromise between the improvement of vascular remanence and intracellular delivery. Maintenance of liposomes pH-sensitivity using DSPE-PEG_{750} will be studied and discussed in this publication. Moreover, few publications have described formulations of long circulating pH-sensitive liposomes (Junior et al., 2007; Momekova et al., 2007; Obata et al., 2009), none has investigated the difference in terms of cellular uptake between classical and pH-sensitive liposomes and no study has proposed a suitable vector for the delivery of nuclear peptides.

The purpose of this study is to propose a suitable vector for the intracellular delivery of a therapeutic peptide called Print3G, while keeping the formulation properties of long-circulating liposomes to effectively deliver the peptide to its site of action. To investigate the benefit of developing a formulation of long circulating pH-sensitive liposomes beside a classical formulation of stealth liposomes, these two formulations were compared in terms of size, shape, charge, encapsulation efficiency, stability and cellular uptake. Confocal laser scanning microscopy (CLSM) and fluorescence-activated cell sorting (FACS) were used to evaluate the intracellular fate of liposomes themselves and of their encapsulated material as a function of time. In order to demonstrate the benefit of the vector, research was expanded to various cell lines: Hs578t human epithelial cells from breast carcinoma, MDA-MB-231 human breast carcinoma cells and WI-26 human diploid lung fibroblast cells.

Firstly, we studied the cellular uptake of liposomes themselves, using NBD-CHOL as a fluorescent marker of the phospholipids bilayer.

Secondly, the delivery of a model molecule, calcein, was investigated. Calcein was chosen as a model molecule for two reasons: (1) its localization in the aqueous compartment of liposomes because of its hydrophilicity similar to Print3G and (2) its self-quenching behavior, often used to study the endosomal escape triggered by pH-sensitive liposomes (Kono et al., 1997; Shi et al., 2002; Hong et al., 2010).

Finally, we studied the intracellular outcome of Print3G, which has to reach the nucleus for its therapeutic action. This helped us to discern the difference between the two formulations in terms of delivery of the entrapped peptide from the endosome to the cytoplasm and even to the nucleus.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC, purity: 98%) was provided by Lipoid (Ludwigshafen, Germany). N-(Carbonyl-methoxypolyethylenglycol-750)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (PEG-750-DSPE, purity 100%) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol (CHOL, purity > 99%), cholesteryl-hemisuccinate (CHEMS) and calcein were purchased from Sigma–Aldrich (Bornem, Belgium). Print3G (purity: 95.39%) biotinylated peptide called Print3G (purity: 98.44%) was acquired from GL Biochem (Shanghai, China). 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (25-NBD-CHOL) was purchased from Avanti Polar Lipids (Pelham, USA). Water was deionized using the Millipore system (18.2 MΩ/cm resistivity) and was filtered through a 0.22 μm Millipore Millipak®-40 disposable filter unit (Millipore Corporation, USA). Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), penicillin–streptomycin, L-glutamine and streptavidin conjugate Alexa Fluor 555 were purchased from Gibco-Life Technologies (Invitrogen Corporation, Paisley, UK). Paraformaldehyde and polysorbate 20 were provided by Merck (Schuchardt and Darmstadt, respectively, Germany). Vectashield mounting medium with DAPI was purchased from Vector Laboratories (Burlingame, CA, USA). All other reagents and solvents were of analytical grade.

2.2. Liposome characterization

Liposome characterization was performed by photon correlation spectroscopy (PCS), zeta potential measures and freeze-fracture electron microscopy to ensure that the selected formulations possessed the required properties of shape and size for *in vitro* studies and further intravenous administration of the encapsulated peptide.

2.2.1. Blank liposomes

Blank unilamellar vesicles, made of SPC:CHOL:PEG-750-DSPE (47:47:6 molar% ratio) for classical stealth liposomes or made of DOPE:CHEMS:CHOL:PEG_{750}-DSPE (43:21:30:6 molar% ratio) for pH-sensitive long circulating liposomes, were prepared by the hydration of lipid film method. Briefly, required amounts of lipids were dissolved in chloroform and evaporated under reduced pressure in a round-bottomed flask to form a thin lipid film. The film was hydrated using a vortex mixer with HEPES-buffered

Table 1
Z-average sizes and zeta potentials of classical and pH-sensitive stealth liposomes ($n > 3$).

	Classical stealth liposomes		pH-sensitive stealth liposomes	
	Z-average size	Zeta potential	*Z*-average size	Zeta potential
Empty liposomes	172.6 ± 14.7 nm	−8.0 ± 3.9 mV	150.4 ± 14.1 nm	−16.3 ± 5.1 mV
Liposomes containing calcein	162.6 ± 4.3 nm	−11.5 ± 3.4 mV	176.2 ± 21.3 nm	−16.6 ± 3.4 mV
Liposomes containing Print3G	163.8 ± 7.2 nm	−6.8 ± 2.8 mV	165.2 ± 1.4 nm	−14.0 ± 3.4 mV

saline, containing 67 mM NaCl and adjusted to pH 7.4 with 0.1 N NaOH solution. The resulting suspension was extruded 5 times through a 0.2 μm Nucleopore polycarbonate membrane (Whatman International Ltd., Maidstone, UK). Final concentrations of lipids were as follows: SPC/CHOL/PEG_{750}-DSPE (36.1:36.1:4, mM) and DOPE/CHEMS/CHOL/PEG_{750}-DSPE (33:16:23:4.5, mM).

2.2.2. Photon correlation spectroscopy (PCS)

Liposome dispersions were diluted 10 times in isotonic HEPES buffer for PCS measurements (HPPS, Malvern Instruments, UK). Measurements were made at 25 °C with a fixed angle of 90°. Quoted sizes represented the average mean for the liposomal hydrodynamic diameter (nm). The polydispersity index (PDI) expressed the size distribution width.

2.2.3. Zeta potential

The zeta potential of liposome formulations was measured with a Zetasizer 2000 DTS52013 (Malvern Instruments, UK) at pH 7.4. Liposome suspensions were diluted 100 times in HEPES buffer pH 7.4, then loaded into a capillary cell mounted on the apparatus and measured 5 times at 37 °C.

2.2.4. Freeze-fracture electron microscopy

Freeze-fracture replicas of blank liposome suspensions were analysed under transmission electron microscopy. In practice, a drop of liposome suspension containing 20% (v/v) glycerol as a cryoprotectant was deposited into a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at −100 °C in shadowing equipment (Balzers® BAF-400) fitted with a freeze-fracture and etching unit. The replicas were examined with a JEOL (JEM-100SX) transmission electron microscope, operating at 80 kV accelerating voltage.

2.3. Determination of Print3G encapsulation efficiency

2.3.1. Print3G encapsulation

The encapsulation of Print3G into the classical formulation of liposomes was made using the freeze-thawing method. Blank liposomes (see Section 2.2.1) were mixed with a 400 μM Print3G solution in HEPES buffer, frozen for 1 min at −196 °C in liquid nitrogen and thawed for 5 min at 30 °C in a water bath. 11 cycles were performed to promote the entry of Print3G into vesicles. Between each cycle, the suspension was mixed for 10 s using a vortex mixer (Ducat et al., 2010).

Concerning the classical formulation, the freeze-thawing method was found to give the best results because it avoids adsorption of Print3G on materials during manufacture. Encapsulation efficiencies were significantly increased in comparison to the hydration of lipid film technique. A design of experiments was built to obtain the best encapsulation efficiency for this formulation and was plainly described by Ducat et al. (2010).

On the contrary, this method could not be used to encapsulate Print3G into pH-sensitive PEGylated liposomes because liposome destruction after freeze-thawing cycles was observed by PCS measurements. High encapsulation efficiency for pH-sensitive liposomes was obtained using the hydration of lipid film method associated with pre-saturation of the extruder by a Print3G solution.

Liposomes were prepared by the hydration of lipid film method described in Section 2.2.1, the hydration of the lipid film was made with a 200 μM Print3G solution in HEPES buffer. Before extrusion, the extruder was saturated with the same solution to avoid any adsorption of the peptide onto extrusion materials. The resulting suspension was extruded five times through a 0.2 μm polycarbonate membrane.

Finally, the obtained suspensions underwent two ultracentrifugation cycles at 35,000 rpm for 3 h and 30 min at 4 °C using the Beckman SW60 rotor (86,496 × *g*) in order to remove free Print3G.

2.3.2. Print3G determination

An HPLC method with UV/visible diode array detector (DAD) was developed and validated for the determination of Print3G using a Hewlett-Packard 1100 series equipment (Agilent Technologies, USA). This method was detailed in our previous study [11]. Ten-μl samples were injected into a Zorbax 300 SB-C18 (3.5 μm, 150 mm × 4.6 mm I.D., Agilent Technologies, USA) in an oven set at 35 °C with a mobile phase containing water and acetonitrile (ACN). Both solvents were acidified with 0.1% of trifluoroacetic acid (TFA). The applied gradient was as follows: 18–22.8% ACN from 0 to 20 min, 100% ACN at 25 min and 18% at 25.2 min. Detection was achieved at absorbance wavelengths of 220 nm and 275 nm.

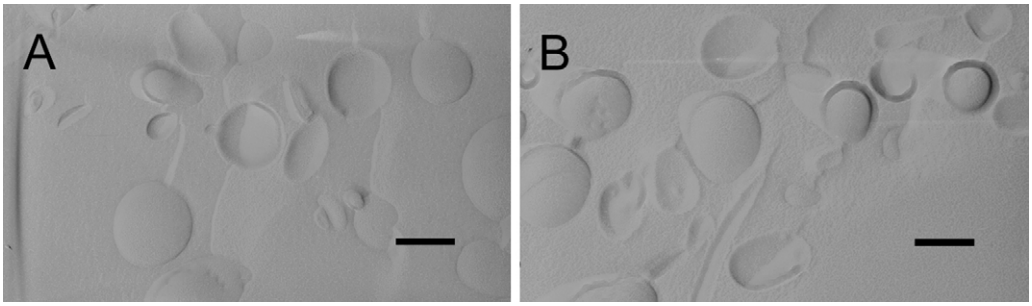

Fig. 1. Micrographs of (A) classical or (B) pH-sensitive PEGylated liposomes as observed on freeze-fracture replicas in transmission electron microscopy. Bars represent 0.125 μm.

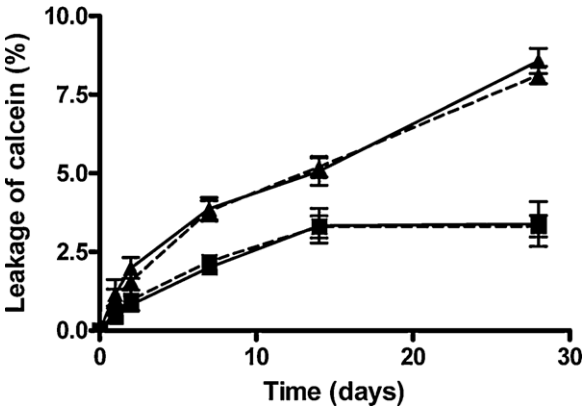

Fig. 2. Leakage of calcein from pH-sensitive liposomes (dotted lines) or standard liposomes (plain lines) as a function of time at 37 °C (full triangles) and 4 °C (full squares) in isotonic HEPES buffer. Data points represent mean values (±S.D.) of three independent experiments.

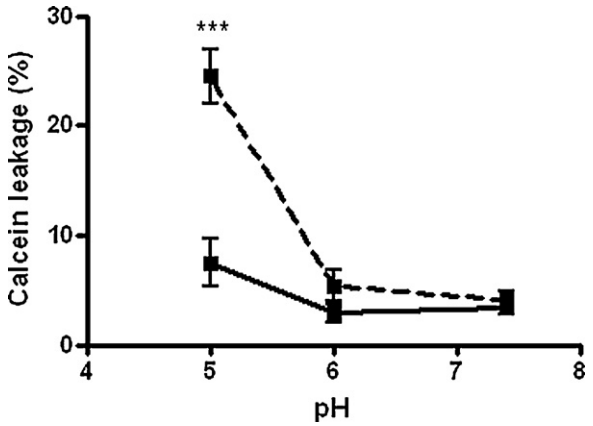

Fig. 3. Leakage of calcein from pH-sensitive liposomes (dotted lines) or standard liposomes (plain lines) as a function of the pH. Data points represent mean values (±S.D.) of three independent experiments.

2.3.3. Encapsulation efficiency

Encapsulation efficiency was expressed in terms of the quantity of peptide loaded into liposomes as a function of the quantity operated. This encapsulation efficiency (EE) was calculated by the following equation (Eq. (1)).

$$\text{EE}\ \ (\%) = \frac{\text{Amount of encapsulated Print3G}}{\text{Total amount of Print3G}} \times 100 \qquad (1)$$

where the "amount of encapsulated Print3G" is the amount of Print3G (in μg) loaded into liposomes and the "total amount of Print3G" is the amount of Print3G (in μg) implemented.

2.4. Stability

Calcein was encapsulated into vesicles in order to study the leakage of a hydrophilic encapsulated material. A 60 mM calcein isotonic solution was used to hydrate the lipid film (see Section 2.2.1) in order to detect a leakage-induced effect. After extrusion, external calcein was removed by 6 ultracentrifugation cycles at 35,000 rpm for 60–45–30–30–30–30 min at 4 °C using the Beckman SW60 rotor (86,496 × *g*).

Encapsulated calcein shows minimal fluorescence, owing to the formation of ground state dimers. Any fluorescence measured will be due to the leakage of calcein out of vesicles and dilution in the exterior aqueous media (Chen and Knutson, 1988). Fluorescence was measured after 0, 1, 2, 7, 14 and 28 days of storage at 4 °C and 37 °C. For the pH sensitivity study, the leakage of calcein as a function of the pH was measured after 6 h under stirring in phosphate buffers at pH 5, pH 6 and pH 7.4. In practice, 100 μl liposome suspension was added to 100 μl HEPES buffered solution or 100 μl of a 4% Triton X-100 solution, for complete liposome destruction, in a 96-well plate. Calcein release from liposomes was measured fluorometrically (SpectraMax Gemini XS); excitation and emission wavelengths were 490 and 520 nm, respectively. The amount of calcein released was calculated using the following equation (Eq. (2)):

$$\%\,\text{calcein released} = \frac{I}{I_t} \times 100 \qquad (2)$$

where I is the fluorescence intensity at 520 nm and I_t is the fluorescence intensity at 520 nm after complete destruction of the liposomes by Triton X-100.

2.5. Cellular uptake of liposomes and of their encapsulated material

2.5.1. Liposomes

In order to follow the intracellular delivery of the encapsulated material, calcein was used as hydrophilic fluorescent marker. These liposomes were prepared as described in Section 2.4, using a 40 mM isotonic solution of calcein. Intracellular penetration of the liposomes themselves was studied using 25-NBD-CHOL incorporated into the bilayer. These vesicles were prepared using the hydration of lipid film method. 300 μM of 25-NBD-CHOL in chloroform was added before evaporation under reduced pressure. Intracellular penetration of Print3G was studied using a biotinylated derivative of Print3G, which was incorporated into liposomes using the same methods as described in Section 2.3.1.

Final concentrations of lipids were 5 times lower than those used for blank liposomes (see Section 2.2.1).

2.5.2. Cell culture

The human cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). Hs578t and MDA-MB-231 were chosen as breast carcinoma cell lines, respectively non-tumorigenic and tumorigenic. The last cell line chosen, WI-26, is an SV-40 immortalized lung fibroblast cell line.

Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 4.5 g/l glucose, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. The three cell lines were seeded on glass coverslips in 24-well plates for CLSM analysis. For FACS studies, Hs578t cells and MDA-MB-231 cells were seeded respectively in 6-well and in 19-well plates.

After 24 h incubation, cells were grown to 70% confluence. The medium was removed before adding liposomes resuspended in serum-free DMEM.

2.5.3. Confocal microscopy analysis

Cells were treated with fluorescently labelled liposomes for various lengths of time (15 min, 2.5 h, 5 h or 15 h) at 37 °C in a humidified 5% CO_2 atmosphere. This step was followed by several washes with phosphate-buffered saline (PBS). Cells were then fixed in 4% paraformaldehyde in PBS for 15 min at 4 °C and rinsed in PBS. Streptavidin conjugate Alexa Fluor 555 was used as binding pair to link and reveal the biotinylated peptide. In practice, cells were permeabilized with 0.2% polysorbate 20 in PBS for 30 min at room temperature, incubated with streptavidin conjugate Alexa Fluor 555 at a dilution of 1:500 (v/v) for 30 min at room temperature

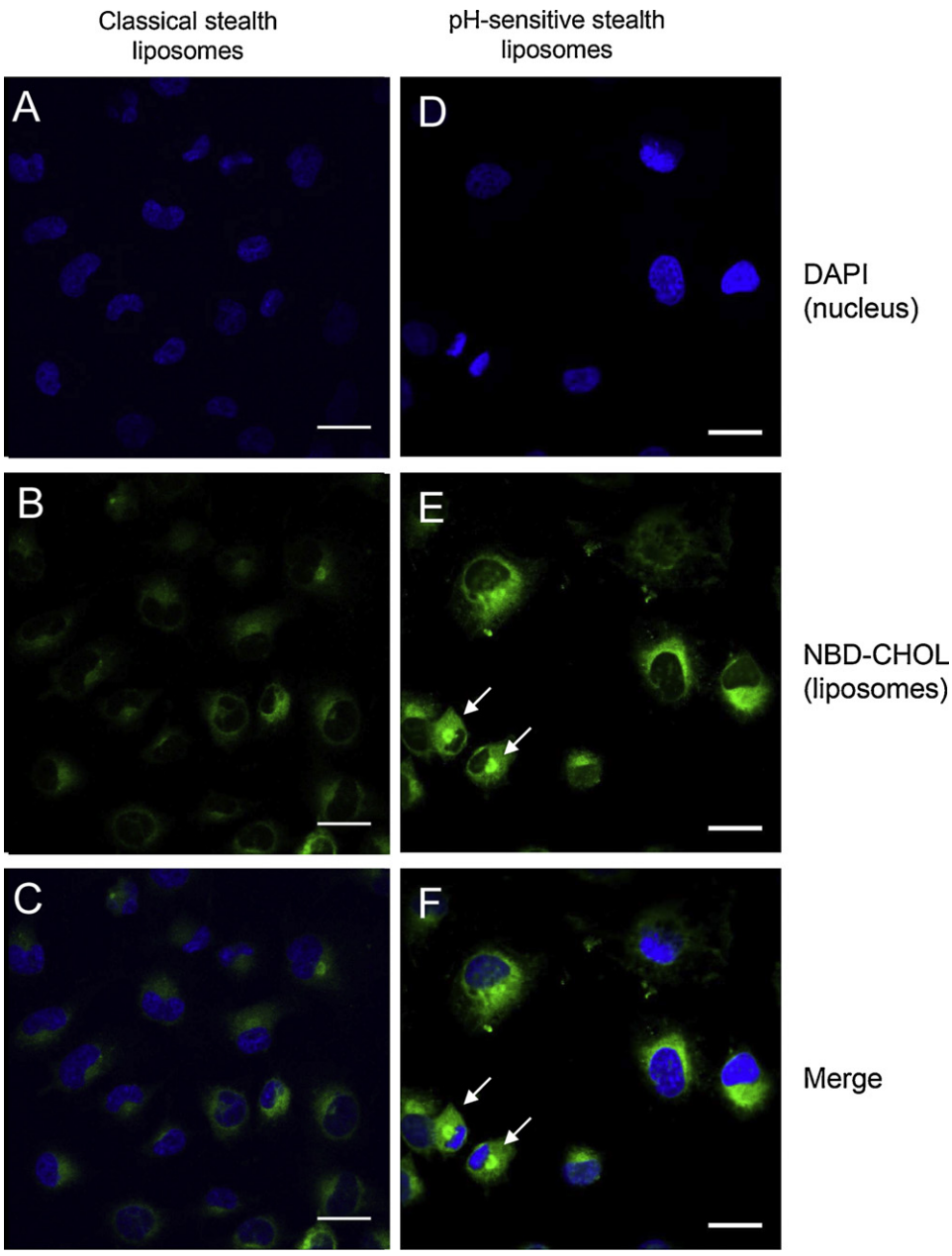

Fig. 4. Confocal laser microscopy analysis of Hs578t cells treated for 2.5 h with classical stealth liposomes incorporating NBD-CHOL (A–C) or with pH-sensitive stealth liposomes incorporating NBD-CHOL (D–F). (A and D) Nuclei blue staining with DAPI; (B and E) green staining of liposomes incorporating NBD-CHOL; (C and F) merge of DAPI and NBD-CHOL channels. Bars represent 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

and washed with PBS. The coverslips were finally mounted with Vectashield medium with DAPI onto glass slides for microscopic observations.

The location of intracellular fluorescence was validated using confocal laser scanning microscope (Olympus FluoView FV100). Several fluorescent molecules were used and observed at several excitation wavelengths: DAPI at 405 nm, calcein and 25-NBD-CHOL at 488 nm, Alexa Fluor 555 at 561 nm.

All optical sections were recorded with the same settings (in particular the laser power and the photomultiplier voltage) for each colour detected. Kalman collection filter 3-frame was used for each acquired image and for each colour.

2.5.4. Flow cytometry analysis

Flow cytometry analysis was conducted on liposomes containing calcein or NBD-CHOL and on empty liposomes without any fluorescent markers (negative controls). Fluorescence intensities and lipid content determination were used as liposome quality control before application on cells. Fluorescence intensities were measured using the SpectraMax Gemini XS; excitation and emission wavelengths were 490 and 520 nm, respectively. Lipid concentration was measured using the phospholipids *C* test (Wako Chemicals, LabAssay Phospholipid, choline oxidase—DAOS method, Wako Chemicals, Neuss, Germany) or the Cholesterol FS test (Dyasis Diagnostic Systems, Holzheim, Germany). Difference between pH-sensitive and classical formulations was less than 10% in terms of fluorescence and lipid concentration.

Cells were treated for 2.5 h with blank liposomes, liposomes containing NBD-CHOL or liposomes encapsulating calcein. After incubation at 37 °C, cells were washed twice with PBS and dissociated with 0.05% trypsin/EDTA. After a centrifugation step at

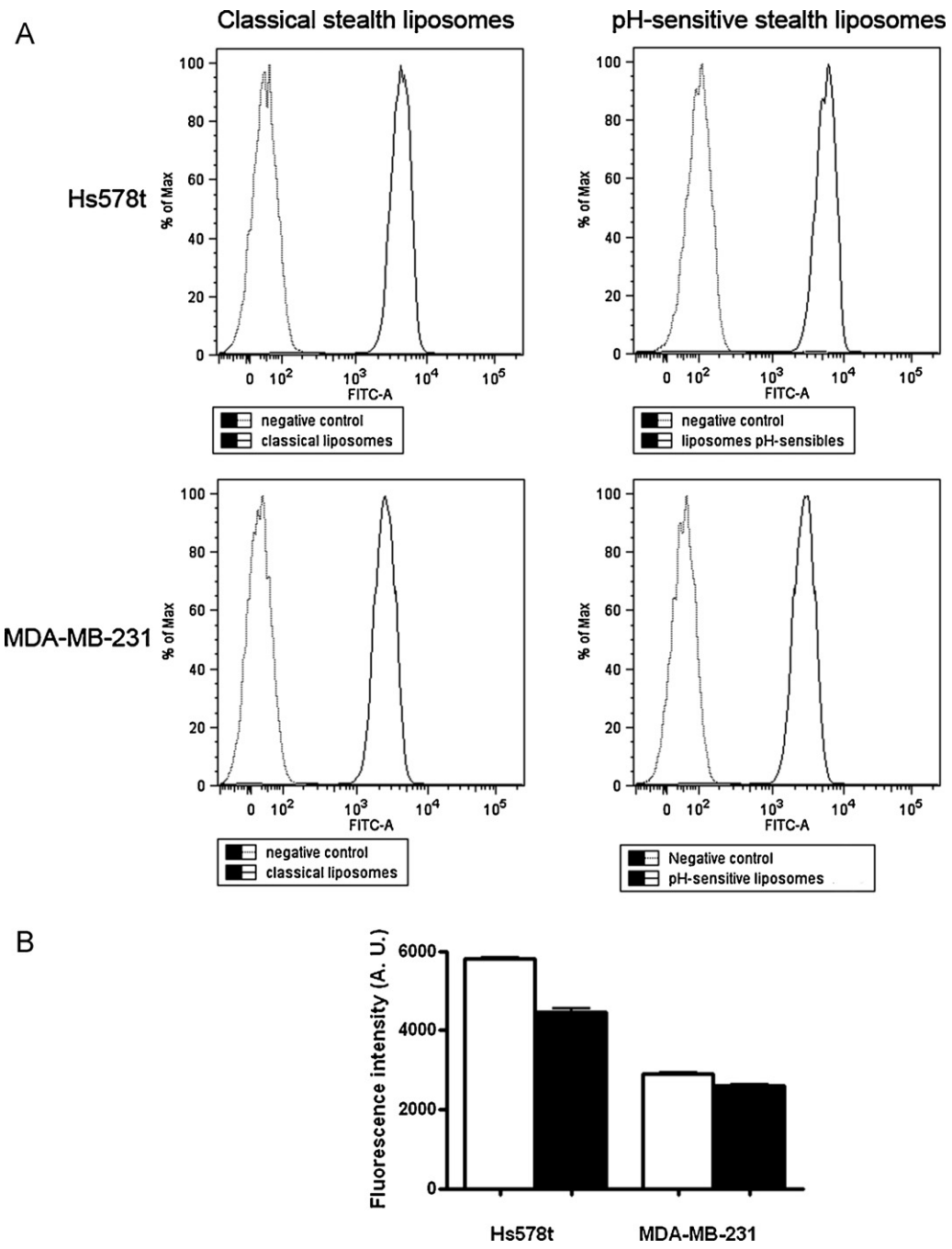

Fig. 5. Flow cytometry analysis of Hs578t or MDA-MB-231 cells treated for 2.5 h with classical or pH-sensitive PEGylated liposomes incorporating NBD-CHOL. (A) Representative FACS normalized histogram of liposomes without NBD-CHOL (doted lines) or NBD-CHOL-incorporating liposomes (plain lines). (B) NBD-CHOL-associated fluorescence (arbitrary unit) in Hs578t cells or MDA-MB-231 cells treated with classical (black) or pH-sensitive (white) liposomes incorporating NBD-CHOL.

1200 rpm for 5 min at 4 °C, supernatants were discarded and cell pellets were washed with cold PBS. Cells were then fixed in paraformaldehyde (4%, v/v) at 4 °C for 10 min and washed twice with cold PBS. Finally, cells were resuspended in 250 μl of cold PBS and kept on ice in the dark.

Analyses were performed using the flow cytometer FACS Canto II (Becton Dickinson, San Jose, CA, USA). The autofluorescence of each cell line was determined with non fluorescent blank liposomes. For calcein-loaded liposomes experiments ($n = 3$), a total of 10,000 events were recorded. For pH-sensitive ($n = 3$) and classical ($n = 2$) formulations of liposomes incorporating NBD-CHOL, a total of respectively 10,000 and 5000 events were recorded. Cell-associated calcein or NBD-CHOL was excited with 488-nm solid state laser (20 mW) and fluorescence was detected using 502LP and 530/30 filters. Data were analysed using FACSDiva and FlowJo software programs.

2.6. Statistical analysis

Assuming Gaussian distribution and homoscedasticity, the statistical significance of the results in the liposome integrity section was tested using the student's *t*-test. Values are presented as mean ± standard deviation. For the measurements of calcein leakage in the stability study, multiple comparisons were performed

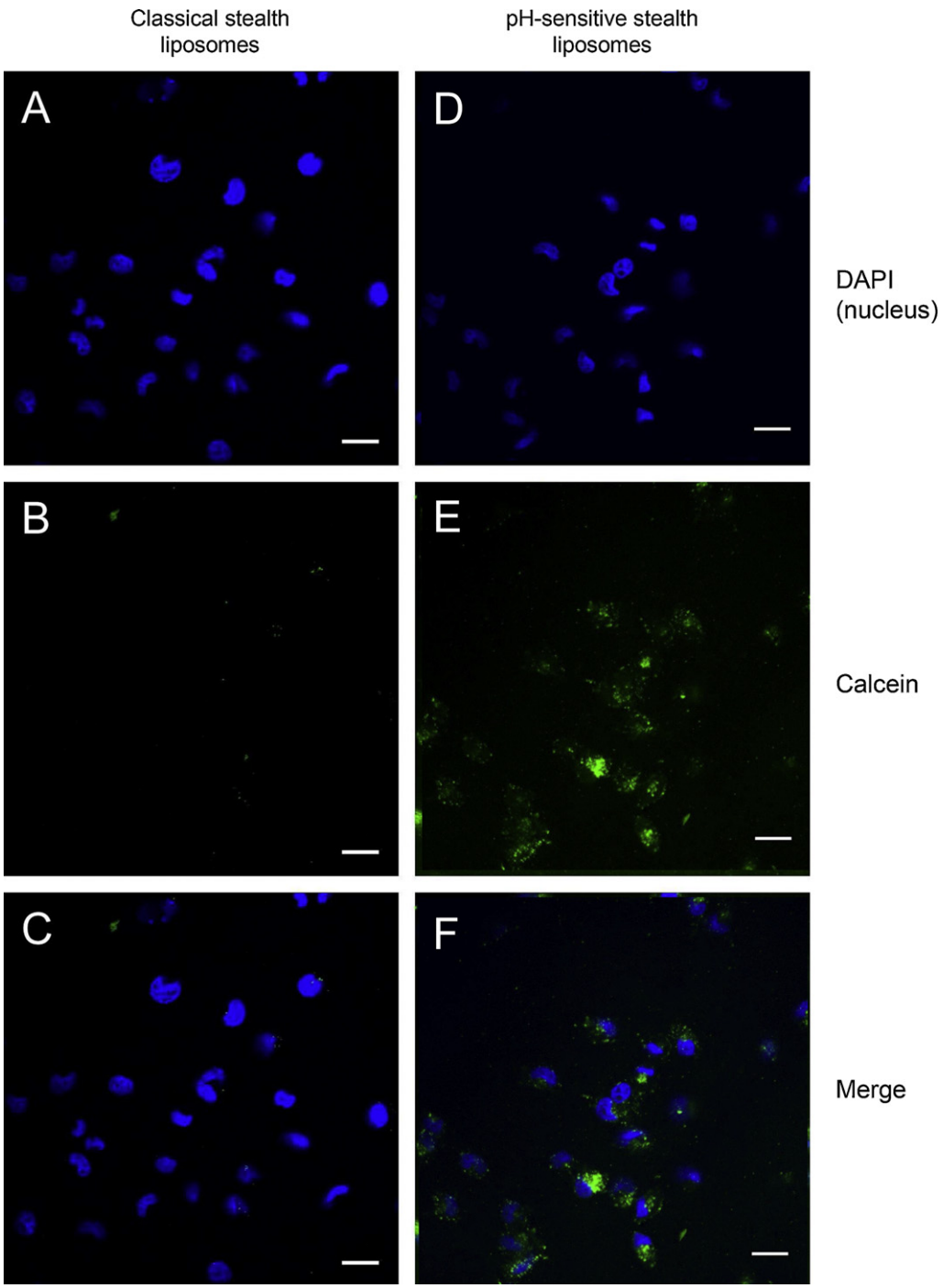

Fig. 6. Confocal laser microscopy analysis of Hs578t cells treated for 2.5 h with classical stealth liposomes (A–C) or with pH-sensitive stealth liposomes (D–F) encapsulating calcein. (A and D) Nuclei blue staining with DAPI; (B and E) green staining of liposomes containing calcein; (C and F) merge of DAPI and calcein channels. Bars represent 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

using a one-way ANOVA. A p value of less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Liposome characterization

Unilamellar liposomes were first characterized using the PCS technique. Data on size and zeta potentials of both formulations of empty liposomes, liposomes loaded with calcein and liposomes encapsulating Print3G are presented in Table 1. Sizes of 172.6 ± 14.7 nm were obtained for blank liposomes of classical composition while pH-sensitive liposomes gave sizes around 150.4 ± 14.1 nm, with a PDI always lower than 0.2 (Table 1). Blank stealth pH-sensitive liposomes are significantly smaller than the classical formulation ($p < 0.01$). Loaded-liposomes were comparable in size regardless of the type of formulation ($p > 0.05$). These sizes were acceptable for the following experiments and were consistent with the concept of passive accumulation in tumor tissues by the EPR effect. Zeta potentials of -8.0 ± 3.9 mV and of -16.3 ± 5.1 mV were respectively obtained for blank classical and

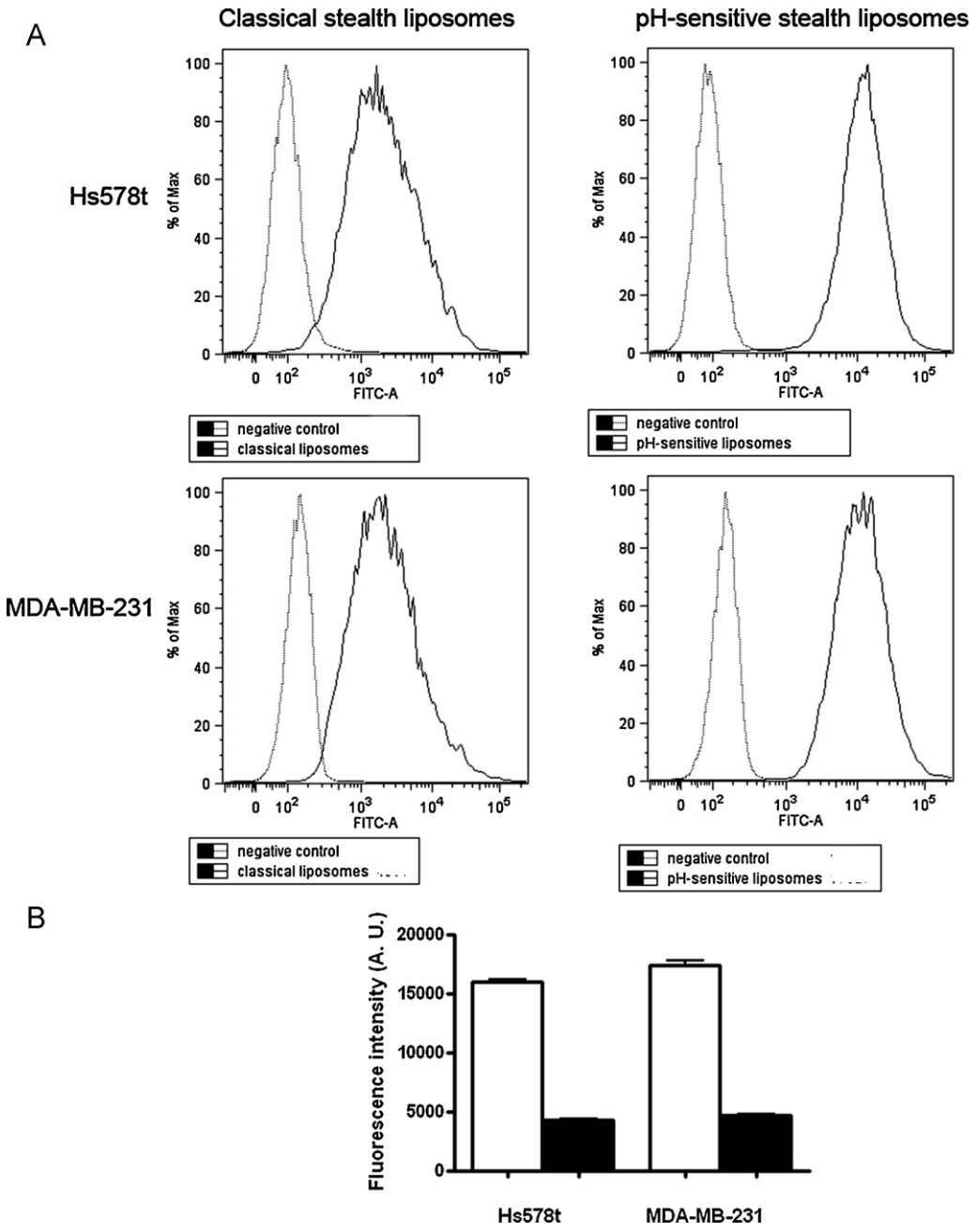

Fig. 7. Flow cytometry analysis of Hs578t or MDA-MB-231 cells treated for 2.5 h with classical or pH-sensitive stealth liposomes encapsulating calcein. (A) Representative FACS normalized histograms of empty (dotted line) or calcein-loaded (plain line) liposome-treated cells. (B) Calcein-associated fluorescence (arbitrary unit) in Hs578t or MDA-MB-231 cells treated with classical (black) or pH-sensitive (white) calcein-loaded liposomes. Results represent mean values (±S.D.) of 3 independent experiments.

blank pH-sensitive stealth liposomes. Liposomes containing calcein or Print3G follow the same tendency. The relatively negative net charge obtained for pH-sensitive liposomes is conferred by the amphiphilic stabilizer (CHEMS), not completely masked by the protective layer constituted by PEGs.

Freeze-fracture electron microscopy allowed the visualization of the shape of the two types of liposomes. Unilamellar vesicles of classical or pH-sensitive stealth liposomes are shown in Fig. 1.

These experiments allowed the visualization of the round shape of vesicles, sometimes elongated, their relatively close size distribution width and their size, close to 130 μm in diameter. PCS gives hydrodynamic diameters, resulting in higher sizes than those given by freeze-fracture electron microscopy.

3.2. Encapsulation efficiency

Print3G is a hydrophilic peptide freely soluble in water, as such it is encapsulated in the inner aqueous cavity of liposomes. In order to encapsulate Print3G into the formulation of classical stealth liposomes, blank liposomes were mixed with a Print3G solution, then freeze-thawed to promote the entry of the peptide into the vesicles. The encapsulation efficiency with this first formulation amounted to $62.68 \pm 2.93\%$ ($n = 3$). This encapsulation technique had been described in a previous study by Ducat et al. (2010), using the experimental design to improve peptide entrapment.

This method could not be used to encapsulate Print3G into pH-sensitive PEGylated liposomes because liposome destruction after

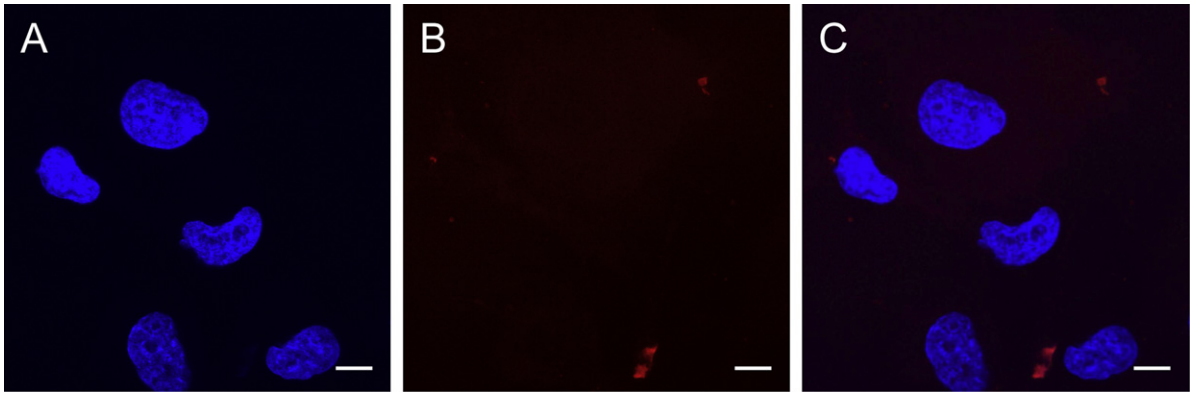

Fig. 8. Confocal laser microscopy analysis of Hs578t cells treated for 5 h with free biotinylated Print3G. (A) Nuclei blue staining with DAPI; (B) free biotinylated Print3G red staining; (C) merge of blue and red channels. Bars represent 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

freeze-thawing cycles was observed by PCS measurements. High encapsulation efficiency for pH-sensitive liposomes was obtained using the hydration of lipid film method associated with presaturation of the extruder by a Print3G solution. The encapsulation efficiency amounted to 90.69 ± 5.65% ($n = 4$). Both formulations possess comparable sizes and shapes and good encapsulation efficiencies of Print3G. Even if two protocols are used, the final formulations are similar. The encapsulation efficiency of Print3G in the pH-sensitive formulation was found to be significantly better than in the classical one ($p < 0.05$). This could be explained by the presence of more negative charges in pH-sensitive liposomes, which could promote the entrapment of Print3G, presenting a theoretical positive charge of +6.1.

3.3. Stability

A one month stability following the leakage of calcein from the two types of liposomes was conducted at physiological temperature and at 4 °C. As shown in Fig. 2, a significantly higher leakage of the encapsulated material was observed at 37 °C than at 4 °C after 30 days ($p < 0.05$). No significant difference was observed between the two formulations. The leakage of calcein did not exceed 3% after one month at 4 °C.

For the study of the influence of pH on stability (Fig. 3), liposomes containing pH-sensitive lipids showed a leakage of calcein significantly higher at pH 5 (ANOVA-1, $p < 0.05$).

However, there was no significant difference between results at pH 6 and pH 7 ($p > 0.05$). Moreover, compared to classical liposomes, the leakage of calcein after 6 h at pH 5 was significantly higher ($p < 0.05$). These results are linked to the composition of liposomes. Long circulating pH-sensitive liposomes contain DOPE which exhibits a conical shape and which is associated with a compound used as a stabilizer of the liposomal membrane: CHEMS. This molecule possesses an inverted cone shape at physiological pH, but, under acidic conditions, the carboxylic acid group becomes protonated and loses this particular shape. The pH decrease destabilizes the liposomal membrane, transiting from a lamellar to a hexagonal phase. The actual data could not be compared with the results found in other studies in the literature because various protocols were followed in those cases to evaluate the pH sensitivity of liposomes. Nevertheless, our results are consistent with those reported by Simoes et al. (2001). We can hypothesize that the pH-sensitivity of this formulation is partially hampered because pH-sensitive liposomes made of only DOPE and CHEMS release almost 85% of their calcein content at pH 5 (Simoes et al., 2001, 2004). Johnsson and Edwards (2001) showed in their studies that adding PEG-lipids to a system made of DOPE alone, could stabilize the lamellar phase. Despite these observations, we measured a significant increase of calcein release by pH-sensitive formulation compared to the classical one. The use of PEG of lower molecular weight than those usually used to obtain long circulating pH-sensitive liposomes, such as PEG_{2000}, could explain why pH-sensitive liposomes containing DSPE-PEG_{750} keep a significant sensitivity at low pH.

So as a compromise must be found between intracellular delivery and protection against opsonization, this pH-sensitivity study allows us expecting a better efficacy of transfection for pH-sensitive formulation than for the classical one, enabling a leakage of the drug into the cytoplasm and avoiding enzymatic degradation.

3.4. Cellular uptake of liposomes and of their encapsulated material

The confocal laser scanning microscopy (CLSM) study allowed evaluating the intracellular fate of liposomes themselves and of their encapsulated material as a function of time and in various cell lines. CLSM offers the major benefit of optically sectioning the cell. Simple fluorescence microscopy can distort the reality and possibly confuse fluorescent compounds in the cytoplasm/nucleus with those adsorbed on the cellular surface. CLSM ensures that liposomes and/or their content have actually penetrated the cell (Mady et al., 2009). Note that all the pictures used to compare formulations were acquired at the same settings (PMT and %laser).

CLSM is frequently associated with Fluorescence Activated Cell Sorting (FACS) to provide quantitative results related to the transfection of vectors in cells. FACS produces simultaneously multiple optical measurements on individual cells at high rates, allowing sorting of cells that meet specific criteria. Herein, FACS study was conducted on Hs578t and MDA-MB-231 cell lines to compare the internalization of classical and pH-sensitive stealth liposomes.

3.4.1. Liposomes containing NBD-CHOL

The penetration of liposomes themselves was studied using NBD-CHOL as a fluorescent marker of the phospholipid membrane. Indeed, cholesterol is the only common compound of both formulations that is inserted in the phospholipids bilayer of classical and pH-sensitive liposomes. NBD-CHOL was added at the same molar ratio in the two formulations. Therefore, it was a suitable compound to label fluorescently the phospholipids membrane of liposomes in order to follow their intracellular fate.

Results obtained using CLSM to evaluate the cellular uptake of NBD-CHOL liposomes by Hs578t cells are presented in Fig. 4. Fig. 4E shows a strong green fluorescence in the cytoplasm of Hs578t cells with a brighter area near the nucleus, following the application of stealth pH-sensitive liposomes. When cells were treated

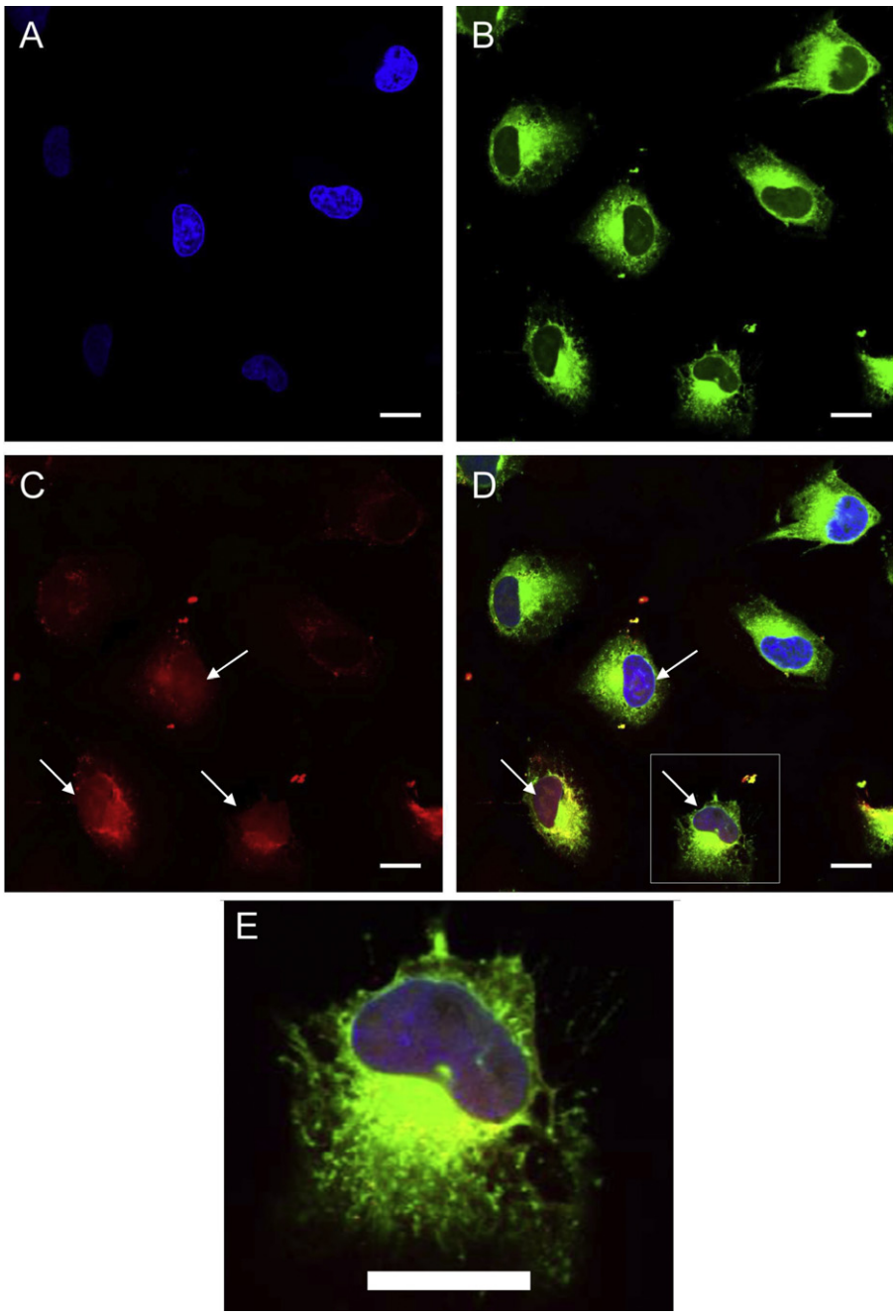

Fig. 9. Confocal laser microscopy analysis of Hs578t cells treated for 2.5 h with long circulating pH-sensitive NBD-CHOL liposomes containing biotinylated Print3G. (A) Nuclei blue staining with DAPI; (B) NBD-CHOL green staining of liposomes; (C) biotinylated Print3G red staining; (D) merge of blue, green and red channels. Bars represent 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

with the classical formulation of stealth liposomes (Fig. 4B), the cytoplasmic fluorescence was weaker and no brighter area was observed near the nucleus. Results obtained for penetration of fluorescent-labelled liposomes within WI-26 cells were similar to those obtained with Hs578t cells while difference between the two formulations was not discernible in MDA-MB-231 cells (data not shown).

To support these CLSM results, FACS study was carried out on Hs578t cells and MDA-MB-231 cells (Fig. 5). Results obtained with Hs578t cells showed that long circulating pH-sensitive liposomes gave fluorescence intensities of 5804 ± 73, higher than those obtained for classical stealth liposomes (4705 ± 109). When liposomes were applied on MDA-MB-231 cells, fluorescence intensities of 2966 ± 12 were obtained using long circulating pH-sensitive liposomes and of 2496 ± 45 for the classical formulation of stealth liposomes. These results confirmed those obtained by confocal microscopy. CLSM and FACS results indicate that long circulating pH-sensitive liposomes penetrate in a higher quantity in Hs578t cells.

This difference could be explained by the fact that the lipids employed in this formulation should allow a better interaction with the cellular membrane (Huth et al., 2006). DOPE is a non bilayer, fusion-promoting lipid, because it can adopt inverted lipid phases and then promote the fusion of lipid bilayers while the presence of PEG could decrease this DOPE capacity (Hafez and Cullis, 2001). Indeed, such a fusion process requires a very close contact between the two membrane structures (cell and liposome). Dos Santos et al. explained that PEG_{750} do not cover completely the

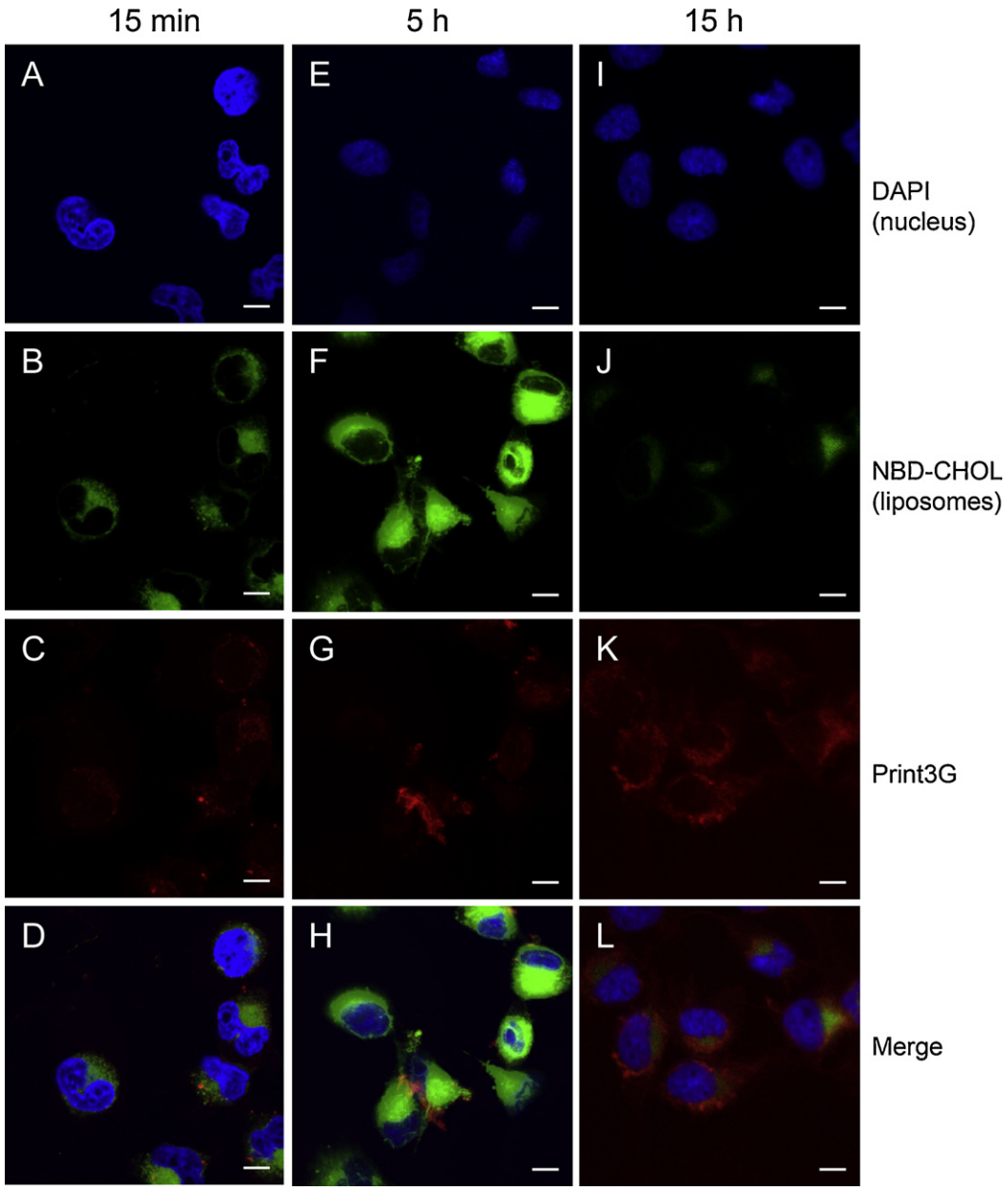

Fig. 10. Confocal laser microscopy analysis of Hs578t cells treated for 15 min (A–D), 5 h (E–H) and 15 h (I–L) with classical stealth liposomes incorporating NBD-CHOL and encapsulating the biotinylated Print3G. (A, E, and I) Nuclei blue staining with DAPI; (B, F, and J) NBD-CHOL green staining of liposomes; (C, G, and K) biotinylated Print3G red staining; (D, H, and L) merge of blue, green and red channels. Bars represent 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

liposome surface, in opposition to PEG_{2000} (Dos Santos et al., 2007). Moreover, their results suggest that a complete surface coverage is not required for PEG to cause increased circulation time. If PEG_{750} does not cover totally the surface of pH-sensitive liposomes, it could remain some regions of interactions between DOPE and cellular/endosomal membrane, but also a significant pH-sensitivity. The use of DSPE-PEG_{750}, of lower molecular weight, could allow keeping an effective interaction of DOPE with the cellular membrane, explaining the better internalization of the pH-sensitive formulation.

3.4.2. Calcein-loaded liposomes

The penetration of calcein-loaded liposomes was studied in order to better understand the intracellular delivery mechanism of an encapsulated material in classical or pH-sensitive stealth liposomes. Hydrophilic model molecule calcein (M.W. = 376; log P = −5.219; 492/517 nm) was entrapped at a self-quenched concentration within the two formulations. The use of calcein as a model molecule could be justified by two ways. Firstly, calcein is encapsulated in the inner cavity of liposomes because of its hydrophilicity. Print3G, which is also water-soluble, is entrapped in the same liposome compartment. Secondly, calcein is a self-quenching fluorescent dye, often used to study the endosomal escape of hydrophilic compounds triggered by pH-sensitive liposomes. Concentration quenching of calcein is a complex phenomenon, based on dimerization and energy transfer to dimer (Chen and Knutson, 1988; Weinstein et al., 1977; Duzgunes et al., 2003). Calcein, encapsulated at high concentration, is virtually non-fluorescent, owing to the formation of ground state dimers. Any fluorescence measured will be due to the leakage of calcein out

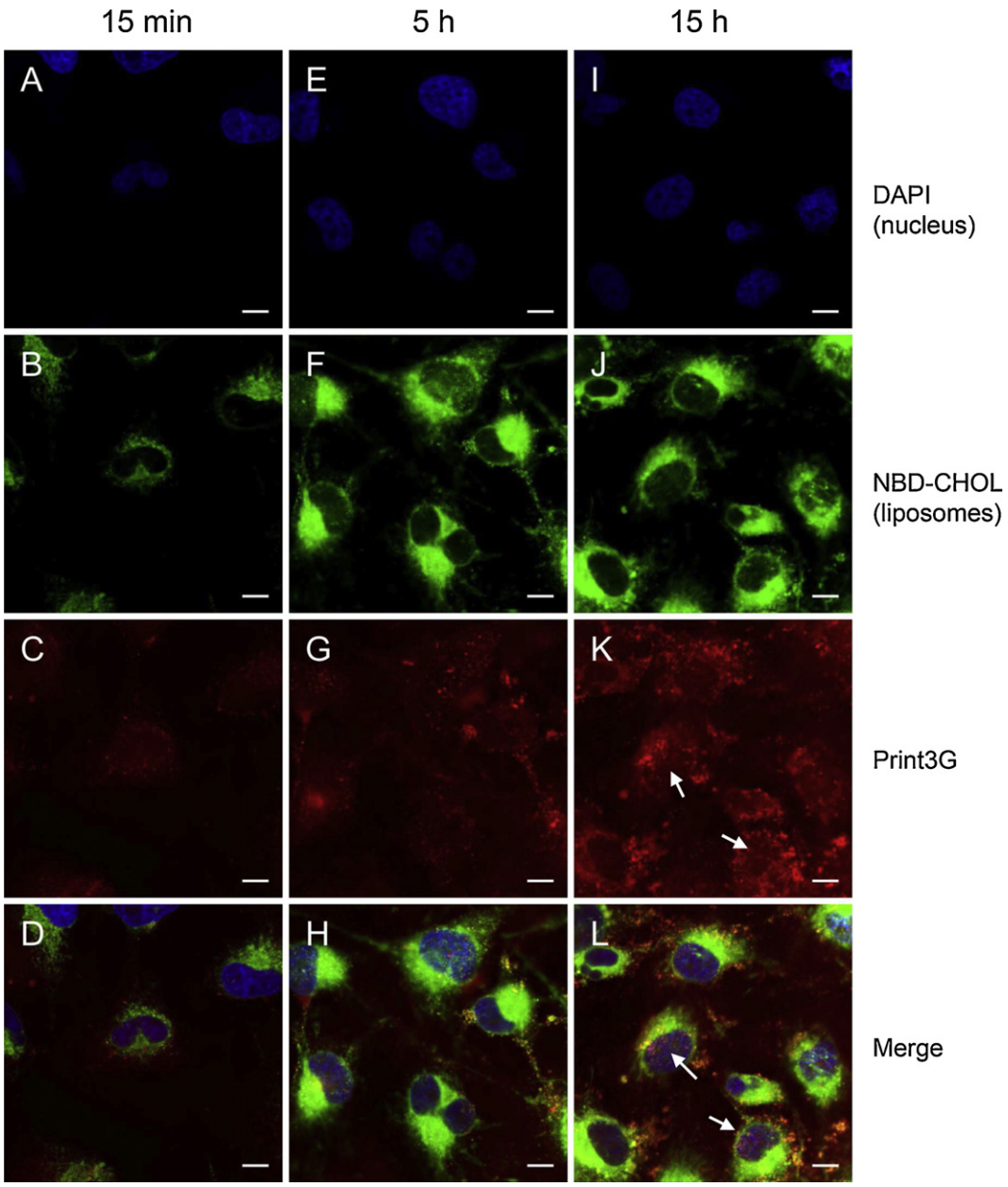

Fig. 11. Confocal laser microscopy analysis of Hs578t cells treated for 15 min (A–D), 5 h (E–H) and 15 h (I–L) with pH-sensitive stealth liposomes incorporating NBD-CHOL and encapsulating the biotinylated Print3G. (A, E, and I) Nuclei blue staining with DAPI; (B, F, and J) NBD-CHOL green staining of liposomes; (C, G, and K) biotinylated Print3G red staining; (D, H, and L) merge of blue, green and red channels. Bars represent 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

of vesicles and dilution in the exterior aqueous media. Regain of fluorescence happens when liposomes are taken up by cells and lysed.

Fig. 6 shows CLSM images illustrating the cellular uptake of calcein-loaded liposomes by Hs578t cells. As shown in Fig. 6B, calcein fluorescence is weak when using classical liposomes. By contrast, the majority of cells were found to be fluorescent when calcein-loaded pH-sensitive liposomes were applied onto cells (Fig. 6). Song et al. (2009) observed this fact using a classical formulation of doxorubicin in PEGylated liposomes. They evoked the possibility of self-quenched doxorubicin presenting a low fluorescence, not discernible in cells. In our study, calcein is encapsulated at a self-quenched concentration and may not be distinguished in the picture. Due to the lowering of the pH, a significant fraction of calcein is delivered from pH-sensitive stealth liposomes, from endosomes to cytoplasm, allowing observing a bright fluorescence.

As it can be also observed in Fig. 6E, fluorescence is sometimes doted, which might suggest that it remains some calcein entrapped in endosomes. But we can conclude that a significant amount of the dye is delivered in cytoplasm because of the bright fluorescence observed. If calcein remained totally in endosomes, the fluorescence should be significantly lower and pictures should be like Fig. 6B, obtained with the classical formulation.

FACS study was conducted on Hs578t cells and MDA-MB-231 cells to confirm results obtained by CLSM study (Fig. 7). Fluorescence intensities in positive Hs578t cells were respectively of $15{,}973 \pm 333$ for cells treated with pH-sensitive stealth liposomes and of 4217 ± 243 for cells treated with classical stealth liposomes. In positive MDA-MB-231 cells, fluorescence intensities amounted to $17{,}357 \pm 716$ for pH-sensitive stealth liposomes and to 4663 ± 96 for the classical formulation. Fluorescence intensity obtained for the pH-sensitive formulation was significantly higher

($p < 0.001$) comparing to the classical one, regardless of the type of cell observed. These results confirmed those obtained using CLSM, the calcein delivery is more efficient using the pH-sensitive formulation. Therefore, calcein is not released in the cytoplasm but remains sequestrated in classical stealth liposomes. On the contrary, pH-sensitive stealth liposomes escape from the endosome by disruption of their membrane, according to their mechanism of action, and then release their encapsulating material in the cytoplasm of cells. Then, calcein could be diluted in the cytoplasm and present a significant fluorescence on confocal pictures.

3.4.3. Liposomes encapsulating Print3G

The two first studies using both CLSM and FACS techniques allowed us to expect a better efficacy of PEGylated pH-sensitive liposomes as Print3G vectors than the classical formulation. However, calcein is not the perfect model molecule to predict the intracellular fate of the peptide and the next step of our study was to investigate the intracellular fate of the peptide, which has to reach the nucleus in order to interact with its target. Biotin was covalently grafted on Print3G in order to follow it to the cell nucleus using streptavidin conjugate Alexa Fluor 555.

3.4.3.1. Free Print3G. Firstly, the penetration of the free biotinylated peptide was evaluated at a concentration equivalent to that present in liposomes used for further experiments. Results are shown in Fig. 8, revealing the weak penetration of the unencapsulated biotinylated peptide.

3.4.3.2. Encapsulated Print3G. Long circulating pH-sensitive NBD-CHOL liposomes encapsulating biotinylated Print3G were then applied onto Hs578t cells for 2.5 h. Results are shown in Fig. 9. As it has been observed in our previous confocal experiments Fig. 9B shows a small region near the nucleus with a brighter fluorescence, indicating an accumulation of NBD-CHOL liposomes at this position. In Fig. 9C, an accumulation of the peptide clearly appears in some nuclei. The use of pH-sensitive liposomes allows to observe a significant amount of peptide escaping from the endosome by disruption of the liposomal membrane. The peptide can then diffuse freely from the cytoplasm to the nucleus of cells.

In order to discern a difference between classical and pH-sensitive long circulating liposomes in terms of sequestrated peptide delivery from the endosome to the cytoplasm and to investigate this phenomenon kinetic, Hs578t cells were incubated with classical or pH-sensitive liposomes for a period of 15 min, 5 h and 15 h. Confocal images obtained with the classical formulation of long circulating liposomes, incorporating NBD-CHOL in the phospholipid bilayer and the biotinylated peptide in their aqueous cavity, are shown in Fig. 10. Comparing Fig. 10B, F and J, the incursion of liposomes by themselves can be followed in the cytoplasm, peaking after 5 h then decreasing, providing an image comparable to those obtained in the first experiments with NBD-CHOL and classical liposomes. Penetration of the peptide (Fig. 10C, G and K) increases slightly but no fluorescence was observed in the cell nuclei.

These experiments were also performed with a formulation of stealth pH-sensitive liposomes. Results are shown in Fig. 11. Fig. 11B, F and J shows an increasing uptake of pH-sensitive liposomes as a function of time with a high fluorescence that remains intense for at least 15 h. The penetration of the peptide is also different from the one observed with classical liposomes: the peptide can enter the cell and reach the nucleus (Fig. 11C, G and K). The presence of Print3G in the nucleus of cells treated with pH-sensitive liposomes and its absence in cell nuclei when treated with the classical formulation still emphasize our arguments. So as for calcein experiments (see Sections 3.3 and 3.4.2), this difference could be explained by the particular mechanism of action of pH-sensitive liposomes, which is efficient to deliver the peptide, avoiding its degradation. While calcein or Print3G remain entrapped in endosome compartment when encapsulated in the classical formulation – observed respectively by a minimal fluorescence for calcein experiments and by a cytoplasm localization for peptide experiments – the pH-sensitive formulation allow the delivery of its encapsulated material in the cytoplasm, resulting respectively in a detectable fluorescence and in a nuclear localization of Print3G. The nucleus localization of the peptide after treatment with pH-sensitive formulation cannot be explained by the polysorbate permeabilization step. Indeed, the same permeabilization step was used when cells were treated with classical formulation but no peptide was observed in the nuclei even 15 h after liposomes delivery.

When experiments were conducted on MDA-MB-231 cell line, the presence of peptide in cells was observed after treatment with pH-sensitive stealth liposomes encapsulating Print3G (data not shown). The peptide was detected in the nucleus of the two cancerous cell lines.

4. Conclusion

Classical and pH-sensitive stealth liposomes possess acceptable size, shape, encapsulation efficiency and stability to be potential vectors for Print3G delivery. However, the use of CLSM and FACS in this study highlights the important benefit of pH-sensitive liposomes over classical ones. Indeed, stealth pH-sensitive liposomes can penetrate by themselves all the cell lines tested and deliver more efficiently hydrophilic materials to the cytoplasm of cancerous cells. This better efficacy could be explained by the particular composition of liposomes allowing a better interaction with cellular membrane and an endosomal escape of entrapped materials. The use of PEG_{750} replacing the widely used PEG_{2000} to formulate long-circulating liposomes is probably the key for the maintenance of a sufficient interaction with cellular membrane and pH-sensitivity. Most importantly, the better capacity of long circulating pH-sensitive liposomes to mediate intracellular delivery allow observing the delivery of the therapeutic peptide to the nucleus of tumorigenic and non tumorigenic breast cancer cells, something not observed with classical liposomes encapsulating Print3G. These results were obtained using liposomes formulated to maintain a long circulation lifetime and our study proved that the penetration of stealth pH-sensitive liposomes remains efficient in spite of their PEGylation. This study proves the benefit of developing stealth pH-sensitive formulations for peptide delivery.

Acknowledgements

This study was supported by the Région Wallonne. We are grateful to P. Compere for helping us to carry out the freeze-fracture electron microscopy study. We are also very thankful to the GIGA Cell Imaging and Flow Cytometry Platform for their technical help in CLSM and FACS studies.

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