International Journal of Pharmaceutics 403 (2011) 230–236

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Polyvalent catanionic vesicles: Exploring the drug delivery mechanisms

Ariane Boudier[a], Pauline Castagnos[a], Elodie Soussan[a], Grégory Beaune[b], Haouaria Belkhelfa[c], Christine Ménager[b], Valérie Cabuil[b], Laila Haddioui[c], Christine Roques[d], Isabelle Rico-Lattes[a,*], Muriel Blanzat[a,*]

[a] Laboratoire IMRCP, UMR CNRS 5623, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex 9, France
[b] UPMC Univ. Paris 06/CNRS/ESPCI/UMR7195, Laboratoire Physicochimie des Electrolytes, Colloïdes et Sciences Analytiques – PECSA, 4 place Jussieu, Paris 75252, France
[c] Fonderephar, 35 chemin des Maraîchers, 31062 Toulouse Cedex 9, France
[d] Laboratoire de Microbiologie Industrielle, Faculté des Sciences Pharmaceutiques, 35 chemin des Maraîchers, 31062 Toulouse Cedex 9, France

ARTICLE INFO

Article history:
Received 15 March 2010
Received in revised form
24 September 2010
Accepted 18 October 2010
Available online 23 October 2010

Keywords:
Catanionic vesicles
Drug delivery
Phagocytic and non-phagocytic cells
Membrane fusion
Endocytosis
Internalisation modelisation

ABSTRACT

Among drug delivery systems, catanionic vesicles now appear as powerful candidates for pharmaceutical applications because they are relatively cheap and easy to use, thus well corresponding to industrial requirements. Using labelled vesicles made of a tricatenar catanionic surfactant, the work reported here aims at exploring the mechanisms by which internalisation into a cell occurs. The study was performed on various cell types such as phagocytic as well as non-phagocytic cells using confocal laser scanning microscopy and flow cytometry. Using various inhibitors, endocytosis and also a passive process, as probably fusion, were highlighted as interaction phenomena between catanionic vesicles and cell membranes. Finally, the interaction modelled with giant liposomes as membrane models confirmed the hypothesis of the occurrence of a fusion phenomenon between the nanovectors and cell membranes. This process highlights the potential of catanionic vesicles for a future pharmaceutical application as a universal drug delivery system.

1. Introduction

The nanoparticle world aims more and more at developing new drug delivery systems. Since 1990s, more or less organised micro- or nano-objects (Moses et al., 2003) following the example of liposomes have become interesting alternatives to classical drugs in the therapeutic approach of various pathologies such as infectious diseases and cancer with commercialised products (Allen and Cullis, 2004; Faraji and Wipf, 2009). Thus liposomes have demonstrated numerous properties interesting the pharmaceutical domain particularly the modification of the pharmacokinetic of the drugs (Allen and Cullis, 2004; Faraji and Wipf, 2009; Khalil et al., 2006; Moses et al., 2003; Pinto-Alphandary et al., 2000).

Among soft matter systems, catanionic vesicles have recently attracted attention. These colloidal systems are vesicles constituted of "catanionic" surfactants, namely characterized by the association of ionic surfactants of opposite charges in aqueous solution. Since they possess a vesicular structure similar to liposomes, catanionic vesicles are as efficient to vectorise hydrophilic and hydrophobic substances and likewise insure protection of encapsulated drugs, reduction of their toxicity and improvement of their efficiency duration (Soussan et al., 2008). In addition, they are easily formulated in aqueous solution by mixing cationic and anionic surfactants, which are considerably cheaper than phospholipids constituting liposomes. Aggregation into vesicles is spontaneous when water is added to the lyophilized monomeric catanionic surfactant (Soussan et al., 2008; Consola et al., 2007; Vivares et al., 2008) whereas liposomes formulation requires a complex preparation using specific material (Yamada et al., 2006; Tsumoto et al., 2009). The catanionic vesicles' formulation in aqueous solution is spontaneous, showing more stable properties on a thermodynamical point of view than liposomes. They are stable in solution up to few days and they can be stored for several years at room temperature in the lyophilized form without any degradation. These catanionic systems can thus be considered as simple and cheap drug carriers of particular interest on an industrial scale.

However, the efficiency of these systems has never been questioned from a mechanistic point of view. Up to now, few works have described the interaction of catanionic vesicles on cells (Kuo et al., 2005, 2008; Park et al., 2008; Soussan, 2007; Vlachy et al., 2009). Nevertheless, to optimize these systems for drug delivery, it is now crucial to elucidate the process involved in the interaction between this new type of catanionic vesicles and cell membranes (Hillaireau

* Corresponding authors: Tel.: +33 561 556 270; fax: +33 561 558 155.
E-mail addresses: rico@chimie.ups-tlse.fr (I. Rico-Lattes), blanzat@chimie.ups-tlse.fr (M. Blanzat).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.10.024

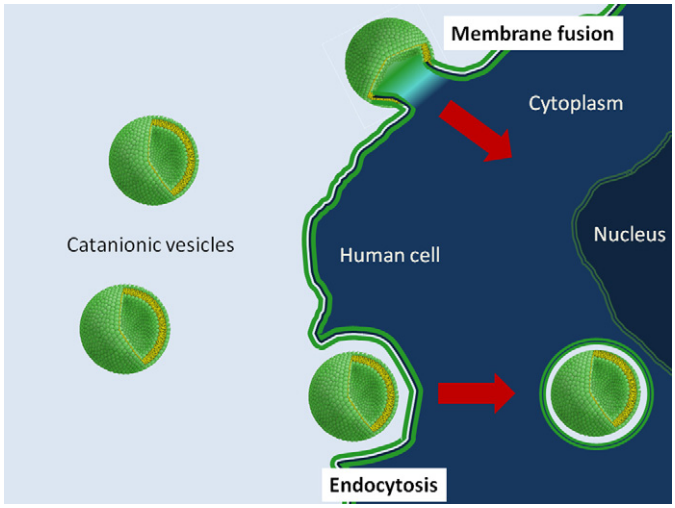

Fig. 1. Interaction between catanionic and cell membranes: fusion and/or endocytosis.

and Couvreur, 2009). As amphiphilic surfactants are comparable to phospholipids (Bonacucina et al., 2009), two pathways of interactions can be imagined between catanionic vesicles and cells: endocytosis and/or fusion (Fig. 1). In this way, various types of cells, primaries, cell lines, characterized or not by a phagocytosis activity were treated with endocytosis inhibitors. A modelisation of the vesicle/membranes interaction with giant liposomes was also performed to study the processes by which the internalisation occurs. Therefore, this study aims at providing insights for going further into the understanding of the mechanisms of drug delivery involving catanionic systems, and to demonstrate the polyvalence in terms of pharmaceutical applications of such a simple, cost effective formulation of catanionic vesicles.

2. Materials and methods

2.1. TriCat synthesis

The tricatenar catanionic surfactant 1-*N*-hexadecylammonium-1-deoxylactitol-bis(R-hydroxydodecylphosphinate), so-called "TriCat", is obtained via a spontaneous acid–base reaction in water between *N*-hexadecylamino-1-deoxylactitol-1 and bis(hydroxydodecyl)phosphinic acid 2, followed by pH measurements until stabilization, already set up in the literature (Soussan et al., 2008).

The precursor of the cationic surfactant, *N*-hexadecylamino-1-deoxylactitol, was synthesized through a reductive amination of the hexadecylamine on lactose monohydrate (Blanzat et al., 1999).

The precursor of the anionic surfactant, the bis-adduct bis(hydroxydodecyl)phosphinic acid, is obtained by an Abramov reaction by the addition of hypophosphorous acid to dodecylaldehyde (Brun and Etemad-Moghadam, 2002).

2.2. FluoCat synthesis

The synthesis was performed using commercially available (molecular probes) 12-(*N*-(7-nitrobenzo-2-oxa-1, 3-diazol-4-yl)amino)dodecanoic acid, product 3 (0.2 mmol), added to *N*-hexadecylamino-1-deoxylactitol, product 1 (0.2 mmol), firstly solubilized in water (15 mL) to give a heterogeneous suspension. The medium was stirred for 24 h at room temperature and the completion of the reaction was confirmed by the disappearance of the insoluble starting material.

FT-IR (KBr) ν = 1562 (COO^- st as); 1407 (COO^- st as).

MS (ESI, m/z): calculated for $C_{46}H_{83}N_5O_{15}$, 946.18; found 946.0 (cation: 377.4, anion: 568.6).

^{1}H NMR (500 MHz, CD_3OD, δ): 0.93 (t, 3H, CH_3), 1.33 (m, 40H, CH_2), 1.62 ($\underline{CH_2}CH_2COO^-$), 1.81 ($\underline{CH_2}CH_2NH_2^+$), 2.20 ($\underline{CH_2}COO^-$), 3.34 ($\underline{CH_2}NH$, $\underline{CH_2}NH_2^+$), 3.52–3.84 (sugar moiety), 4.15 (NH), 6.38 (NH_2^+), 8.57 ($\underline{CH}CNO_2$).

2.3. Other reagents

Other reagents were purchased at Sigma (France). Milli Q water (Millipore, France) was used into the experiments.

2.4. Vesicle formulation

TriCat was put in water or in phosphate buffer solution (PBS) (pH 7.4, 150 mM), stirred and then sonicated (Vibra Cell, Bioblock Scientific with a titanium probe, pulse rate: 30%, intensity: 3×) for 15 min. The vesicles were also prepared with a TriCat/FluoCat (19/1) mixture according to the same protocol. In the case of cell biology tests, the samples were prepared extemporaneously.

2.5. Transmission Electron Microscopy

The formation of the vesicles was observed through Transmission Electron Microscopy (TEM) using a JEOL JEM 1011 electron microscope, operating at 100 kV. Mixtures of catanionic associations in PBS (10^{-3} M) were applied on copper grids (Formvar), negatively stained with sodium phosphotungstate (pH 7.5) (2% (wt/vol)).

2.6. Particle size and zeta-potential analyses

Dynamic Light Scattering (DLS) was performed on a Malvern Instruments Nano ZS, using a He–Ne laser (633 nm), at a scattering angle of 173° and at 25.0 ± 0.1 °C. The hydrodynamic mean diameter of the nanoparticles was determined using the software provided by Malvern Instruments. The Contin model was applied to obtain size data. All the auto-correlation function fits were checked and found to be in accordance with the experimental data. Zeta-potential measurements were performed on the same apparatus, using an electrophoretic light scattering technique.

2.7. Fluorescence spectrophotometry

Fluorescence spectra were recorded using the apparatus Photon Technology International equipped with a xenon lamp EIMAC of 175 W. The excitation wavelength was set at 488 nm and the slits were fixed at 2 nm.

2.8. Culture of bovin chromaffin cells

After isolation (for complete protocol see Sol et al., 2004), cells were cultured in DMEM/F12 (Invitrogen, France) with Foetal Calf Serum (FCS) (5%), penicillin (50 IU/mL) and streptomycin (50 mg/mL) at a cell concentration of 0.5×10^6/mL. Culture medium was renewed every 4 days.

2.9. Culture of VAESBJ cells

Cells, provided by Cabri (Germany), were cultured at 2×10^5 cell/mL in DMEM: FCS (10%), glutamine (2 mM), penicillin (50 IU/mL) and streptomycin (50 mg/mL), non-essential amino acids (0.1 mM). Culture medium was renewed every 2 days.

2.10. Culture of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from buffy coats prepared from healthy donors (EFS, Toulouse, France) by density gradient centrifugation on Ficoll-Paque (LONZA, Belgique). PBMCs were resuspended in RPMI 1640 medium supplemented with FCS (10%) and then allowed to adhere for 2 h in a 6 well plate at 3×10^6 cell/mL. Non-adherent cells were removed and adherent cells were cultured in RPMI 1640 supplemented with serum (10%), penicillin (50 IU/mL), streptomycin (50 mg/mL) and glutamine (2 mM).

2.11. Culture of human monocyte derived macrophages

Macrophages were obtained from PBMCs after 4 days of culture in RPMI 1640 supplemented with FCS (10%), penicillin (50 IU/mL), streptomycin (50 mg/mL) and glutamine (2 mM).

2.12. Vesicle/cell interaction

The vesicle final concentration was fixed at 2.5×10^{-5} M on the cells cultured without FCS.

2.13. XTT test

This test was performed to determine the working concentration. Cell proliferation was determined using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-arboxanilide (XTT) test. After 3 h of incubation, the cells were washed and 50 μL of a DMEM solution, without Red Phenol, containing XTT (0.5 mg/mL) and coenzyme Q (40 μL/mL) were added. After 3 h of incubation, 100 μL of a sodium dodecyl sulphate solution (10%) was added. The reaction was then read on a Polarstar Galaxy spectrophotometer (BMG Labtech, France) at a wavelength set at 450 nm.

2.14. Flow cytometry

Cells were treated with catanionic vesicles into the cell culture medium without FCS. The analyses were then performed using flow cytometry Cyan ADP (Beckman Coulter, USA) at an excitation wavelength of 488 nm.

2.15. Internalisation inhibition

Macrophages and VAESBJ cells were pre-treated with uptake inhibiting agents: A, amiloride (3 mM); C, chlorpromazine (10 μg/mL); F, filipin (5 μg/mL); and ACF, the mixture of the previous amiloride + chlorpromazine + filipin at the same concentrations; Lac, lactose (0.4 mol/L); Gal, galactose (0.4 mol/L) and Glu, glucose (0.4 mol/L), 1 h before introducing TriCat/FluoCat vesicles. The agent concentration was maintained throughout the experiment. The same results were obtained in two independent sets of experiments on 20,000 cells each.

2.16. Synthesis of the magnetic liposomes

The preparation of magnetoliposomes has been described elsewhere (Filion and Phillips, 1997). Encapsulation of a magnetic fluid inside liposomes was performed using a spontaneous swelling procedure. The first step was to prehydrate the phospholipid film with a colloidal solution of maghemite (γFe_2O_3) nanoparticles and the second step was to swell it with pure water.

A small mass of perfectly dry powder of cis-1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, around 1 mg) was placed in a glass Petri dish. A total of 10 μL of the magnetic fluid was added ([Fe] = 1.8 M, conductivity around 1 mS) and the mixture was spread and sheared with a gloved finger to obtain a fat, oily, orange film. This film was presumably a lamellar phase swelled with charged particles. Immediately following the shearing, 1.5 mL of distilled water was poured onto the fatty film to start the spontaneous swelling of the liposomes. The preparation was placed in a water bath at 45 °C for 20 min and observed in a customized cell: FluoCat vesicles and GUV mixtures were placed between two cover glasses.

2.17. Confocal laser scanning microscopy

As far as the confocal laser scanning microscopy analyses are concerned, cells were adhered onto Lab-Teck slides (Nunc, Germany) for 30 min at 37 °C and fixed with paraformaldehyde (4%) for 15 min at room temperature. After two washings, cells were incubated with NH_4Cl (0.5 M) for 15 min at room temperature and finally washed twice with PBS buffer. Slides were mounted according to the manufacturer specifications with the mounting medium Mowiol. Confocal laser scanning microscopy was performed using a Leica SP2 AOTF (Leica, Germany) confocal laser scanning microscope equipped with a 40× oil immersion objective. FluoCat vesicles were excited with the 488 nm line of a He–Ne laser Soft LEICA.

Concerning the liposomes, the incubation with TriCat/FluoCat vesicles was performed for 20 min at concentrations of 0.4×10^{-3} M of DOPC and 5×10^{-4} M of the TriCat/FluoCat (19/1) mix before observation on the confocal microscope into a customized cell.

3. Results and discussion

3.1. Catanionic vesicle formulation and characterization

Coming from renewable resources which provide biocompatibility and non-toxicity, the sugar-based catanionic amphiphiles that we have developed result from a proton exchange between an amino-sugar derived surfactant, *N*-hexadecylamino-1-deoxylactitol (product 1 of Fig. 2), and an amphiphile bearing an acidic function, bis(hydroxydodecyl)phosphinic acid (product 2 of Fig. 2) leading to the formation of a tricatenar catanionic surfactant, 1-*N*-hexadecylammonium-1-deoxylactitol-bis(α-hydroxydodecyl)phosphinate (TriCat) (Fig. 2) (Vivares et al., 2008).

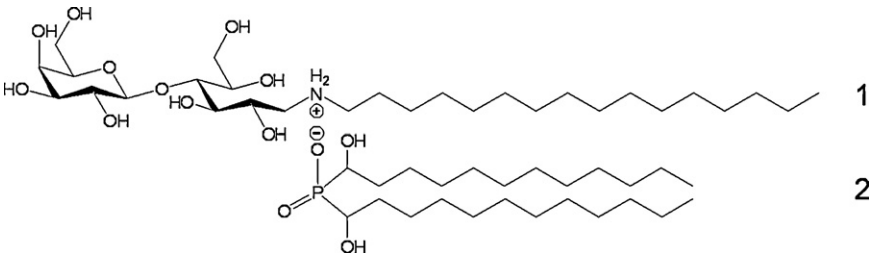

Fig. 2. Chemical structure of 1-*N*-hexadecylammonium-1-deoxylactitol-bis(α-hydroxydodecyl)phosphinate (TriCat) employed in this study to formulate catanionic nanovectors.

Table 1
Physicochemical characteristics of catanionic vesicles: dynamic light scattering analyses.

	TriCat concentration [M]	Solvent	Hydrodynamic diameter [nm]	Polydispersity index [–]
TriCat	1×10^{-3}	Water	125	0.3
TriCat/FluoCat 19/1	1×10^{-3}	Water	149	0.3
TriCat/FluoCat 19/1	2.5×10^{-5}	Water	146	0.3
TriCat/FluoCat 19/1	2.5×10^{-5}	PBS[a]	195	0.3

[a]Phosphate Buffer Solution (pH 7.4, 150 mM).

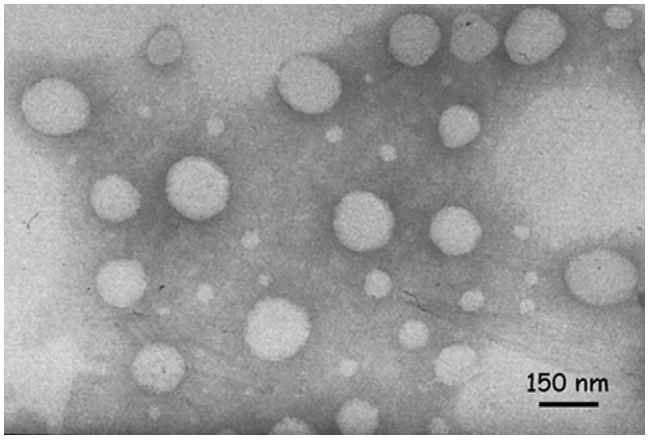

Fig. 3. TEM image of vesicles obtained spontaneously with TriCat at 1×10^{-3} M in Phosphate Buffer Solution (pH 7.4, 150 mM).

As previously published, TriCat is characterized by the ability to spontaneously form stable vesicles in an aqueous medium, water or phosphate buffer as checked by Transmission Electron Microscopy (TEM) (Fig. 3). Former freeze-fracture micrographs have also proved a unilamellar structure for these vesicles (Soussan et al., 2008). These vesicles had a narrow distribution in the nanosize range and were less than 200 nm in diameter as assessed by dynamic light scattering (Table 1).

In addition, to label vesicles, a fluorescent catanionic amphiphilic molecule, *N*-hexadecyl-ammonium-1-deoxylactitol 12-(-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoate (FluoCat) (Fig. 4), derived from the aminosugar 1, was synthesized following the protocol described in Section 2. When considering the UV/vis absorption spectrum of this fluorescent probe, the bathochrome shift from the maximal absorption wavelength of 488 nm when the molecule was free in aqueous solution, to 482 nm in the presence of catanionic vesicles revealed the changing of environment of the probe into a more ordered structure, and thus its insertion inside the amphiphilic bilayer of vesicles. The spectrofluorimetric characterization showed $\lambda_{ex} = 488$ nm and $\lambda_{em} = 550$ nm without any pH-dependence, compatible with flow cytometry or confocal laser scanning microscopy detection.

From Table 1, one can also see that sizes of vesicles with or without the FluoCat probe inserted in their bilayer (at a given TriCat concentration of 1×10^{-3} M in water), or whatever the concentration (1×10^{-3} M or 2.5×10^{-5} M, with the given composition of TriCat/FluoCat 19/1 in water), remain stable (ranging around 140 nm) with a polydispersity index of about 0.3 in each case which is satisfying since their formulation is spontaneous. From this match of sizes whatever the composition and/or concentration in water, one can thus consider that TriCat vesicles are stable to FluoCat insertion inside their bilayers in water and that labelled vesicles of TriCat/FluoCat are stable to dilution in water.

When comparing sizes of TriCat/FluoCat vesicles formulated in water and in PBS after dilution from 1×10^{-3} M to 2.5×10^{-5} M, one can see a slight change in range from 150 nm to 200 nm when changing the medium, which is not significant considering the rather large distribution of values.

Additional experiments of surface tension measurements have also been performed to confirm vesicles stability to dilution. A sample of TriCat vesicles was formulated at the concentration of 1×10^{-3} M in water, showing a surface tension of 30 mN/m. It was then diluted by 40 in water, hence its concentration at 2.5×10^{-5} M which is under its CAC. If the vesicles were not stable to dilution, the surface tension of the sample would have reached the value of about 40 mN/m, which is obtained for monomers at this concentration. However, the surface tension of the sample remained stable around 72 mN/m during at least one day. This observation can be explained by the stability of vesicles under dilution. Indeed, vesicles stayed intact in the solution. However, the monomer concentration was divided by 40, namely 1.25×10^{-6} M. At this concentration of monomers, it is thus normal that the surface tension reaches the value of water.

In addition to their stability to external modifications, these sugar-based catanionic vesicles have proved their potential as drug delivery systems with the ability to encapsulate drug models of various hydrophilicity and to release them (Soussan et al., 2009, 2008; Consola et al., 2007).

3.2. Cytotoxicity study

Prior to starting the cellular uptake of vesicles, a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-arboxanilide (XTT) cytotoxicity test was performed to determine the work-

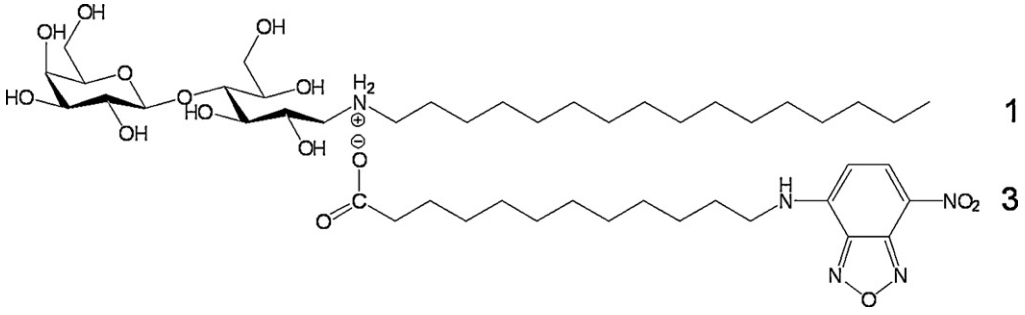

Fig. 4. Chemical structure of *N*-hexadecylammonium-1-deoxylactitol 12-(-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoate (FluoCat), the fluorescent probe encapsulated inside catanionic vesicles.

ing concentration on a keratinocyte-like cell line, VAESBJ. The vesicles showed cell activity with satisfactory cytotolerance at low concentrations (74% and 64% viability for 5×10^{-6} M and 2.5×10^{-5} M, respectively) whereas higher concentrations led to increased toxicity (up to a viability of 31% viability at 1×10^{-4} M). The cytotoxicity at the highest concentrations may have been the consequence of cell apoptosis following a caspase-independent pathway as previously published concerning another catanionic system on macrophage lines (Kuo et al., 2008, 2005). Therefore, the concentration of 2.5×10^{-5} M was chosen to pursue the study with the uptake of vesicles.

3.3. Interaction with cells

TriCat vesicles labelled with the fluorescent probe FluoCat entrapped inside their bilayer – mimicking an amphiphile active principle – were used for biological assays, in order to track their behaviour in cells thanks to various fluorescence techniques. TriCat/FluoCat vesicles were tested on various cell types: human lines such as VAESBJ cells, characterized by a keratinocyte-like phenotype, and also on primary cells such as bovine chromaffin cells (Sol et al., 2004), human peripheral blood mononuclear cells and human monocyte derived macrophages. The results presented in Fig. 5 show the confocal laser scanning microscopy which was performed on each cell type. Intracellular fluorescence was observed whatever the cell considered. The staining was perinuclear and diffuse as well as punctuated on all the cells. The vesicles were therefore able to affect phagocytic and also non-phagocytic cells. As the fluorophore moiety is carried by an amphiphilic molecule similar to TriCat, this study allowed following the fate of the catanionic bilayers. The hypothesis of its solubilization in the cytosol or incorporation into several cytoplasmic organelles, as it has been described for other amphiphilic molecules (Savic et al., 2003) can be considered.

3.4. Internalisation study

Then, the mechanisms of entry into phagocytic and non-phagocytic cells were further explored using various endocytosis inhibitors, cold temperature and sugar solutions. The vesicular uptake by cells (human monocyte derived macrophages and the keratinocyte cell line) was measured by flow cytometry detection (Fig. 6A and B). These two types of cells were studied independently under several inhibition conditions (Fig. 6C and D). In each experiment, forward scatter and side scatter were checked and the vesicles did not modify neither the size nor the structure of the cells contrary to what was described with other positively charged catanionic vesicles (Kuo et al., 2008).

As classically described, the endocytosis inhibitors used in these experiments were amiloride to inhibit macropinocytosis pathway, chlorpromazine for the inhibition of the clathrin internalisation and also filipin as a caveolae uptake disruptive (Khalil et al., 2006). A combination of the three substances was used to highlight simultaneously macropinocytosis, clathrin and caveole independent pathways, whereas a 4 °C condition was set up to explore the fusion process (Huth et al., 2006) while all active processes corresponding to endocytosis were blocked. A change of behaviour is observed whether the cells are phagocytic (macrophages) or not (keratinocytes).

3.4.1. Phagocytic cells

As far as the macrophages were concerned, all the inhibitors tested led to a decrease of 30–70% of vesicle uptake, which clearly indicates that the macropinocytosis, clathrin and caveolae pathways were used for the catanionic vesicles' entry into these phagocytic cells. Moreover, it is noteworthy that the incubation

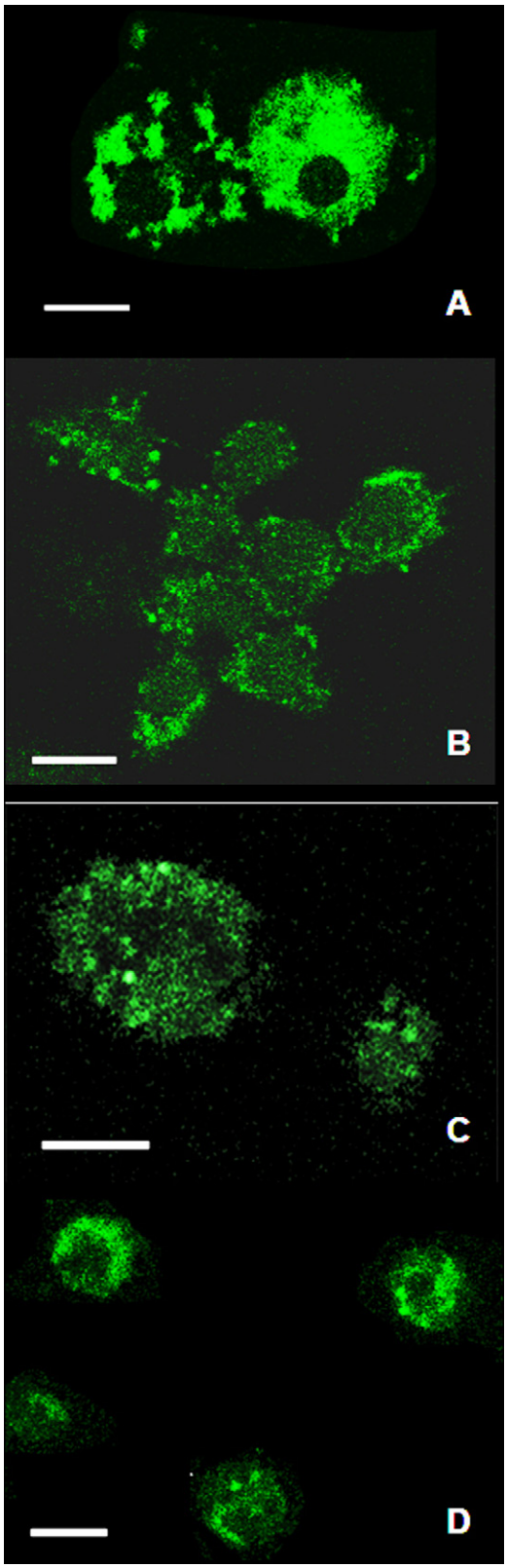

Fig. 5. Uptake of TriCat/FluoCat vesicles (19/1) at a concentration of 2.5×10^{-5} M, followed by confocal laser-scanning microscopy (Leica SP2) (40× oil objective, 488 nm excitation), by: bovin chromaffin cells (A), VAESBJ cells (B), monocyte derived macrophages (C), peripheral blood mononuclear cells (D). Scale bar is 10 μm.

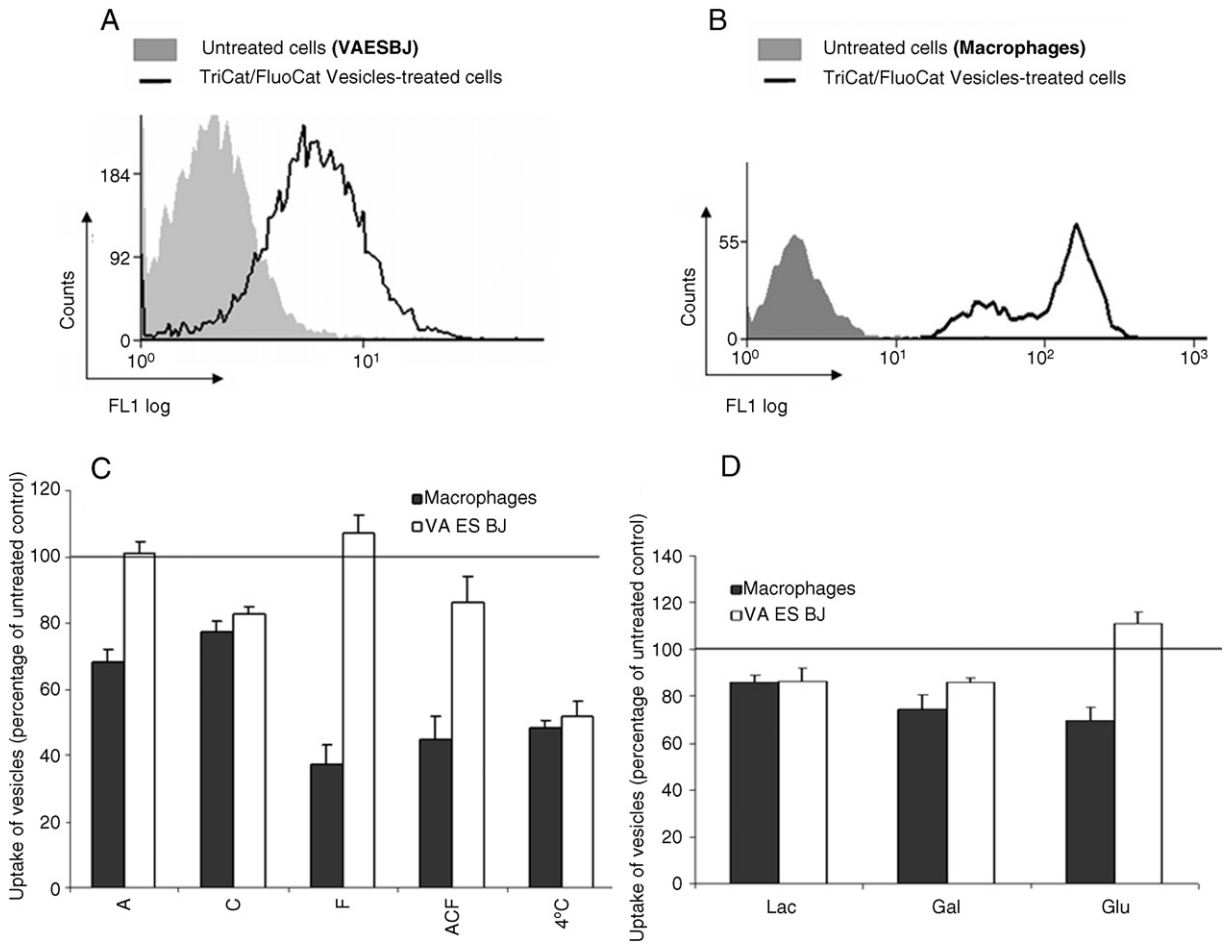

Fig. 6. (A) and (B) Typical result of the uptake of TriCat/FluoCat vesicles by VAESBJ cells (A) and macrophages (B) measured by flow cytometry on a Cyan ADP Beckman Coulter apparatus (FL-1 channel corresponds to 488 nm excitation). (C) and (D) Uptake inhibition of TriCat/FluoCat vesicles detected by flow cytometry (mean fluorescence detected by FL-1 channel) of the preparation condition as a percentage of the fluorescence given by the untreated control (cells incubated with vesicles without inhibitor). The same results were obtained in two independent sets of experiments on 20,000 cells each. Plain line corresponds to negative control without vesicles. (C) A = amiloride, C = chlorpromazine, F = filipin, ACF = amiloride + chlorpromazine + filipin, and (D) Lac = lactose, Gal = galactose, Glu = glucose.

with the three inhibitors combined or the incubation at 4 °C led to the same inhibition of 50% in vesicle uptake. As all active processes are blocked at low temperature (Huth et al., 2006), this result first suggests that macropinocytosis, clathrin and caveolae pathways are the only means of internalisation as far as endocytosis pathway is concerned.

In addition, for these two experiments, the inhibition of vesicle entry into macrophages was not total, proving that a non active process occurred, probably membrane fusion – fusion being not inhibited at low temperatures since it is a passive process (Huth et al., 2006).

The saturation of the cell sugar receptors before the addition of the sugar-coated TriCat vesicles led to a decrease in the uptake of the vesicles by macrophages, suggesting a possible specific recognition between vesicles and cell sugar receptors, followed by a clathrin internalisation (Khalil et al., 2006). The results were in accordance with the physiology of the phagocytic cells.

3.4.2. Non-phagocytic cells

The results obtained with the keratinocyte-like cells (Fig. 6) were also interesting since neither inhibitor nor sugar solution led to a decrease of the uptake of catanionic vesicles, in accordance with the non-phagocytic phenotype of such cells. In contrast, incubation at 4 °C decreased the uptake without inhibiting it completely. Again, the fusion phenomenon can be strongly hypothesized.

This study clearly attests that, whatever the cell type, catanionic vesicles are able to penetrate using the most appropriate mechanism, endocytosis and/or membrane fusion. The universality of this system is no longer utopic, as it is noteworthy that catanionic vesicles can easily encapsulate hydrophilic (Soussan et al., 2008) or hydrophobic (Consola et al., 2007) drugs in their structure.

3.5. Modelisation of the interaction between catanionic vesicles and phospholipid membranes

Finally, to go further into the understanding of the possible fusion between catanionic vesicles and cell membranes, catanionic/phospholipid bilayer interaction was modelled using giant DOPC liposomes as previously published (Beaune et al., 2009). The giant cis-1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposome synthesis is detailed in Section 2. The experiment was performed using TriCat/FluoCat vesicles incubated with such liposomes and observed using confocal laser scanning microscopy. Fig. 7 clearly shows a homogeneous fluorescent signal situated on the liposome membranes. This phenomenon can be explained by the fusion between DOPC membranes and catanionic vesicles as they are amphiphilic. These results correlate well with the cell

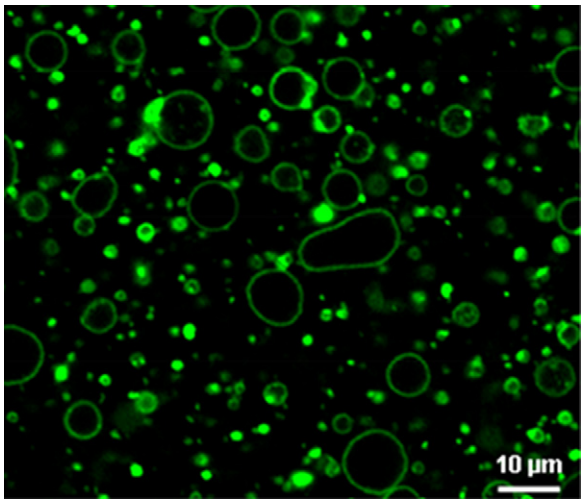

Fig. 7. TriCat/FluoCat vesicle interaction with giant DOPC liposomes.

observations made previously and also with the work already published (Beaune et al., 2009). Indeed, in this latter, this kind of interaction between giant liposomes and vesicles was strongly hypothesized, after showing that the interaction between GUVs and TriCat occurred on a vesicular – and not monomeric – state of the TriCat surfactant.

This fusion process has already been observed in the case of liposomes (Bonacucina et al., 2009; Huth et al., 2006) or lipoplexes (Zuhorn and Hoekstra, 2002). Nevertheless, as far as we know, it has not yet been reported for catanionic vesicles. This fusion phenomenon is crucial as it allows a drug, encapsulated in the vesicle core, to be delivered to its intracellular target and highlights the universality of the system on cells. With such a system able to affect any cell type, pharmaceutical applications in infectious diseases (especially to cure pathologies induced by intracytoplasmic organisms), cancer and obviously gene therapy can easily be imagined.

4. Conclusion

In conclusion, this paper highlights the great effectiveness of cheap, easy-to-use catanionic vesicles. They are indeed able to affect phagocytic and also non-phagocytic cells, human cell lines or primary cells, after endocytosis and/or fusion processes. Further study will deal with the validation of this apparently universal system with the encapsulation of various drugs of therapeutic interest.

Acknowledgement

The ANR is acknowledged for funding support. The authors thank the Toulouse RIO Imaging platform for putting the confocal laser scanning microscope at their disposal.

References

Allen, T.M., Cullis, P.R., 2004. Drug delivery systems: entering the mainstream. Science 303, 1818–1824.
Beaune, G., Soussan, E., Blanzat, M., Rico-Lattes, I., Cabuil, V., Ménager, C., 2009. Interaction between catanionic vesicles and giant magnetic liposomes. C. R. Chim., 1–7.
Blanzat, M., Perez, E., Rico-Lattes, I., Promé, D., Promé, J.-C., Lattes, A., 1999. Langmuir 15, 6163–6169.
Bonacucina, G., Cespi, M., Misici-Falzi, M., Palmieri, G.F., 2009. Colloidal soft matter as drug delivery system. J. Pharm. Sci. 98, 1–42.
Brun, A., Etemad-Moghadam, G., 2002. New double-chain and aromatic (alpha-hydroxyalkyl) phosphorus amphiphiles. Synthesis 10, 1385–1390.
Consola, S., Blanzat, M., Perez, E., Garrigues, J.C., Bordat, P., Rico-Lattes, I., 2007. Design of original bioactive formulations based on sugar-surfactant/non-steroidal anti-inflammatory catanionic self-assemblies: a new way of dermal drug delivery. Chem. A Eur. J. 13, 3039–3047.
Faraji, A.H., Wipf, P., 2009. Nanoparticles in cellular drug delivery. Bioorg. Med. Chem. 17, 2950–2962.
Filion, M.C., Phillips, N.C., 1997. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. Biochim. Biophys. Acta 1329, 345–356.
Hillaireau, H., Couvreur, P., 2009. Nanocarriers' entry into the cell: relevance to drug delivery. Cell. Mol. Life Sci. 66, 2873–2896.
Huth, U.S., Schubert, R., Peschka-Süss, R., 2006. Investigating the uptake and intracellular fate of pH-sensitive liposomes by flow cytometry and spectral bio-imaging. J. Control. Release 110, 490–504.
Khalil, I.A., Kogure, K., Alkita, H., Harashima, H., 2006. Uptake pathways and subsequent intracellular trafficking in non viral gene delivery. Pharmacol. Rev. 58, 32–45.
Kuo, J.H.S., Jan, M.S., Chang, C.H., Chiu, H.W., Li, C.T., 2005. Cytotoxicity characterization of catanionic vesicles in RAW 264.7 murine macrophage-like cells. Colloids Surf. B 41, 189–196.
Kuo, J.H.S., Chang, C.H., Lin, Y.L., Wu, C.J., 2008. Flow cytometric characterization of interactions between U-937 human macrophages and positively charged catanionic vesicles. Colloids Surf. B 64, 307–313.
Moses, M.A., Brem, H., Langer, R., 2003. Advancing the field of drug delivery: taking aim at cancer. Cancer Cell 4, 337–341.
Park, J., Rader, L.H., Thomas, G.B., Danoff, E.J., English, D.S., DeShong, P., 2008. Carbohydrate-functionalized catanionic surfactant vesicles: preparation and lectin-bnding studies. Soft Mater. 4, 1916–1921.
Pinto-Alphandary, H., Andremont, A., Couvreur, P., 2000. Targeted delivery of antibiotics using liposomes and nanoparticles: research and applications. Int. J. Antimicrob. Agents 13, 155–168.
Savic, R., Luo, L., Eisenberg, A., Maysinger, D., 2003. Micellar nanocontainers distribute to defined cytopasmic organelles. Science 300, 615–618.
Sol, J.C., Sallerin, B., Larrue, S., Li, R.Y., Jozan, S., Tortosa, F., Mascott, C., Carraoue, F., Tafani, M., Lazorthes, Y., 2004. Intrathecal xenogeneic chromaffin cell grafts reduce nociceptive behavior in a rodent tonic pain model. Exp. Neurol. 186, 198–211.
Soussan, E., 2007. Conception de vésicules catanioniques dérivées de sucre et étude de leur mécanisme de délivrance de principes actifs. Université Paul Sabatier – Toulouse III, Toulouse.
Soussan, E., Mille, C., Blanzat, M., Bordat, P., Rico-Lattes, I., 2008. Sugar-derived tricatenar catanionic surfactant: synthesis, self-assembly properties, and hydrophilic probe encapsulation by vesicles. Langmuir 24, 2326–2330.
Soussan, E., Cassel, S., Blanzat, M., Rico-Lattes, I., 2009. Drug delivery by soft matter: matrix and vesicular carriers. Angew. Chem. Int. Ed. 48, 274–288.
Tsumoto, K., Matsuo, H., Tomita, M., Yoshimura, T., 2009. Efficient formation of giant liposomes through the gentle hydration of phosphatidylcholine films doped with sugar. Colloids Surf. B 68, 98–105.
Vivares, D., Soussan, E., Blanzat, M., Rico-Lattes, I., 2008. Sugar-derived tircatenar catanionic surfactant: self-assembly and aggregation behavior in the cationic-rich side of the system. Langmuir 24, 9260–9267.
Vlachy, N., Touraud, D., Heilmann, J., Kunz, W., 2009. Determining the cytotoxicity of catanionic surfactant mixtures on HeLa Cells. Colloids Surf. B 70, 278–280.
Yamada, A., Yamanaka, T., Hamada, T., Hase, M., Yoshikawa, K., Baigl, D., 2006. Spontaneous transfer of phospholipid-coated oil-in-oil and water-in-oil microdroplets through an oil/water interface. Langmuir 22, 9824–9828.
Zuhorn, I.S., Hoekstra, D., 2002. On the mecanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? J. Membr. Biol. 189, 167–179.