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ORIGINAL PAPER

Dictyostelium Extracellular Vesicles Containing Hoechst 33342 Transfer the Dye into the Nuclei of Living Cells: A Fluorescence Study

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Abstract Cells of the eukaryotic unicellular microorganism *Dictyostelium discoideum* are constitutively resistant to vital staining of their nuclei by the DNA-specific dye Hoechst 33342. By studying the mechanisms of this resistance, we evidenced that these cells expel vesicles containing the dye for detoxification (Tatischeff et al., *Cell Mol Life Sci*, 54: 476–87, 1998). The question to be addressed in the present work is the potential use of these extracellular vesicles as a biological drug delivery tool, using Hoechst 33342 as a model of a DNA-targeting drug. After cell growth with or without the dye, vesicles were prepared from the cell-free growth medium by differential centrifugation, giving rise to two types of vesicles. Negative staining electron microscopy showed their large heterogeneity in size. Using fluorescence techniques, data were obtained on the dye loading and its environment inside the vesicles. By UV video-microscopy, it was demonstrated that the dye-containing vesicles were able to deliver it into the nuclei of naive *Dictyostelium* cells, thus overcoming their constitutive resistance to the free dye. A vesicle-mediated dye-transfer into the nuclei of living human leukaemia multidrug resistant K562r cells was also observed.

Keywords Extracellular vesicles · Fluorescence · Hoechst 33342 · Drug delivery · *Dictyostelium discoideum*

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Introduction

Dictyostelium discoideum (*Dd*) is an ancestral non-pathogenic amoeba, at the border of the plant and animal kingdoms. When growing on bacteria or in axenic medium, these eukaryotic cells, (10 μm in diameter) dividing by mitosis, have many characteristics of animal cells. They are compared to lymphocytes for motility and to macrophages for phagocytosis. (Fore more information on *Dictyostelium*, *cf.* http://dictybase.org).

As a DNA-specific dye, labeling the nuclei of most living mammalian cells, free Hoechst 33342 (HO342) (Fig. 1) is entering *Dd* cells, but the vital dye does not permeate the nuclear membrane. By questioning the mechanisms used by *Dd* cells to get rid of this xenobiotic, we showed by fluorescence techniques that they expel vesicles containing HO342 into the cell growth medium, as a detoxification mechanism [1]. The fluorescence properties of HO342 have been described when associated with DNA [2] and/or with the sodium dodecyl sulfate (SDS) detergent [3]. The study of the fluorescence characteristics of HO342 transported by *Dd* vesicles could shed light on its environment in these vesicles and on their properties as a potential drug delivery tool.

Efficient drug targeted delivery inside pathological cells is indeed a general problem [4–6] encountered both in the

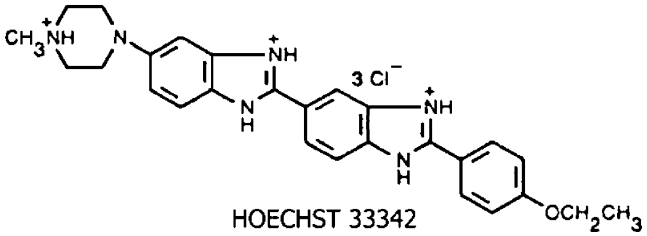

Fig. 1 Formula of Hoechst 33342

treatment of drug resistant tumors [7] and for antigen or antisense therapy [8].

The main focus of the present work was to show that the extracellular vesicles could mediate the transfer of HO342 into the nuclei of naive living *Dd* cells, never brought in contact with the dye before, and thus overcome their constitutive resistance to the labeling of their nuclei by the free dye. With the aim of questioning a possible vesicle-mediated dye transfer to the nuclei of human mammalian cells[1], a first experiment was performed with human leukaemia K562r cells, which are both resistant to antitumoral chemotherapy and to HO342 vital staining [9].

Taking into account the conservation of many essential cell functions from *Dictyostelium* to mammalians, the vesicles externalized by this early-diverged protist could be used as a valuable model for clarifying the complexity of the yet largely unknown vesicle-mediated eukaryotic intercellular communication [10].

Experimental

Growth of *Dictyostelium* cells and vital staining by HO342

If not otherwise specified, chemical reagents were from Sigma (L'Isle d'Abeau Chesnes, France). Vegetative *Dd* cells, Ax-2 strain, were grown in suspension on a gyratory shaker (150 rpm) at +22 °C, in HL5 semi-defined medium [11, 12], containing antibiotics (penicillin (50 U/ml) and streptomycin (50 U/ml)) (Biomedia, Boussens, France). In contrast with mammalian cells, *Dd* cells are grown without fetal calf serum. For proper oxygenation, each suspension was grown in an Erlenmeyer containing five times the suspension volume. *Dd* cells are able to grow and divide at the expense of soluble or particulate nutrients. Cells were generally used in the late exponential phase of growth.

A stock solution of HO342 (1 mg/ml) (Molecular Probes Europe, Leiden, the Netherlands), was prepared in de-ionized water, filter sterilized (0.22 μm) and stored at −20 °C. In all the experiments, the dye was added to the culture medium when initiating the cell culture at 3×10^6 vegetative cells/ml. Most experiments were carried out with vegetative cells incubated with 10 μg/ml of HO342 during 24 h. In order to check the influence of the cell incubation time with the dye, vegetative cells were also incubated, as a function of time, with HO342 (10 μg/ml). Comparison of the growth curves with HO342 or without the dye indicated that HO342 was not toxic for *Dd* cell growth at this concentration. Vesicles were prepared after 24, 72 and 95 h of growth after controlling the cell morphology and density by light microscopy. These times were chosen as representative of late exponential, early stationary and more stationary—but non-apoptotic—cells. In order to check for the influence of the initial HO342 concentration, cultures of *Dd* cells, initiated in the presence of different concentrations of HO342 (2.2 μg/ml to 17.4 μg/ml), were grown in parallel during 24 h and vesicles were then prepared from the different cultures.

Preparation of *Dictyostelium* vesicles

Cells grown with HO342 or without were harvested. Cell-free media were obtained by centrifugation of the cell suspensions at successively $700\times g$ for 5 min and $2{,}000\times g$ for 10 min (+4 °C). After centrifugation of the $2{,}000\times g$ supernatant at $12{,}000\times g$ for 20 min (+4 °C) the pellet was used as V12 *Dd* vesicles. In order to verify if further purification of vesicles could be obtained by centrifugation, the $12{,}000\times g$ supernatant was centrifuged at $105{,}000\times g$ for 1 h (+4 °C) and the pellet was used as V105 *Dd* vesicles. Pellets were re-suspended and concentrated (×50 or ×20) in a Tris buffer (40 mM Tris–HCl, pH 7.4) with 250 mM sucrose, 5 mM dithiothreitol, 2 mM EDTA and protease inhibitors. Vesicles, prepared from the cell growth medium in presence of HO342, were named V12H and V105H, whereas the control vesicles without the dye were named V12C and V105C. All vesicles were highly stable, as they did withstand repeated liquid nitrogen freeze–thaw cycles without breaking. They could be kept at +4 °C at least 6 weeks without releasing the dye. Both types of vesicles, V12 and V105, were compared for their protein content, their dye transport property and their in vitro dye transfer efficiency.

Growth of human leukaemia K562r cell lines

The erythroleukaemia K562 cells were maintained in suspension in a 25 cm^2 Corning flask for tissue culture (VWR, Fontenay-sous-Bois, France), using RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine (2 mM) and antibiotics (penicillin (50 U/ml) and streptomycin (50 U/ml)) (VWR, Fontenay-sous-Bois,

[1] Pending European Patent (No.WO2005004925), 20-01-2005, "Extracellular vesicles from non-pathogenic amoeba useful as vehicle for transferring a molecule of interest to an eukaryotic cell," DRITT-SAIC, Paris Université Pierre et Marie Curie.

France). Cells were grown in a 5% CO_2 and 95% humidified air atmosphere, at +37 °C. The resistant K562r cell line, expressing the multidrug P-glycoprotein, was selected from the normal sensitive K562s cell line as described [13]. In order to maintain P-glycoprotein expression, the medium of K562r cells was supplemented with colchicine (60 ng/ml). The cells at a density of about 10^6 cells/ml were harvested by centrifugation at 300×*g* for 5 min and re-suspended at 10^7 cells/ml in RPMI 1640 medium with 10% fetal calf serum.

Protein analysis

Quantification of the vesicular proteins was obtained by the method of Peterson [14]. Protein electrophoretic patterns were obtained by SDS-PAGE (12% acrylamide)[15]. Sypro-red staining (Molecular Probes Europe, Leiden, the Netherlands), with sensitivity similar to silver nitrate staining, was used for protein visualization.

Preparation of HO342-loaded liposomes

Egg phosphatidylcholine and cholesterol were mixed to prepare *L*arge *U*nilamellar *V*esicles (LUV) [16], in the presence of HO342 (100 μg/ml) in potassium phosphate buffer (17 mM pH 6.8). The excess free dye was eliminated by Bio-Gel PD-10 column filtration (Bio-Rad, Marnes-la-Coquette, France).

Electron microscopy (negative staining)

Twenty microliters of V12C and V105C vesicles concentrated (×50) were loaded for 30 s on formvar coated nickel grids. Grids were stained for 10 s with a droplet of 0.2% (w/v) aqueous uranyl acetate, dried and then observed with a Philips CM12 electron microscope at an accelerating voltage of 80 kV. Many different fields were observed to obtain an estimation of the vesicle size range.

Absorption spectroscopy

The concentration of HO342 (Mr=616), kept as an aqueous stock solution (1 mg/ml) at −20 °C, was controlled before use on a UV–Visible Varian DMS 70 spectrophotometer, assuming a molar absorbance of ε=45,000 M^{-1} cm^{-1} at 350 nm (Handbook of Fluorescent Probes, Molecular Probes).

Fluorescence spectrofluorimetry

Steady state fluorescence emission and excitation spectra were recorded with an experimental set-up, automated with a MacIIci computer and Labview software for data acquisition [17]. Kaleidagraph was used for the data treatment. The cuvet (10×10 mm), with a 3×3 mm centered quartz optical path (Hellma France, Paris) was positioned with two perpendicular micrometers. Slits of 2 mm-width were used for the McPherson excitation monochromator (McPherson, Chelmsford, USA) and in- and out slits of respectively 1.8 and 1 mm width were used for the Bausch & Lomb emission monochromator (Bausch & Lomb, Analytical Systems Div., Rochester, USA). All fluorescence measurements were made at +22 °C. All the spectra were uncorrected for wavelength variations in spectral transmission and recorded from 360 to 720 nm, using a 350 nm excitation wavelength in the HO342 first absorption band. The sample absorbance at this excitation wavelength was usually less than 0.1 in order to eliminate inner filter effects. The grating second order of Rayleigh diffusion of the 350 nm excitation wavelength was measured at 700 nm and used as a marker of diffusing components, namely the vesicles.

HO342 fluorescence was first studied in the concentration range of 4 μg/ml to 44 μg/ml in potassium phosphate buffer (17 mM pH 6.8). The HO342-DNA interaction was studied at a [base pair (bp)]/[dye] ratio of 2.3, by adding HO342 (14.3 μM, final concentration) to a solution of DNA (33 μM final concentration in base pairs, Mr=610) in potassium phosphate buffer (17 mM pH 6.8). The DNA of *Clostridium perfringens* was chosen for its (A/T) base composition similar to the DNA of *Dd*. The HO342-lipid interaction was studied with liposomes. The HO342-SDS interaction was also studied, together with the influence of SDS 2% to DNA-bound or lipid-bound HO342. Indeed, to study the fluorescence spectra of the HO342-loaded vesicles, disruption of the vesicles was obtained using the anionic detergent SDS at the final concentration of 2%, i.e. above its *C*ritical *M*icelle *C*oncentration (CMC=8.1 mM).

Video-microscopy

The morphological appearance of growing *Dd* cells was controlled by phase contrast microscopy with an Olympus BHA transmission microscope (Olympus France, Rungis). UV fluorescence microscopy was performed with the same BHA Olympus microscope, equipped with a 100 W mercury lamp and an ultraviolet-blue fluorescence excitation block. For video-microscopy, images were recorded by a self-made video set-up, with the following additional components: a C adaptation (Olympus France, Rungis), a Black & White analogic CCD camera and a Black & White video monitor (Optophotonics, Eaubonne, France), a Dazzle Hollywood DV-Bridge for analogic-digital conversion (New Edge, Boulogne Billancourt, France) and an Apple iMac G3 with a FireWire input. Data acquisition was achieved through NIH Image 1.62.

To study the transfer of HO342 from vesicles to the nuclei of living cells, 10 μl of cells (10^7 cells/ml in the

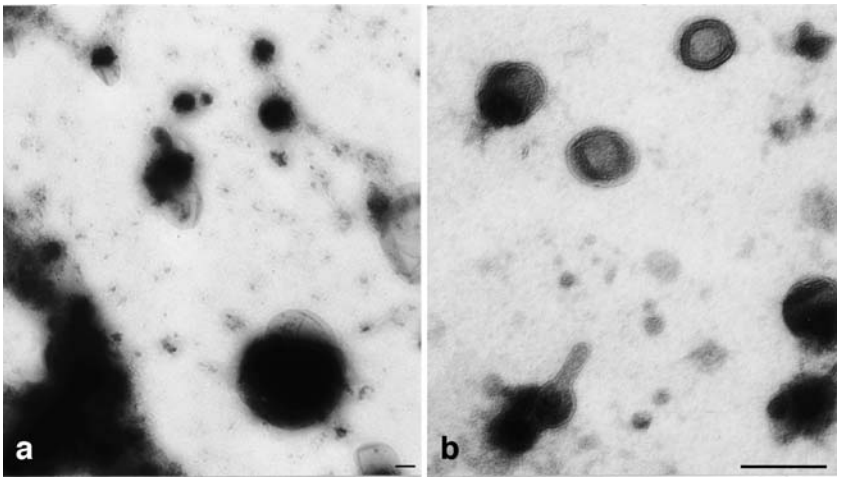

Fig. 2 Negative staining electron microscopy of *Dictyostelium* extracellular vesicles. **a** V12H **b** V105H vesicles (×50). Bars=200 nm

growth medium) and 10 µl of concentrated (×50) V12H or V105H vesicles in the vesicle medium were layered on a microscope slide with a 20×20 mm cover glass and observed as a function of time.

Results

Characteristics of the vesicles expelled by *Dd* cells

Extracellular vesicles of *Dd* cells (V12H and V105H, V12C and V105C), concentrated from the growth medium (see "Experimental" for details), have been analyzed by negative staining electron microscopy. Whether prepared in the presence of HO342 or not, all observed organelles were limited by a lipid-like bilayer membrane (Fig. 2). The vesicle size appeared heterogeneous, ranging from about 60 to 500 nm in diameter, as estimated in different observed fields. In the V12C and the V105C preparations, small round-shaped vesicles containing electron-dense material were present, as well as empty vesicles with a folded-up membrane.

The protein content of both V12C and V105C control vesicles, as obtained by Peterson method, was found equal to 0.37+/−0.04 mg/ml for two different preparations (concentrated ×10). SDS-PAGE (12% acrylamide) electrophoretic patterns (Fig. 3) show that the V12C and V105C vesicles were comparable with polypeptides of 97, 66–60, 45, and 35–30 kDa.

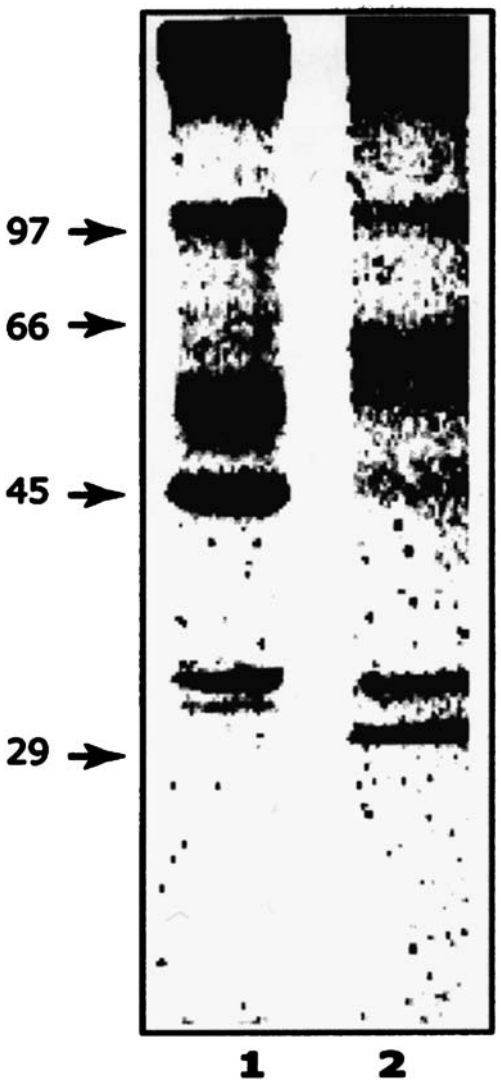

Fig. 3 SDS-PAGE of *Dictyostelium* control vesicles. *1* V12C *2* V105C vesicles. Fifty micrograms of proteins from control vesicles (×50) were layered per well and run on 12% SDS-PAGE. Proteins were visualized by SYPRO-red staining

Emission spectra of HO342 in different environments

In order to relate the measurements of the HO342 fluorescence characteristics in *Dd* vesicles to HO342 fluorescence data [2, 3], we defined in the present experimental conditions the dye fluorescence properties in different environments. HO342 could be in interaction with the lipid bilayer of the

vesicles or with the associated nucleic acids, DNA or RNA [1], or possibly in the aqueous environment of the vesicular lumen. Since we used the anionic detergent SDS, in order to destroy the lipid bilayer of the vesicles, the influence of the detergent on the emission spectra of HO342, previously described [3], was also presently studied in the different environments.

Figure 4a shows the emission spectra of HO342 in buffer (pH 6.8). As shown for two different concentrations, 8 and 44 μg/ml, the fluorescence emission spectra displayed a maximum of very weak intensity at 475 nm. In the presence of SDS 2%, HO342 exhibited a high fluorescence intensity (Fig. 4b), increased by a factor of about 150 as compared to the dye in buffer. The observed emission maximum was slightly blue shifted from 475 to 470 nm. When interacting with (*Cp*) DNA, HO342 (8 μg/ml) exhibited also a higher fluorescence intensity, increased by a factor of about 85, with a blue-shifted emission peak at 452 nm. Addition of SDS 2% to HO342-DNA complex induced a small increase in fluorescence intensity of about 2, with a noticeable red shift of the emission peak from 452 to 470 nm (Fig. 4c). HO342-containing LUV exhibited also an increase in fluorescence intensity and a blue shifted emission peak at 447 nm, when compared to the dye in buffer. In the presence of SDS 2%, a significant red shift of the emission peak from 447 to 470 nm was observed but with a small decrease in fluorescence intensity (Fig. 4d).

Emission spectra of the HO342-loaded *Dd* vesicles

The spectra of the HO342 loaded vesicles were then obtained and related to the ones of HO342 in the different environments studied. The V12H and V105H concentrated (×50) vesicles exhibited similar emission spectra between 360 and 720 nm with a maximum around 450 nm, in between those observed for the HO342-DNA and HO342-lipid complexes, but with a lower intensity for the V105H vesicles. The control V12C and V105C vesicles (×50) displayed only background fluorescence between 390 and 500 nm. It is worth noting the relatively large diffusion component at 700 nm, due to the vesicular nature of the material (Fig. 5a,b).

To compare the spectra of the dye loaded in the *Dd* vesicles with the ones of HO342 in interaction with DNA, lipids and/or SDS, vesicles were disrupted by SDS 2%, as monitored by the large decrease of the 700 nm diffusion peak (Fig. 5c,d). In these conditions, HO342 freed from the V12H and V105H vesicles exhibited a fluorescence

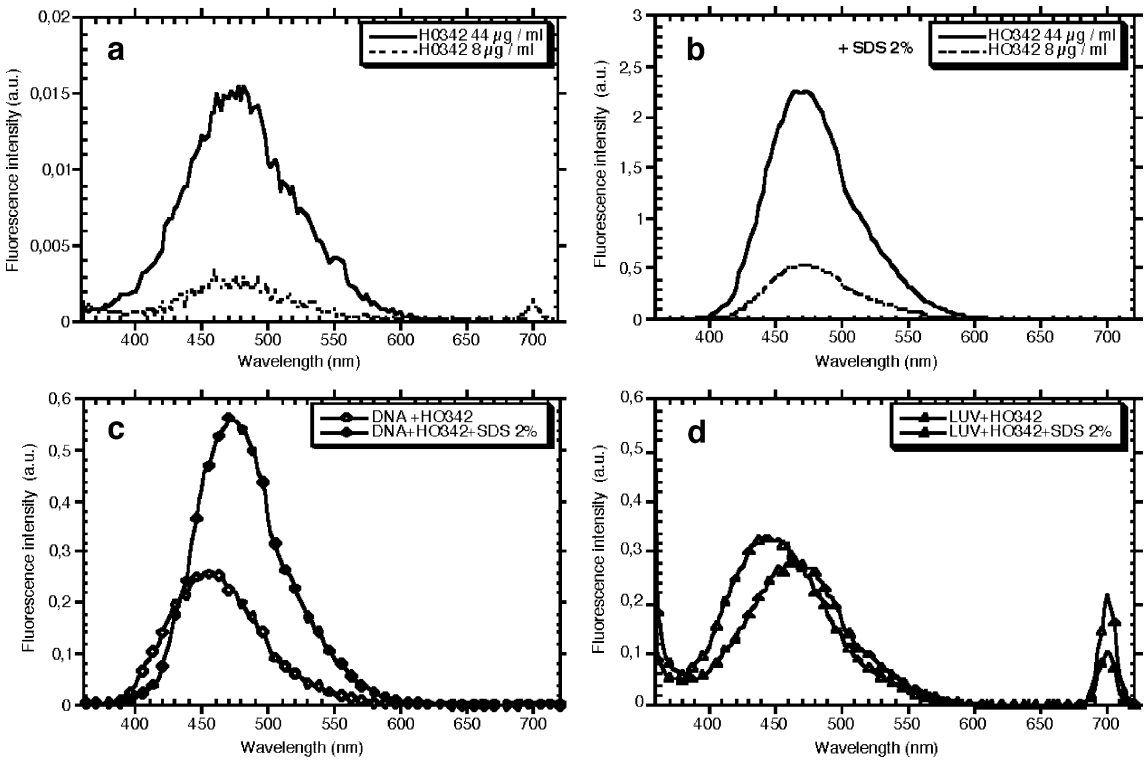

Fig. 4 Fluorescence emission spectra of HO342, free in buffer or complexed with DNA, lipids and SDS detergent. **a** HO342 at two different concentrations in potassium phosphate buffer (17 mM pH 6.8); **b** HO342 at the same two concentrations complexed with SDS 2% (final concentration) in the same buffer; **c** HO342 (8 μg/ml) added to (*Cp*) DNA at a [base pair (bp)]/[dye] ratio of 2.3 and further complexed with SDS 2%; **d** HO342 associated with LUV liposomes and further complexed with SDS 2%. All spectra were obtained with an excitation wavelength of 350 nm

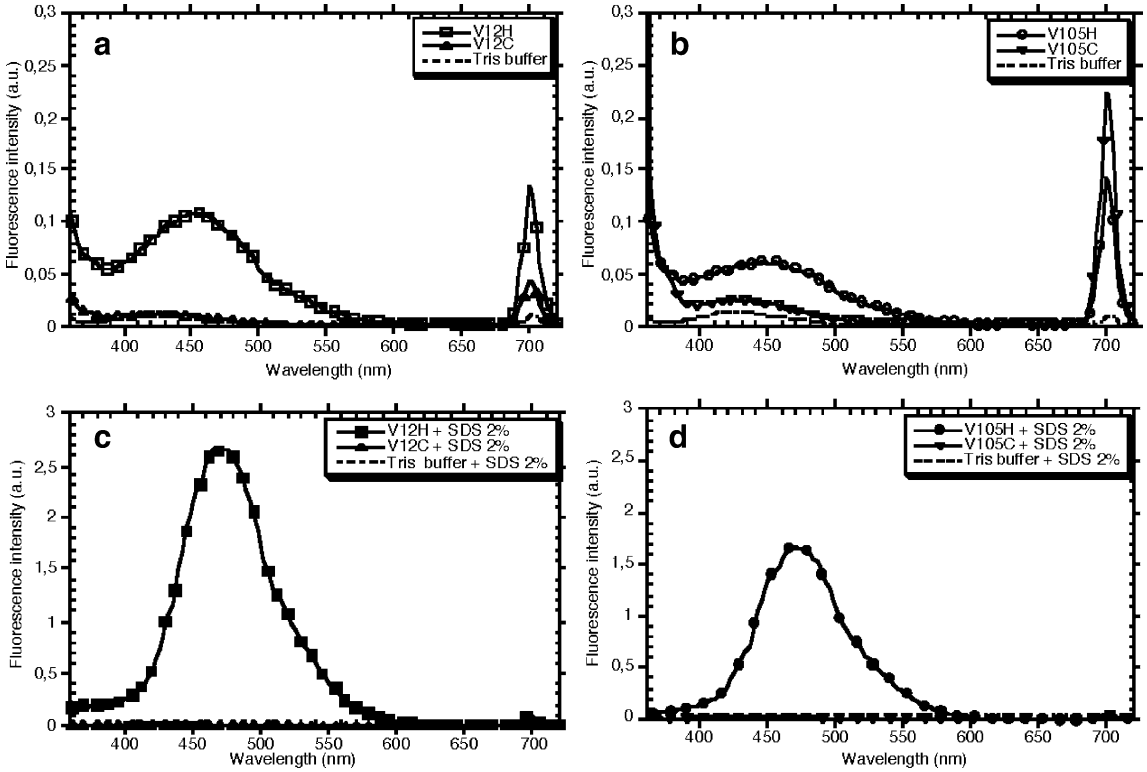

Fig. 5 Fluorescence emission spectra of *Dictyostelium* extracellular vesicles. **a** V12H and V12C vesicles, concentrated (×50) from the growth medium of cells respectively incubated with or without HO342; **b** V105H and V105C vesicles (×50); **c** V12H and V12C vesicles (×50) disrupted by SDS 2% (final concentration); **d** V105H and V105C vesicles (×50) disrupted by SDS 2% (final concentration). Emission spectra of Tris-buffer are shown in all parts of the figure. All spectra were obtained with an excitation wavelength of 350 nm

emission peak red-shifted at 470 nm, instead of the maximum at 450 nm observed for HO342 in the intact vesicles. Its intensity compared to the one in buffer, was increased by a factor of about 25 for both types of vesicles, lower than the one observed for HO342 interacting only with SDS 2% (×150). In contrast, no effect of SDS 2% was observed on the fluorescence characteristics of the control V12C and V105C vesicles. These data confirmed that it is actually HO342 carried by the vesicles that was characterized by spectrofluorimetry, and that the V105H vesicles had a lower HO342 content than the V12H vesicles.

In order to follow the vesicle loading of HO342 (10 μg/ml) as a function of cell incubation time with the dye, V12H vesicles (×20) were prepared after 24, 72 and 95 h. The vesicles were studied by their fluorescence emission spectra, both with and without addition of SDS 2%. Without SDS, the intensity of the 450 nm emission maximum as well as the 700 nm diffusion peak were increasing with an incubation time higher than 24 h, suggesting a higher amount of externalized vesicles (Fig. 6a). In the presence of SDS, the fluorescence maximum was shifted to 470 nm with the same increase of intensity after 24 h (Fig. 6b) than the one observed above (Fig. 5c,a) for the same incubation time.

In order to check for the influence of the initial HO342 concentration in the cell medium, V12H vesicles were prepared after 24 h of cell incubation in the presence of different concentrations of HO342 ranging from 2.2 to 17.4 μg/ml. The different fluorescence emission spectra of the V12H vesicles (×20), did not exhibit any noticeable intensity variation (Fig. 6c). However, only vesicles obtained from the medium at the highest HO342 concentration (17.4 μg/ml) exhibited an important increase of fluorescence intensity after SDS (2%) disruption (Fig. 6d).

Dd vesicles loaded with HO342 mediate the dye transfer to the nuclei of living cells

Transfer of HO342 to naive Dd cells

The transfer of HO342 from the V12H vesicles (×50) to the nuclei of naive *Dd* cells grown without HO342 was studied by video-microscopy. Three different fields were observed at different times, either with white light to control the cell morphology (Fig. 7a,c,e), or under UV excitation for fluorescence observation of the nuclei (Fig. 7b,d,f). After 5 min (a,b), very few fluorescent structures appeared, but after 35 min (c,d) and 65 min (e,f), almost all the nuclei were heavily stained within the living cells. As a control, concentrated (×50) V12C vesicles were added to naive *Dd* cells and no fluorescent structures were observed after

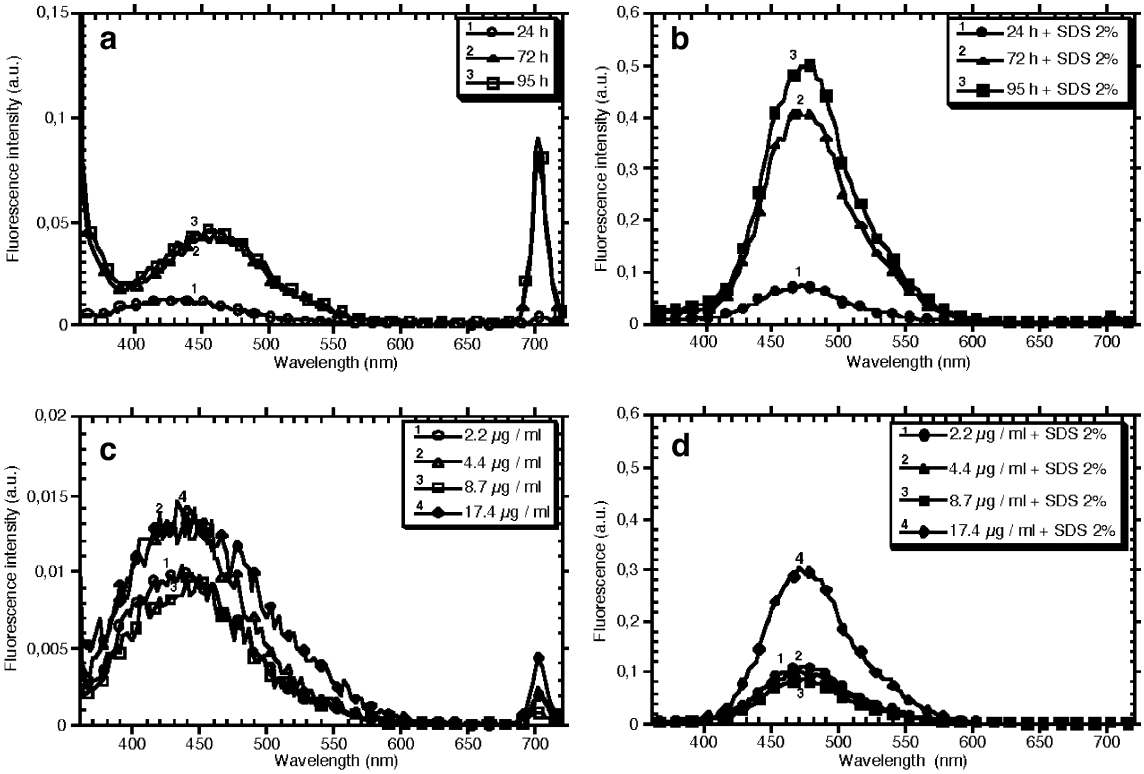

Fig. 6 Fluorescence analysis of HO342 loading in the *Dictyostelium* V12H vesicles. **a** Influence of incubation time of the cells with HO342 (10 μg/ml) on the fluorescence emission spectra of the V12H vesicles (×20); **c** Influence of incubation of the cells with different concentrations of HO342 during a 24 h period on the fluorescence emission spectra of the V12H vesicles (×20). In both cases, fluorescence emission spectra were recorded after vesicle disruption by SDS 2% (**b**, **d**). All spectra were obtained with an excitation wavelength of 350 nm

60 min (data not shown). In the same experimental conditions, the HO342-containing LUV (Fig. 4d), were not efficient for the transfer of HO342 to naive *Dd* cells, even after 60 min (data not shown).

The V105H vesicles (×50), although containing apparently less HO342 than the V12H vesicles, as shown by fluorescence spectrofluorimetry (Fig. 5b), were also able to mediate HO342 transfer towards the nuclei of living *Dd*

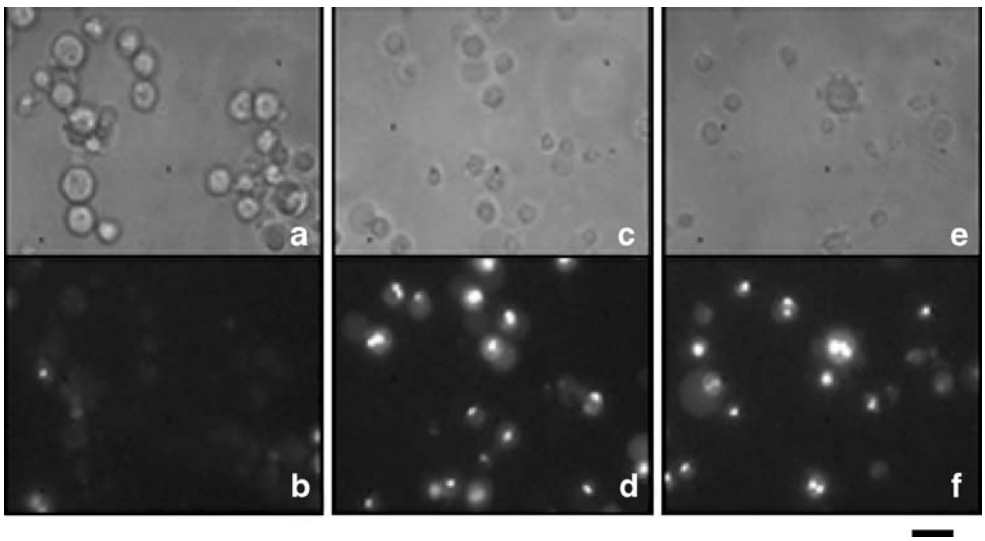

Fig. 7 Transfer of HO342 mediated by the V12H vesicles to the nuclei of naive *Dictyostelium* living cells. *Dd* cells in cell growth medium were incubated in the presence of concentrated (×50) V12H vesicles in vesicle medium (see "Experimental"). Three different fields, representative of the whole preparation, were observed at different times. Observations were performed by white light microscopy, either with phase contrast (×40 CP objective) (**a**), or without phase contrast (×40 UV objective) (**c**, **e**). The same three fields were successively observed by UV fluorescence microscopy (×40 UV objective) (**b**, **d**, **f**). Bar=20 μm

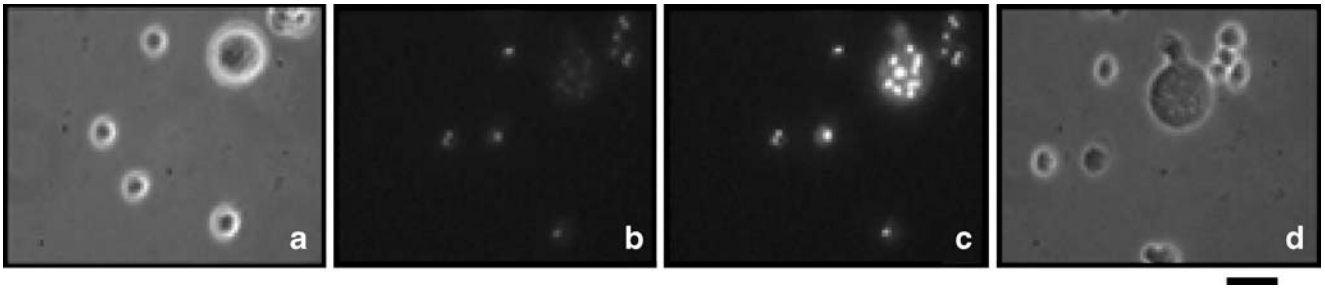

Fig. 8 HO342 transfer mediated by the V105H vesicles to the nuclei of naive *Dictyostelium* living cells as a function of time. A single field of *Dictyostelium* cells in cell growth medium was incubated in the presence of V105H vesicles (×50) (see "Experimental") and observed, either by phase contrast (×40 CP objective) at 5 min (**a**) and 3 h 50 (**d**), or by UV fluorescence microscopy (×40 UV objective) at times 2 h 30 (**b**), and 3 h 30 (**c**). Bar=20 μm

cells. The kinetics of dye transfer was observed on a single field by UV excitation in the time range of 8 min to 3 h 30. No fluorescence of the nuclei was observed before 2 h 30 (Fig. 8b). Only after 3 h 30, could the labeling of the nuclei (Fig. 8c) be compared with the one observed after 35 min in the presence of V12H vesicles (×50) (Fig. 7d). Observation by phase contrast microscopy (Fig. 8a and d) showed a morphological modification of the living cells during the long observation time, but without any feature of cell necrosis.

Transfer of HO342 to living human leukemia K562r cells

Despite their lower HO342-loading capacity, the V105H vesicles appeared very similar to the V12H vesicles by all other characteristics. Therefore, we used the V105H vesicles, being further purified than the V12H vesicles, to check their ability to transfer HO342 to the nuclei of human leukemia living cells. This was investigated by adding V105H vesicles (×50) to multidrug resistant K562r cells. Phase contrast microscopy (Fig. 9a,c,e,g) showed that the cells were still refringent after staying about 4 h under a cover glass. Two of the cells had a quite normal appearance (Fig. 9c,g), whereas some blebs were observed on the two others (Fig. 9a,e). The efficient transfer of HO342 to the cell nuclei was observed under UV excitation after about 4 h. The simultaneous observation of the same cells with white light and UV excitation (Fig. 9b,d,f,h) clearly showed that the HO342-stained nuclei were inside the K562r cells.

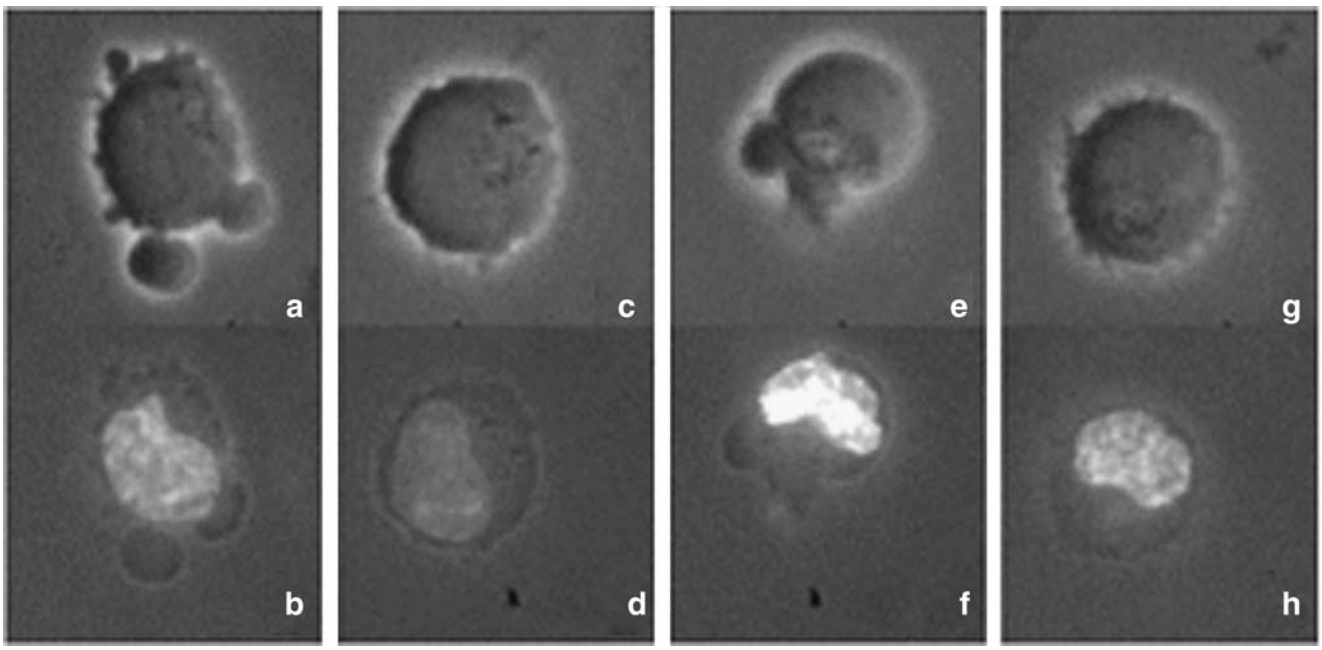

Fig. 9 Transfer of HO342 mediated by the *Dictyostelium* V105H vesicles to the nuclei of human leukemia living K562r cells. Cells were incubated (v/v) with V105H vesicles (×50) (see "Experimental"). Four different K562r cells are shown, in the range of incubation time 3 h 40–4 h 10, as observed either with phase contrast (×40 CP objective) (**a**, **c**, **e**, **g**) or with both white light and UV fluorescence microscopy (×40 UV objective) (**b**, **d**, **f**, **h**). Bars=20 μm

Discussion

In a previous work, we have shown the externalization of vesicles by *Dd* cells, obtained after a 105,000×*g* centrifugation of the cell-free culture medium, both as a detoxification mechanism when cells were grown in presence of HO342, and as a constitutive secretion process in the absence of HO342. The first characterization of these vesicles by SDS-PAGE, electron microscopy and lipid analysis gave some indications on their protein content, their morphological aspect and the nature of the lipid membrane [1].

In this work, in order to further characterize these vesicles at different steps of purification, we obtained and studied the pellet of a 12,000×*g* centrifugation of the cell-free culture medium in comparison with the one obtained after a further 105,000×*g* centrifugation of the 12,000×*g* supernatant. From the electrophoretic patterns and the electron micrographs of both these vesicles, no significative difference could be described. Therefore, proteomic of the *Dd* vesicles is now in progress to shed some more light on the nature of these vesicles.

The specific aim of the present work was to study the ability of the vesicles expelled by the non-pathogen unicellular eukaryotic microorganism, *Dictyostelium discoideum*, to transport a DNA specific dye to nuclei of living cells. Previous studies by spectrofluorimetry gave some data on the fluorescence properties of HO342 in interaction with DNA [2, 3], as well as in interaction with the anionic detergent SDS [3]. The interaction of the dye with lipids has also been reported [18], but without any spectral data. In the present work, we compared the fluorescence spectral properties of HO342 transported by the *Dictyostelium* vesicles, with the ones of the dye either in buffer or complexed with DNA, lipids and/or SDS in the same conditions. Such a comparison permitted to approach the dye chemical state inside the vesicles. When the dye was transported by the vesicles, as compared to the dye in aqueous solution, a blue shift in the maximum intensity from 475 to 450 nm, was observed, indicating a higher energy level of the dye excited state inside the vesicles, as well as a higher value of the fluorescence intensity. Such fluorescence characteristics are to be related to the ones of the dye complexed either with DNA or with lipids, which exhibits a similar blue shift with a maximum, respectively at 452 nm for DNA and 447 nm for lipids. These results strongly suggest that, with 10 μg/ml HO342 for cell incubation, most of the dye transported by the *Dictyostelium* vesicles is complexed with either DNA and/or lipids. To compare the spectra of the dye loaded in the *Dd* vesicles, with the ones of HO342 in interaction with the studied biomolecules, DNA and lipids, and/or with SDS, the vesicles were disrupted by SDS 2% (Fig. 5c,d). In these conditions, the HO342 freed from the V12H and V105H vesicles exhibited a fluorescence emission peak red-shifted at 470 nm instead of the maximum at 450 nm observed for HO342 associated to the undisrupted vesicles, indicating a lower energy level of the excited state of the dye-SDS complex. Such an effect could be ascribed to the large difference in the dipole moment of the solvent in interaction with the dipole moment of the dye-excited state. It is worth noting that the fluorescence intensity increase (×25), due to the addition of SDS to the vesicle suspension, was much lower than the one observed for the dye interacting with SDS in buffer (×150), but ten times higher than the one observed when SDS was added to the dye-DNA complex (×2.5). By addition of SDS to the dye-lipid complex, no increase in intensity was observed, leaving therefore the total increase of the dye fluorescence in the vesicles in presence of SDS, as resulting of a part complexed with DNA and a part free in the vesicle medium.

The data obtained as a function of the cell incubation time with the dye demonstrated that the higher amount of dye loading the vesicles was obtained between 24 and 72 h. The results on the dye concentration dependence in the growth medium suggested a process of HO342 vesicle loading comprising two steps: below about 9 μg/ml, all the dye is complexed with DNA and/or lipids; above this threshold free HO342 is present in the vesicles. These results will be of use for the optimization of the vesicle preparation as a new tool for drug delivery.

In contrast with most mammalian cells, but mammalian stem cells [19, 20], naive *Dictyostelium* cells are constitutively resistant to the vital staining of their nuclei [1]. The present data of video-microscopy demonstrate the ability of both the V12H and the V105H vesicles to transfer the dye to the nuclei of naive *Dictyostelium* cells. However, the dye transfer occurred with a higher rate for the V12H vesicles than for the V105H vesicles. Thus, when comparing the fluorescence spectral properties of HO342-transporting vesicles at the two different steps of preparation as well as their HO342-transfer efficiency, it appeared that the 105,000×*g* pellet of vesicles had a lower amount of HO342 loaded as well as a lower ability to transfer the dye to the nuclei of naive *Dictyostelium* living cells than the 12,000×*g* vesicles, suggesting that the V12H vesicles are better vectors for HO342 transfer. These observed differences between the two types of vesicles were not explained at the present stage of the study. For further optimization of the vesicle preparation as a tool for drug delivery, some more steps of purification as well as protein identification of both types of vesicles have to be performed.

A possible use of *Dictyostelium* vesicles as vectors able to reach the nuclei of non homologous cells was studied with human leukemia, K562r, multidrug resistant cells, known to be also resistant to HO342 vital staining of their nuclei [9]. Independently of the large difference in size of

the DNA and the nuclei of human cells as compared to *Dictyostelium* cells, the transfer of HO342 mediated by the *Dictyostelium* vesicles to the nuclei of K562 cells was efficient at a rate comparable to the one observed with the same vesicles to the nuclei of *Dictyostelium* cells. This indicates that the vesicle-mediated dye delivery was able to overcome, as well the constitutive resistance of *Dictyostelium* cells as the drug-induced resistance in K562r cells, for the vital nuclei staining.

These data suggest for the *Dictyostelium* extracellular vesicles a role in shipping intercellular molecular information. Such a role could be compared to the one of mammalian cell extracellular vesicles like exosomes [21, 22] involved in different functions and namely in the transfer of antigens [23] .The externalization of microvesicles of different size, origin and function, is a property of many mammalian cell types [10, 24, 25].Whatever their origin, all the extracellular vesicles are examples of « vesicles shipping extracellular messages » [26], and « triggering intercellular communication » from one cell to another [27]. A possible analogy with the budding of exosomes from multivesicular bodies of mammalian cells fusing with the plasma membrane [23] could be suggested for the cell externalization of vesicles. The mechanism of dye transfer to the nuclei of living cells by the *Dictyostelium* extracellular vesicles remains to be addressed. Two putative mechanisms have to be further explored: either a fusion of the vesicles with the plasma membrane and entry of the dye into the cells by reaching the endocytosis pathway and then the nuclear membrane or entry of the vesicles themselves by pinocytosis—or macropinocytosis—and then fusion with the nuclear membrane. These questions will be addressed in our further works.

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