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# Assessment of liposome disruption to quantify drug delivery *in vitro*

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ABSTRACT

Efficient liposome disruption inside the cells is a key for success with any type of drug delivery system. The efficacy of drug delivery is currently evaluated by direct visualization of labeled liposomes internalized by cells, not addressing objectively the release and distribution of the drug. Here, we propose a novel method to easily assess liposome disruption and drug release into the cytoplasm. We propose the encapsulation of the cationic dye Hoechst 34580 to detect an increase in blue fluorescence due to its specific binding to negatively charged DNA. For that, the dye needs to be released inside the cell and translocated to the nucleus. The present approach correlates the intensity of detected fluorescent dye with liposome disruption and consequently assesses drug delivery within the cells.



## 1. Introduction

Over the past few decades, there has been increasing interest in the use of liposomes for drug delivery. They represent one of the most widely researched methods for improving circulation time, bioavailability and targeting in numerous therapeutics [1,2]. The maximum effect of a drug can only be achieved by strictly controlling its specificity for the target cell.

However, other details of the drug delivery systems, such as efficient liposome disruption within cells, are often technically more challenging. Although drug behavior depends on the chemical properties of the compound in question, its release is also affected by other factors. The nature of individual liposomes as drug delivery systems, depends on physico-chemical properties such as size, shape, and chemistry, all of which determine the rate of disruption of the liposomes [3,4].

There is an increasing need to develop a system that can be used to assess the disruption of the liposomes into the cell. Fluorescence can be used to visualize labeled proteins, DNA and RNA in order to determine their release/localization in the cells [5]. The approach using labeled compounds is not always straightforward as the constructs must be developed. This process is time consuming and the detection limit is usually low unless the fluorescent materials aggregate or associated with specific cellular components [6]. Furthermore, the fluorescent markers have been extensively used for tracking drug delivery [7–9] but are not indicative of liposome disruption, not assuring release of drugs in the cell cytoplasm.

Hoechst 34580 (*N*,*N*-Dimethyl-4-[5-(4-methyl-1-piperazinyl)[2,5′-bi-1*H*-benzimidazol]-2′-yl]benzenamine trihydrochloride) (Fig. 1) is a fluorescent dye that is excited by ultraviolet light at 357 nm, and emits blue/cyan fluorescent light with an emission maximum at about 490 nm. Fluorescence is enhanced upon binding to double-stranded DNA [10]. This bisbenzimidazole derivative is a supravital minor groove-binding DNA stains with AT selectivity. The dyes bind to all nucleic acids, but AT-rich double stranded DNA enhances the fluorescence by more than ~2-fold when compared to GC-rich strands [11]. Because of this increase in fluorescence, Hoechst 34580 is used for the quantification of DNA and particularly for staining the nuclei of living and fixed cells.

In the present study, we intended to establish a method to assess the disruption of liposome drug delivery system *in vitro* using Hoechst 34580 as an imitating drug. The present approach enabled us to correlate the intensity of a fluorescent dye with the disruption of the liposome and consequent drug delivery in cell cytoplasm (Fig. 2).

*Abbreviations:* DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DSPE-MPEG, N-(carbonyl methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; EPC, egg phosphatidylcholine; PBS, phosphate buffered saline.

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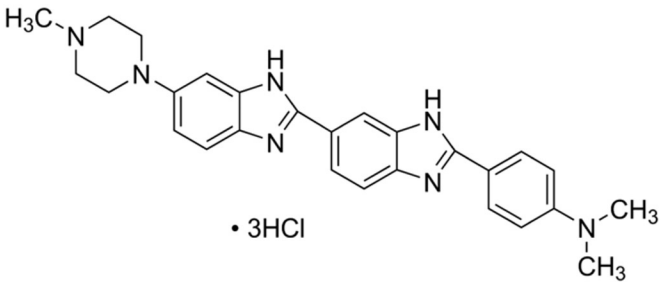


**Fig. 1.** Chemical structure of Hoechst 34580.

## 2. Materials and methods

### 2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), egg phosphatidylcholine (EPC) and N-(carbonyl methoxypolyethylene glycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE–MPEG) were obtained from Lipoid GmbH (Germany), and DSPE–PEG–FA was obtained from Avanti Polar Lipids (USA). Cholesterol and Hoechst 34580 were obtained from Sigma (USA). NucRed® Live 647 ReadyProbes® was obtained from Invitrogen (USA).

### 2.2. Liposome preparation

Liposomes composed of DOPE/EPC, cholesterol and DSPE–MPEG (57: 38: 5, molar ratio) were prepared as previously described [12]. Briefly, known amounts of DOPE/EPC, cholesterol and DSPE–MPEG were dissolved in chloroform in a 50 mL round-bottom flask. The organic solvent was evaporated using a rotary evaporator followed by additional evaporation under reduced pressure by a high-vacuum system to remove remaining traces of chloroform. The resultant dried lipid film was dispersed in phosphate buffered saline (PBS) buffer containing 38 μg/mL Hoechst 34580. The mixture was vortex-mixed at a temperature greater than the phase-transition temperature (room temperature) to yield multilamellar vesicles, which were then extruded (extruder supplied by Lipex Biomembranes Inc., Vancouver, Canada) through 200 nm pore size polycarbonate filters (Nucleopore) followed by several passages through 100 nm polycarbonate filters (Nucleopore) to form large unilamellar vesicles. The dye not incorporated into liposomes was removed from the samples after passage through a gel filtration chromatography column (GE Healthcare).

### 2.3. Leakage assay

After 1 week of preparation, liposomes were subjected to a new passage through a gel filtration chromatography column (GE Healthcare). The eluate contain eventual free Hoechst 34580 dye, released from liposomes, was collected. The following procedure was used to detect the free dye, adapted from Silvander M. and Edwards K., 1996 [13]. The initial emission spectra from eluate collected after gel column separation of liposomes was measured. The spectra of Hoechst 34580 was also obtained, at the same concentration inside liposomes. Then DNA was added in excess (5 mg/mL) to bind all free dye. The addition of DNA brings about an enhanced fluorescence intensity if free Hoechst 34580 is presented in solution, binding to supravital minor groove of DNA. A Synergy Mx Multi-Mode Reader from BioTek (USA) was used to perform the fluorescence measurements. Emission spectra was performed after excitation at 357 nm.

### 2.4. Cell culture conditions

The human colonic epithelial cell line (Caco-2) (ATCC, HTB-37) was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate supplemented with 10% (*v*/*v*) of fetal bovine serum (FBS), 1% (*v*/*v*) penicillin/streptomycin solution and 1% (*v*/*v*) non-essentials amino acids. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. Caco-2 cells were routinely subcultured over 7 days, and the culture medium was replaced every 2 days. Human peripheral blood mononuclear cells were isolated from buffy coats of blood donors from the Immunohaemotherapy Department of Hospital de São João (Porto, Portugal) by centrifugation over Lymphoprep (Axis-Shield), and monocytes

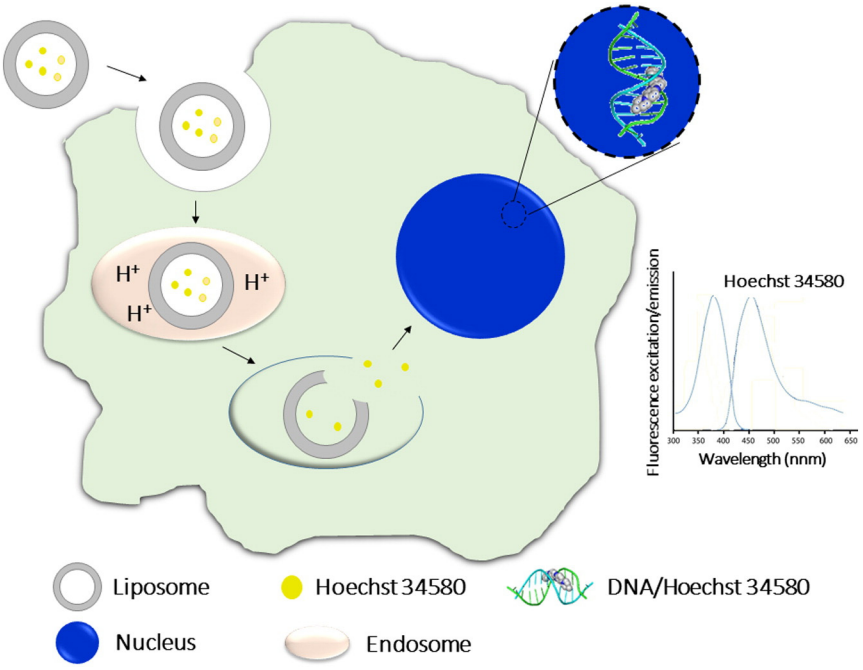


**Fig. 2.** Schematic representation of proposed method to simulate liposomal drug release *in vitro*.