The Fluorosome™ technique for investigating membrane on- and off-loading of drugs by β-CD and sonicated SUV

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Received 25 October 2001; accepted 19 November 2001

First published online 6 March 2002

Edited by Maurice Montal

Abstract The application of the Fluorosome technique to test drug delivery systems is described. Fluorosomes, egg phosphatidylcholine liposomes with bilayer embedded fluorophores, were employed to investigate the ability of sonicated small unilamellar vesicles (sSUV) and β-cyclodextrins (β-CD) to deliver drugs into or extract drugs from the fluorosome's phospholipid bilayer. The addition of phloretin to a fluorosome suspension resulted in fluorescence reduction reflecting phloretin entering the bilayer and quenching fluorophore fluorescence. Subsequent addition of sSUV to phloretin pretreated fluorosomes showed an increase in fluorescence reflecting phloretin extraction from the fluorosome membrane. Sequential additions of β-estradiol loaded β-CD to fluorosomes as well as the addition of β-estradiol alone resulted in fluorescence reduction due to β-estradiol insertion into the membrane. Further addition of pure β-CD resulted in a fluorescence increase indicating β-estradiol extraction from the fluorosome membrane.

Key words: Fluorosome; Nitrobenzoxa-1,3-diazolyl fluorophore; Diphenylhexatrienyl propanoyl fluorophore; Drug delivery system; Cyclodextrin; Sonicated small unilamellar vesicle

1. Introduction

In the past, the major focus in drug research has been the development of drugs with new or increasingly potent biological activities. While this is still the case, the development of new formulations emphasizing drug delivery systems is becoming of increasing importance in enhancing drug effectiveness and safety [1,2]. Free doxorubicin, an antibiotic and antitumor drug, for example, is pumped out of multidrug resistant cancer cells by P-glycoprotein. Doxorubicin loaded nanoparticles are able to adsorb onto the tumor cell and progressively release the encapsulated drug, the high local drug concentration gradient favoring the intracellular penetration of doxorubicin [3].

Drug delivery systems can be formed variously from lipids, carbohydrates, proteins, or synthetic polymer systems in a large variety of configurations [1,2,4–6], for example, drug entrapment in vesicles or liposomes [1,5,7]. Moreover, lipid systems such as sonicated small unilamellar vesicles (sSUV) are used to extract cholesterol from lysosomes [8]. Cyclodextrins (CD), water soluble cyclic oligosaccharides containing a minimum of six D-glucopyranose units with hydrophobic cavities [9], are also used as drug delivery systems in pharmaceutical applications [4]. β-CD can be used to manipulate and mediate cellular cholesterol content [10–14] as well as in removing cholesterol from homogenized milk [15].

Lipid bilayer membranes are key objects in drug research with regard to the interaction of drugs with membrane bound receptors, drug targeting, penetration, and permeation of cell membranes, and the use of liposomes in micro-encapsulation technologies for drug delivery [6,16].

In view of the increasing rate of development in the area of drug delivery, a rapid in vitro system to investigate the ability of a drug delivery vehicle to deliver drugs into a membrane bilayer would be beneficial. In previous studies, we described a rapid in vitro fluorescent assay, the Fluorosome technique, for measuring the entry rates of molecules into membranes [17,18]. Fluorosomes are small unilamellar vesicles, with a fluorescent dye embedded in their bilayers. The decrease of fluorescence induced by the presence of drug molecules in the bilayer is used to measure the bilayer entry of such molecules. It might be expected that the extraction of drug molecules from the membrane should result in a fluorescence increase and therefore monitor bilayer exit after initial insertion. The Fluorosome technique requires only nanomol quantities of test molecules and can measure a wide variety of membrane entry rates. In this paper we demonstrate the potential of the Fluorosome technique as an empirical in vitro method to characterize the entry of a drug from a carrier moiety into a membrane bilayer as well as drug removal from a membrane by a carrier vehicle. For this purpose we employed sSUV and β-CD as model carrier vehicles.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC, 26.3 mM, in chloroform) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). β-DPH-HPC (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) and NBD-C_6-HPC (2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phospho-

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Abbreviations: β-CD, β-cyclodextrin; β-DPH-HPC, 2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethyl sulfoxide; egg PC, egg phosphatidylcholine; MLV, multilamellar vesicles; NBD-C_6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; sSUV, sonicated small unilamellar vesicles; Tris, tris(hydroxymethyl)aminomethane

0014-5793/02/$22.00
PII: S0014-5793(02)02512-7

choline) (Molecular Probes, Eugene, OR, USA) were dissolved in chloroform (HPLC-grade, Sigma-Aldrich, Milwaukee, WI, USA) to concentrations of 355 μM and 1.7 mM, respectively. Phloretin and β-estradiol (Sigma, St. Louis, MO, USA) were dissolved to 10 mM and 40 mM, respectively in dimethyl sulfoxide (DMSO, ICN Biomedicals Inc., Aurora, OH, USA) and were stored in brown bottles at 277 K.

10 mM Tris–HCl pH 7.4 buffer was made of ultrapure tris(hydroxymethyl)aminomethane (Tris, T.J. Baker, Philipsburg, NJ, USA), filtered through 0.2 μm GTTP Isopore™ membranes (Millipore, Bedford, MA, USA) to prevent dust and stored at 277 K.

2.2. Preparation of β-CD solutions

Solutions of 10 mM and 13.3 mM β-CD (Sigma) were prepared in ultrapure water (pH 5.6, conductance of 18.2 MΩcm, Photronix, Medway, MA, USA) immediately before use. β-Estradiol loaded β-CD were prepared by mixing 300 μl of a 13.3 mM β-CD solution in ultrapure water with 20 μl of a 40 mM β-estradiol solution in DMSO together with an additional 200 μl DMSO and stirred overnight. The β-CD/β-estradiol solution was used within 12 h of preparation.

2.3. Preparation of sSUV

sSUV were prepared by adding 300 μl of a 26.3 mM egg PC/chloroform solution into a 6 ml flat bottom glass tube, evaporating the chloroform under a nitrogen flow and placing the resulting film under vacuum (0.1 mm Hg) for 3 h. The resulting film was hydrated with 10 mM Tris buffer pH 7.4 for 5 min and agitated to form multilamellar vesicles (MLV). 500 μl of the MLV suspension was transferred to another 6 ml glass tube, placed in ice water to prevent overheating during sonication and sonicated using the sonicator microtip (Sonic Dismembrator model 300, Fisher Scientific, Bohemia, NY, USA) at 35% full power for 25 min until a clear sSUV solution was obtained. To avoid membrane fusion, the sSUV were stored at 277 K and used within 1 day.

2.4. Preparation of fluorosomes

β-DPH-HPC/egg PC fluorosomes: chloroform solutions of 300 μl 26.3 mM egg PC and 10.7 μl 355 μM β-DPH-HPC were pipetted into a glass tube (giving a 0.05 mol% fluorescence dye to phospholipid ratio). NBD-C_6-HPC/egg PC fluorosomes: chloroform solutions of 300 μl 26.3 mM egg PC and 20 μl 1.7 mM NBD-C_6-HPC were pipetted into a glass tube (resulting in a 0.43 mol% fluorescence dye to phospholipid ratio). The organic solvent was evaporated under a flow of nitrogen. The resulting film was put under vacuum (0.1 mm Hg) for 3 h and taken up in 600 μl ultrapure water (NBD-C_6-HPC fluorosomes) or 600 μl 10 mM Tris buffer pH 7.4 (β-DPH-HPC fluorosomes). After 5 min the film was agitated to form MLV. Unilamellar vesicles of a homogeneous size were formed by use of a membrane extrusion device (Liposofast®, Avestin, Ottawa, ON, Canada). Using this device the MLV were subjected to 31 passes through a polycarbonate filter with 100 nm pores. Fluorosome size was confirmed by photon correlation photometry to be tightly centered around 100 nm (Coulter N4 submicron particle analyzer, Coulter Electronics, Hialeah, FL, USA). Approximately 500 μl of the fluorosome solution was diluted with water to a final volume of 100 ml. To avoid photobleaching, all preparation steps were carried out under aluminum foil. All fluorosome suspensions were stored in aluminum foil wrapped bottles at 277 K and used within 1 week.

2.5. Fluorescence spectroscopy

Measurements were performed using 3 ml aliquots of fluorosome suspension at 293 K in a FluoroMax II (SPEX, Edison, NJ, USA) under intense stirring. Spectrophotometer settings for experiments with β-DPH-HPC/egg PC fluorosomes were: excitation 356 nm, emission 428 nm, excitation slit bandpass 1 nm, emission slit bandpass 6 nm, and for the NBD-C_6-HPC/egg PC fluorosomes: excitation 465 nm, emission 538 nm, excitation slit bandpass 1 nm, emission slit bandpass 6 nm. The fluorescence emission of the fluorosomes was monitored for a minimum 1000 s prior to the addition of drug solutions to the cuvette to establish a baseline. A 10 μl syringe (Hamilton, Reno, NV, USA) with a custom made extra long needle was used to add up to 5 μl of the β-estradiol solution as well as the β-CD solution to the NBD-C_6-HPC/egg PC fluorosomes through an injection port on the spectrophotometer. A pipettor (Pipetman®, Gilson, distributed through Rainin, Woburn, MA, USA) was used to perform the experiments with the β-DPH-HPC/egg PC fluorosomes. For this purpose, the measuring chamber was protected against light by a custom made lid. The addition of 5 μl or less pure DMSO to the fluorosomes showed no effect on the fluorescence signal or on the fluorosome size as determined by the Coulter submicron particle analyzer. Emission values were acquired every second to ensure a significant acquisition of data points.

3. Results

3.1. Phloretin/sSUV system

Unilamellar egg PC vesicles with 0.05 mol% β-DPH-HPC as fluorescent probe (β-DPH-HPC/egg PC fluorosomes) were prepared and used to examine changes in fluorescence intensity after the addition of phloretin and sSUV. The results are presented in Fig. 1 as fluorescence vs. time graphs. Fig. 1A–D shows the actual fluorescence intensities, while in Fig. 1E, a summary of the previous frames is given for comparison purposes.

Due to the photobleaching of β-DPH-HPC, baseline fluorescence intensity decreases in the course of an experiment in a non-linear manner (3% within the first 1000 s). In Fig. 1A,B, circle-ended arrows indicate the addition of 0.5 μl aliquots of a 10 mM DMSO solution of phloretin (5 nmol phloretin) to a fluorosome suspension. The additions of phloretin result in rapid and large decreases in fluorescence intensity. Sequential additions of sSUV to the fluorosome system (regular arrows) after the initial addition of phloretin result in sequential increases in fluorescence. In Fig. 1A, following an initial addition of two additions of sSUV, a second addition of phloretin results again in a decrease in fluorescence.

Fig. 1C,D shows control experiments, demonstrating that the addition of sSUV (2×8 μl, Fig. 1C) or pure DMSO (2×1 μl, Fig. 1D) to the fluorosome preparation results in no change in fluorescence.

The normalized fluorescence compilation summarizing all measurements (Fig. 1E) demonstrates that the same baselines are obtained for the reference experiments with sSUV and DMSO as well as for the phloretin experiments prior to the addition of phloretin. The additions of 5 nmol phloretin to individual fluorosome suspensions result in the same magnitude of fluorescence decrease. Additionally, fluorescence increases equivalently for both experiments after additions of 8 μl sSUV to the phloretin loaded fluorosome suspension.

3.2. β-Estradiol/β-CD-system

Unilamellar egg PC vesicles with 0.43 mol% NBD-C_6-HPC as fluorescence probe (NBD-C_6-HPC/egg PC fluorosomes) were prepared and used to examine the changes in fluorescence intensity after the addition of β-estradiol and β-CD. The results are presented in Fig. 2 as fluorescence vs. time graphs. Fig. 2A–C are actual measured fluorescence intensities, while Fig. 2D, a compilation of all NBD-C_6-HPC/egg PC fluorosome experiments, is given for comparison purposes.

Due to the photobleaching of NBD-C_6-HPC, baseline fluorescence intensity decreases in the course of an experiment in a non-linear manner (1% within the first 1000 s). In Fig. 2A, the circle-ended arrow indicates the addition of 1 μl of 40 mM (40 nmol) β-estradiol as a DMSO solution to a fluorosome suspension. The addition of β-estradiol results in a rapid and large decrease in fluorescence intensity, which is reversed in part by the two additions of 5 μl of the β-CD solution indicated by regular arrows.

Fig. 2B shows an experiment where β-CD preloaded with β-

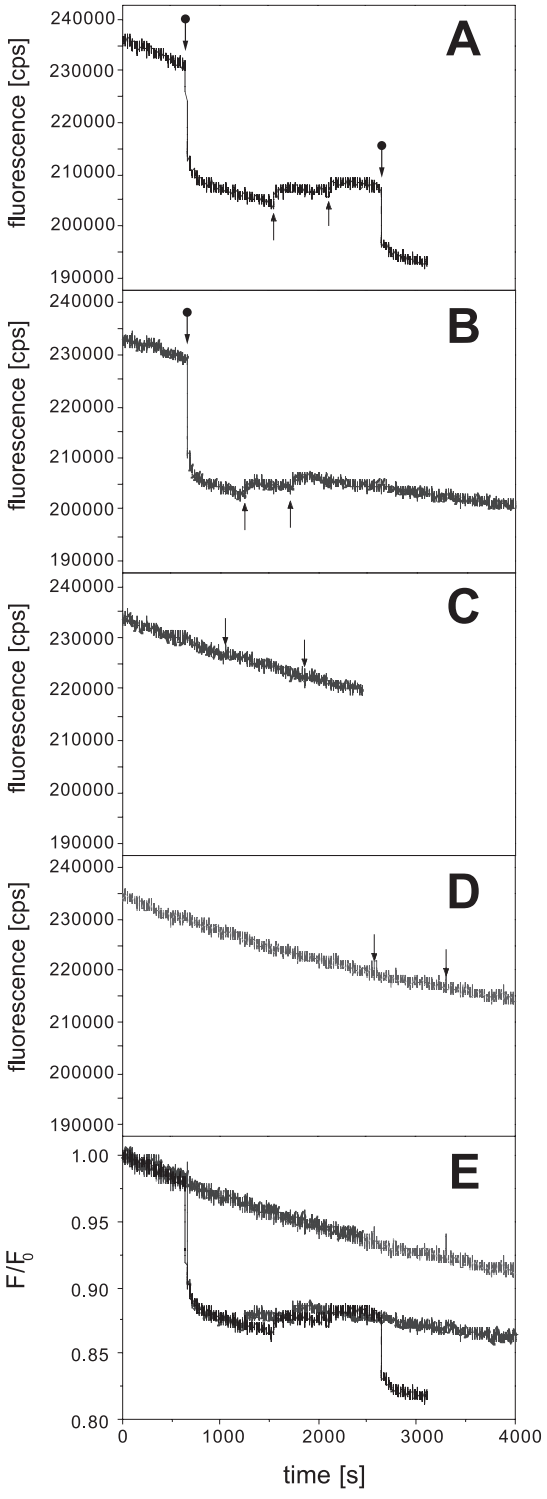

Fig. 1. Fluorescence intensity vs. time resulting from phloretin insertion into the membranes of β-DPH-HPC/egg PC fluorosomes and its subsequent extraction by sSUV. A,B: Addition of 5 nmol phloretin in DMSO (circle-ended arrows) results in a rapid decrease in fluorescence reversed by subsequent applications of 8 μl sSUV suspension (normal arrows). Fluorosomes are sensitive to a second addition of phloretin even after sSUV addition (A). C,D: Control additions (arrows) of 2×8 μl sSUV (C) and 2×1 μl DMSO (D) lead to no change in fluorescence signal. E: Composite normalized fluorescence for the previous panels.

estradiol was added in aliquots (arrows) to a fluorosome suspension to a final β-estradiol content of 40 nmol. All additions resulted in decreases in fluorescence intensity. In Fig. 2C the arrows indicate additions of pure β-CD solution, which lead to no change in fluorescence intensity. Pure DMSO also results in no reduction of the fluorosome fluorescence (data not shown).

Fig. 2D shows a composite plot of Fig. 2A–C with the fluorescence normalized; it may be noted that the presence

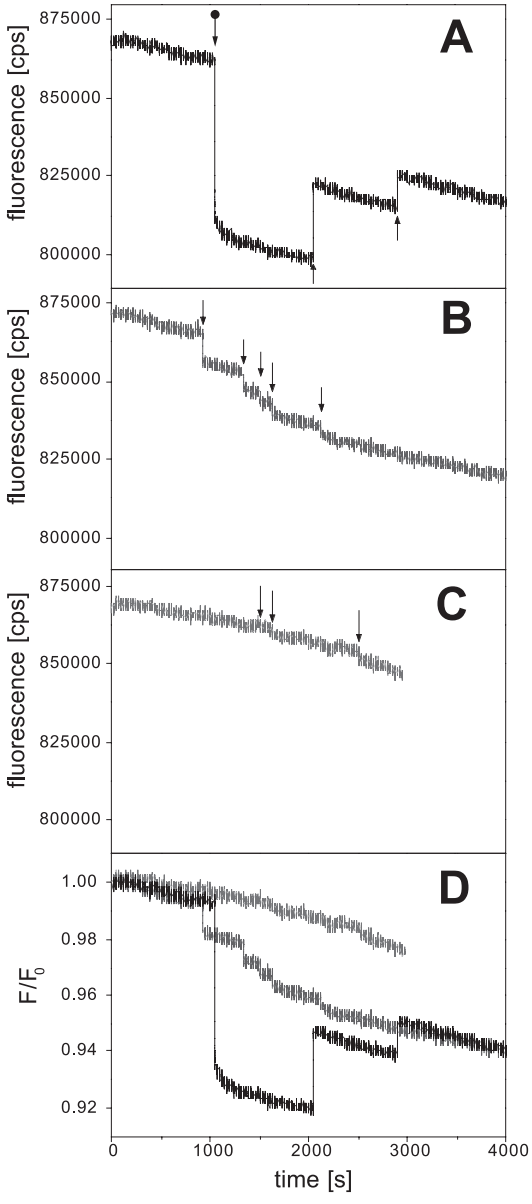

Fig. 2. Fluorescence intensity vs. time resulting from β-estradiol insertion into the membranes of NBD-C_6-HPC/egg PC fluorosomes and its subsequent extraction by β-CD. A: Addition of 40 nmol β-estradiol as a DMSO solution (circled-ended arrow) results in a rapid decrease in fluorescence. Successive additions of 5 μl β-CD solution (normal arrows) lead to progressive increases in fluorescence intensity. B: Additions of preincubated β-CD and β-estradiol result in decreases in fluorescence intensity. C: Control additions (arrows) of untreated β-CD result in no significant change in fluorescence. D: Composite normalized fluorescence for the previous panels. An equal normalized fluorescence intensity obtains for the experiments shown in A and B in which the final amounts of β-estradiol are the same.

of 40 nmol β-estradiol in a NBD-C_6-HPC fluorosome suspension results in the same value of fluorescence in the presence of approximately equivalent amounts of β-CD, regardless of the manner the β-CD addition was made.

4. Discussion

We describe the use of the Fluorosome technique to investigate drug delivery or drug extraction from membrane bilayers using sSUV and β-CD. The previously published procedure for fluorosome preparation [17,18] was altered by preparing an egg PC/fluorophore film prior to MLV formation, which enables the usage of complex fluorophores and ensures complete incorporation of fluorophore molecules into the membrane. The use of fluorosomes employing fluorophores not covalently bound to anchor phospholipids is not suitable for studies on drug delivery vehicles, since unbound fluorophores can be extracted form the bilayer by drug delivery vehicles. The fluorosomes used in this study contained either of two different fluorophores, β-DPH-HPC or NBD-C_6-HPC. These were chosen to demonstrate the versatility of the Fluorosome technique for investigating a variety of drugs or drug delivery vehicles, even in those cases where the drug or drug delivery system possesses an intrinsic fluorescence overlapping in wavelength that of a specific fluorophore. Other fluorophores, in addition to the two described in this paper, can be used in the preparation of fluorosomes.

Our results using sSUV loaded with β-estradiol are similar those of Noy and Xu [19] who used unilamellar egg PC vesicles with 2 mol% NBD-DPPE as both acceptor and donor vesicles to examine the transfer of retinol between model membranes. Their system had the fluorophore anchored at the headgroup region of the phospholipid bilayer. Thus the retinol molecules were detected at the exterior hydrophilic region of the model membrane, in contrast to our system where the drugs are detected only after they enter the interior of the bilayer itself.

Both fluorosome systems, β-DPH-HPC/egg PC and NBD-C_6-HPC/egg PC, show slowly decreasing fluorescence intensities due to photobleaching of the fluorescence dyes, however this gradual photobleaching does not obscure fluorescence changes resulting from the insertion of phloretin and β-estradiol into the model membranes. Reference experiments with pure DMSO as well as aqueous solutions as used for the sSUV preparation and β-CD solution showed no effect on the fluorescence of the fluorosomes. β-CD solutions and sSUV suspensions were also tested for their influence on fluorosome fluorescence, both showing no effect on the fluorescence signal.

β-DPH-HPC/egg PC fluorosomes were used to demonstrate their ability to monitor the extraction of phloretin from membrane bilayers by sSUV. The removal of phloretin by sSUV is seen as a reversal of the fluorescence quenching initially brought about by the entry of phloretin molecules into the fluorosome membrane.

NBD-C_6-HPC/egg PC fluorosomes were employed to demonstrate their ability to monitor the extraction of β-estradiol from membrane bilayers by β-CD. To demonstrate the ability of fluorosomes to monitor drug transfer from drug delivery systems into membrane bilayers, β-CD loaded with β-estradiol was added to a fluorosome suspension. Sequential additions of β-CD/β-estradiol resulted in a reduction of fluorescence signal, as did the addition of β-estradiol alone. Normalized fluorescence intensities were the same for a final amount of β-estradiol of 40 nmol in both experiments, despite the manner in which β-estradiol and β-CD were added to the fluorosome suspension: the hormone inserted into the fluorosome membrane and was extracted by β-CD in the first experiment, the β-estradiol was transferred from β-CD to the fluorosome membrane in the second experiment.

In conclusion, the Fluorosome technique should prove valuable in the design, characterization, and development of drug delivery systems. It has the potential to provide a simple, sensitive in vitro means, at an early phase of development, for characterizing the ability of drug delivery vehicles to release molecules into membranes or extract them from membranes.

Acknowledgements: This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG). Fluorosome™ is a registered trade mark of GLSynthesis Inc., http://www.glsynthesis.com. The Fluorosome™ technique together with associated methodologies is patent pending.

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