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Fluorescent lipid based sensor for the detection of thymidine phosphorylase as tumor biomarker

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ABSTRACT

5-Fluorouracil (5-FU) is a chemotherapic drug widely employed to treat a wide range of solid tumors. Unfortunately, it has a narrow therapeutic window and the level of its target enzymes in biological fluids of patients can vary considerably. On these premises, a new fluorescent lipid based sensor for the detection of thymidine phosphorylase, one of the target enzymes of 5-FU, was developed, to optimize patient treatment. Both cationic and anionic fluorescent liposomes containing both an amphiphile tail-tagged with a pyrene residue and a 5-FU derivative were investigated. The effect of the presence of a bulky quencher (the bromine atom) covalently linked to the end of the alkyl chain of the anionic component on the emission signal was also evaluated. The interaction of liposomes with the target enzyme induces the occurrence of a fluorescent signal, at an extent that depends on the formulation, due to the variation of the excimer/monomer ratio. In particular, a promising specific result was obtained upon the interaction of the target enzyme with liposomes formulated with DOPC, the cationic fluorescent surfactant, the 5-FU derivative and 11-bromoundecaonic acid at 5/1/1/3 molar ratio. Langmuir compression isotherms allowed clarifying the influence of lipid organization on the response of the sensor.

1. Introduction

Thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) are three proteins involved in the metabolism of pyrimidines and are the target of a potent chemotherapeutic agent, 5-fluorouracil (5-FU), widely employed in the treatment of some of the most frequently occurring malignant tumors (breast, colon, and skin cancer). Their presence or their absence in biological fluids is indeed related to a specific state of health and their easy and fast detection is a problem of major concern. In fact, for patients affected by DPD deficiency (5–8%) 5-FU can even be fatal at the very first dose [1]. In fact, being DPD the rate limiting enzyme in extracellular 5-FU catabolism, its deficiency increase the bioavailability of 5-FU active metabolites causing an increase of the toxicity of the drug. Moreover, the drug can have severe toxic effects if patients are overdosed (about 15%) whereas it will have a reduced therapeutic efficacy if patients are underdosed (about 50%) [2]. Thus it is clear the need for a rapid and accurate detection of the activity of these enzymes before and during 5-FU treatment to individuate patients who cannot be treated and to set the optimal doses of treatment. At present, simple, reliable and cheap screening methods to dose the activity of the three enzymes before and during the treatment with 5-FU do not exist.

A successful chemosensor requires the assembly of one or more receptor units together with a transducer part in a geometrically defined pattern to allow the fast and reliable identification of the presence or the absence of manifold target molecules. Liposomes can be sensor elements acting as transducer components that support amphiphilic sensing materials (*i.e.* a fluorescent probe) and the receptor molecule [3–5]. Liposomes, combined with fluorescent compounds, received considerable attention as sensors for chemical and biological detection because they generate a quick signal (thus reducing the diagnosis time), are very sensitive and are relatively inexpensive [6,7]. Pyrenyl amphiphiles, due to their ability to form excimers (E) characterized by spectral emission at different wavelength with respect to the monomer (M), can be exploited as fluorescent probes for sensing applications [8], because

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the variation of E/M ratio can indicate lipid rearrangement upon the interaction of the lipid assembly with a certain target molecule.

Herein we report an investigation on the development of a new fluorescent lipid-based sensor to detect the presence of the TP. TP was chosen as model target enzyme of 5-FU in this investigation because is the only one commercially available. The sensor is constituted by mixed liposomes in which the sensing element is **1**, a cationic amphiphile tagged on the hydrophobic tail with a pyrene moiety, and the recognition element is **2**, a 5-FU derivative (Chart 1). Upon the interaction of the target enzyme with 5-FU anchored on lipid membrane (hence on the liposome surface) lipids should reorganize and segregate in domains according to their charge features. This reorganization should induce a variation of the E/M ratio due to the consequent variation of the distance among the pyrene moieties located in the hydrophobic region of the bilayer. In fact, it is well known that E formation is due to the collision of a pyrene molecule in the ground-state with an excited one or to the direct excitation of a preformed dimer [9]. Thus it is clear that spatial proximity between two pyrene moiety in the bilayer can significantly change upon lipid rearrangement and thus, as a consequence, the binding of the target enzyme to its substrate anchored to the bilayer can strongly affect E/M ratio. It must keep in mind that E formation is a complex phenomenon that depends, besides on the distance between the two fluorophore, on their relative orientation and on their local mobility, on M and E decay processes (such as internal quenching) and E formation and dissociation rate constant [10–12]. It is clear that all these factors can considerably vary in response to lipid reorganization, thus contributing to the sensitivity of the system. Obviously, liposome surface charge plays a pivotal role in the interaction with the enzyme, thus both cationic and anionic fluorescent liposomes were investigated. Liposomes were formulated with a natural phospholipid, 1,2-dioleoyl-*sn*-glycerophosphocholine (DOPC), **1**, **2** and, in the case of anionic liposomes, also lauric acid (12-A) or 11-bromoundecanoic acid (11-BrA), all reported in Chart 1. DOPC was chosen as major component because, being an unsaturated phospholipid, makes the lipid bilayer fluid thus facilitating the eventual lipid rearrangement upon the binding of the protein. The addition of 12-A allowed evaluating the influence of surface potential on the transducing properties of the sensor. It is clear that, being the external surface of all proteins characterized by the presence of charged aminoacids that generate an electrostatic potential, the anionic surface potential of the aggregates generated by the presence of an anionic component influences the interaction of the enzyme with liposome membrane [13,14] causing a different electrostatic perturbation (thus a different lipid rearrangement or segregation in domains) with respect to cationic ones that can be reflected in the induced fluorescent signal. The replacement of 12-A with 11-BrA allowed studying the effect of a bulky fluorescence quencher in the lipid bilayer on the E/M ratio before and after the binding of TP to 5-FU decorating the surface of fluorescent liposomes. All experiments were run also in the presence of bovine serum albumin (BSA) to evaluate the specificity of the sensor in the presence of a common plasma protein different from the target enzyme. The most interesting formulations were also investigated by Langmuir compression isotherms to determine how and to what extent the organization and the capability of lipid bilayer to interact with the target enzyme are influenced by the molecular structure and by the amount of lipid components.

Our fluorescence experiments show that the presence of the 5-FU derivative, **2**, does not affect the interaction of TP with cationic liposomes (*i.e.* does not confers to them specificity toward the target enyme). On the other hand, no interaction, or at least any variation of the E/M signal, was observed upon the interaction of TP with the anionic formulations. On the contrary, in the presence of liposomes in which a bromine atom (a quencher) is present in the hydrophobic part of the bilayer a significant variation of the fluorescent signal was observed in the presence of TP whereas neglectable variation were induced upon the addition of BSA, the negative control in our investigation. It seems by Langmuir trough experiments that the specific formulation containing 11-BrA features a high fluidity, properties that is crucial in this liposome based sensor in which the optical response in the presence of the target enzyme relies on lipid rearrangement. It is evident that the response of the investigated fluorescent lipidic sensor is strictly related to the complex interplay between lipid organization, bilayer fluidity and surface charge and on its variation in the presence and in the absence of the target enzyme.

Table 1
Composition of the investigated liposomes formulations (in molar ratio).

DOPC/**1**/**2**	DOPC/12-A/**1**/**2**	DOPC/11-BrA/**1**/**2**
9:1:0	6:3:1:0	6:3:1:0
8:1:1	5:3:1:1	5:3:1:1
		5:1:4:0
		3:3:4:0

2. Experimental section

2.1. Instrumentation

Steady-state and time-resolved fluorescence experiments were carried out on a Fluoromax-4 Horiba-Jobin Yvon spectrofluorimeter.

UV measurements were carried out on a Cary 300 UV–vis double beam spectrophotometer (Varian Australia PTY Ltd., Mulgrave, Vic., Australia).

Liposomes were prepared using a Branson Digital sonifier 250 with microtip probe (3 mm) and with an acoustic sound enclosure.

DLS measurements were performed with a Malvern Nano-ZetaSizer, equipped with a 5 mW HeNe laser (wavelength = 632.8 nm) and a digital logarithmic correlator.

Isotherm compression measurements were performed using a Langmuir Minitrough (KSV Instruments Ltd, Helsinki) in Teflon with 325 mm of length and 75 mm width and total area of 24,380 mm enclosed in a plexiglass box to reduce surface contamination.

2.2. Materials

DOPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). TP recombinant from *Escherichia coli*, BSA, phosphate-buffered saline (PBS; Aldrich; 0.01 M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl; pH 7.4), 12-A, 11-BrA and all reagents employed for the synthesis of **1** and **2** were purchased from Sigma-Aldrich. **1** and **2** were prepared as previously described [15,16].

2.3. Methods

2.3.1. Liposomes preparation and characterization

A lipid film was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solutions containing the DOPC, **1**, **2** in the absence or in the presence of 12-A or 11-BrA at different molar ratio (Table 1). Films were stored overnight under reduced pressure ($3.9\,10^{-4}$ atm) and PBS was added to the lipid film to obtain a 1 mM lipid dispersion. Solutions were heated and vortex-mixed. The suspension was sonicated for 15 min at 40W while cooled in an ice bath.

2.3.2. Determination of the size of the aggregates

1 mL of 1 mM sonicated liposomes was analyzed using DLS measurements. The normalized intensity autocorrelation functions