

Ethidium Bromide and SYBR Green I Interact with Lipid Micelles

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Abstract

Ethidium Bromide and SYBR Green I are usual examples of ligands that specifically binds to DNA by intercalation. Here we describe that micelles of gangliosides, a lipidic family of sialoglycosphingolipids which are the most important lipids in central nervous system, are able to interact with both, EtBr and SYBR Green I and fluoresce after UV light excitation. The interaction is not affected by the presence of high saline concentration (1M NaCl), a condition which dissociate potential electrostatic interaction between amino and carboxylic acid groups present in EtBr and GM1 respectively; instead, increase the fluorescence of EtBr/GM1 micelles. This result suggests that the EtBr is located inside the hydrophobic core of the micelle. Moreover, the SYBR Green I associated ganglioside micelles does not emit green light as with DNA, but there was a change in the light spectrum towards higher wave lengths, now emitting orange light. It was also found that the interaction with these dyes is not affected when the micelles were preloaded with other molecules (paclitaxel albumin). Moreover, further studies showed that this interaction is not observed with other ionic and nonionic classic detergent micelles, and even the addition of these molecules to GM1 micelles interferes with the described EtBr / GM1 interaction.

Keywords: Ethidium bromide; Fluorescence; GM1 micelles; SYBR Green I

Abbreviations: CMC: Critical Micelle Concentration; CPC: Cetyl pyridinium chloride; D: Deoxicholate; GD1a/GD1b: Disialogangliosides; DNA: Deoxyribonucleic acid; Doxo: Doxorubicin; EDTA: Ethylene diamine tetra acetic acid; EtBr: Ethidium Bromide; GM1: Monosialogangliosides; NP40: Nonidet P40; RNA: Ribonucleic acid; SDS: Sodium dodecylsulphate; SYBR Green I: N,N'-dimetil-N-N-propilpropano-1,3-diamina; TOP2A: Topoisomerase II; TX100: Triton X100.

Introduction

EtBr is an aromatic compound with a heterocyclic moiety that emits orange-red light at 605 nm when exposed to 285 nm UV light, especially when bound to DNA (30-fold increase). This property determines that one of the more traditional applications of EtBr is to detect DNA from agarose gels. Another use that has been given to EtBr is as a general indicator of associations of DNA-dependent and DNA-independent protein. These associations DNA-dependent protein were selectively inhibited by EtBr in the precipitation reaction with no apparent effect on DNA- independent protein association [1]. Photophysical properties of this fluorescent dye after interaction with DNA were extensively studied by Cosa et al. [2] and the work of Karapetian et al. [3] revealed at least three types of complexes of EtBr with ds-DNA.

Due to the largely described strong mutagenic activity of EtBr [4], new molecules with the same photophysical properties, but less dangerous had been developed. This dyes molecules were the bisbenzimidazole or indole-derived stain known as Hoechst 33342, Hoechst 33258 and 49,6-diamidino-2-phenylindole, phenanthridinium stains (propidium iodide) and cyanine dyes like Pico Green, YOYO-1 iodide, SYBR Gold and SYBR Green I. Between them, SYBR Green I, an asymmetrical cyanine dye that binds to DNA and the resulting DNA-dye-complex absorbs blue light (λ_{\max} =497 nm) and emits green light (λ_{\max} =520 nm) [5] is a high-sensitivity reagents that has demonstrated to be much less mutagenic than EtBr [6]. This stain binds preferentially to double-stranded DNA, but will stain single-stranded DNA as well as RNA although with lower performance.

In previous works, we described the spontaneous incorporation of oncological drugs in nanomicellares structures of gangliosides [7-9]. Gangliosides are lipids composed by a common molecule of ceramide with a hydrophilic head composed by two to four sugars and one to three sialic acids. This specific structure gives Ganglioside specific high affinity association with a very low CMC of around to 10^{-8} to 10^{-10} M [10]. We found that lipid micelles of GM1 were able to load hydrophobic and hydrophilic oncological drugs through a hydrophobic interaction. Between the oncological drugs studied the anthracycline Doxo shares with the above mentioned dyes molecules the ability to interact with DNA. Doxo, a metabolite of *Streptomyces peucetius* var. *Caesius* [11], is a chemotherapeutic agent developed in the 1970s [12] that is used in the treatment of a wide range of cancers. Multiple mechanisms have been proposed to explain the cytostatic and cytotoxic actions of anthracyclines [13]. The main mechanism of the Doxo antineoplastic activity is thought to be associated with the intercalation of the planar anthracycline chromophore group between two base pairs of the DNA, leading to inhibition of the DNA synthesis or poisoning of TOP2A. Other suggested mechanisms for Doxo cytotoxicity include free radical formation, lipid peroxidation, and direct membrane effects. In addition to being a commonly used antineoplastic agent, Doxo is a popular research tool due to its inherent fluorescence associated with the central anthracycline chromophore group, which has been proposed for

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diagnostic uses [14]. Thus, the question whether the micellar structures could load any of these dyes emerged, which could provide important methodological advantages for the study and characterization of micellar systems. To this end, we studied whether EtBr and SYBR Green I could interact with lipid micelles and fluorescence. According to the above, to study the interaction between intercalating agents with micelles, we test the behavior of EtBr and SYBR Green I with different micelle structures by electrophoresis in agarose gels.

Materials and Methods

Materials

Monosialogangliosides purified from pig brain was gently provided for TRB Pharma S.A. (Buenos Aires, Argentina). Disialoganglioside GD1a and GD1b were purified in the lab by Dr Valeria Heredia (Córdoba, Argentina). Sodium dodecylsulphate, Triton X100, Nonidet P40, Cetyl pirydinium chloride and Deoxicholate were from Sigma Chemical Co. (Buenos Aires, Argentina). DNA standard 100 to 1500 bp were purchased from Promega (Buenos Aires, Argentina). SYBR Green I nucleic acid gel stain was obtained from Molecular Probes (Eugene, OR) 10.000X.

Methods

Ganglioside micelle preparation: Micelles of purified monosialogangliosides were prepared by dissolving the gangliosides in bidistilled water at a final concentration of 20 mg.mL⁻¹. The solutions were maintained at 4-8 °C for 24 h and then they were centrifuged at 50,000 × g for 15 min and the supernatant was filtered through 0.22 μm. GM1 Micelles are stable in solution for at least 4 months.

Agarose gel electrophoresis: Agarose gel at 1.5% was prepared with 40 mM Tris Acetic acid and 1 mM EDTA (TEA) buffer pH 8. Samples were loaded and running at 3.8 V during 60 min in OWL electrophoresis chamber (Thermo Scientific). The SYBR staining was conducted at the point of seeding while EtBr was made by immersion of the gel after de run in 0.5 μg/mL BrEt for 30 min. Then samples were irradiating with UV light with a filter tray at 312 nm in an Image Master VDS, Pharmacia Biotech. The color photograph was taken with a Lumix Panasonic camera model DMC-ZS7.

Results

Interaction of EtBr with GM1 micelles

We evaluated the binding of EtBr to GM1 micelles in two different conditions:

- By incubation of EtBr with increasing concentration of GM1 micelles and then subjected to agarose gel electrophoresis
- By incubation of pre running GM1 micelles with EtBr, as in the DNA detection method

In both cases, EtBr fluorescence was observed with intensity directly proportional to the concentration of GM1 (Figure 1). Moreover, higher intensity of fluorescence was observed when the EtBr-GM1 interaction took place prior to electrophoresis run than when the interaction occurred after running the GM1. The detection limit reaching by this method was of 0.8 μg of ganglioside.

Interaction of SYBR Green I with GM1 micelles

The interaction of GM1 with other dye, SYBR Green I, was also evaluated. The results clearly showed that SYBR Green I not only interacts with GM1 micelles but also produce a change in the

fluorescence spectra. In order to obtain a better understanding about this interaction, we performed a comparative study of fluorescence emission from the interaction of EtBr and SYBR Green I with DNA and with GM1 micelles. As expected, the orange fluorescence emitted by the interaction of EtBr with DNA or GM1 micelles was observed at 605 nm. However, while the interaction of SYBR Green I with DNA produces the classical green fluorescence emission at 520 nm, after interaction with GM1 micelles an orange emission at 605 nm was observed (Figure 2). This difference between the behaviors of SYBR Green I dye with DNA vs. GM1 micelles could be explained by a change in the environment where SYBR Green I is located in GM1 micelles, since the inside of GM1 micelles highly hydrophobic [7] and more hydrophobic than in DNA, and this condition produce a shift in the emission spectra.

We also analyzed whether DNA and GM1 micelles samples pre stained with SYBR Green I, were capable of interacting with EtBr and emit fluorescence upon excitation with UV light. The result shows that GM1 micelles pre stained with SYBR Green I emit high intensity after incubation with EtBr, but DNA pre-stained not (data not shown). Although these results for GM1 micelles do not allow us to distinguish whether it is a replacement of SYBR Green I by the EtBr or if there is a simultaneous interaction of both dyes, the huge increase of the intensity after the addition of EtBr suggest that both dyes, SYBR Green I and EtBr, are involved in the interaction.

Effect of ionic strength on the interaction of EtBr with DNA/GM1 micelles

Considering that EtBr and GM1 have ionic groups in its structure, two primary and a tertiary amine groups in EtBr and one sialic acid as anionic group in the GM1, this open the possibility that the electrostatic interaction was the driven force for the complex formation. Here we evaluated the effect of ionic strength on EtBr-GM1 micelle interaction and compared it with the EtBr- ds-DNA interaction. GM1 and DNA were run in agarose gel and next were incubated in a solution of BrEt (10 μg/mL) with or without 1 M NaCl. The results of Figure 3 shows that upon the binding to EtBr, the presence of 1 M NaCl produce a clear reduction in the fluorescence of DNA but a highly enhancement of fluorescence of GM1 micelles. These results strongly suggest that the interaction of EtBr with GM1 micelles is not of electrostatic nature but rather hydrophobic.

Interaction of EtBr with other micellar structures

Considering the results obtained with the micelles of GM1, was assessed whether the interaction was specific of these micelles or also

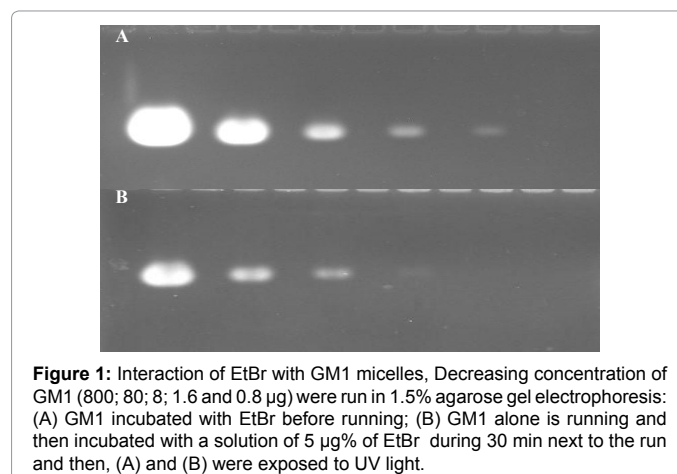


Figure 1: Interaction of EtBr with GM1 micelles, Decreasing concentration of GM1 (800; 80; 8; 1.6 and 0.8 μg) were run in 1.5% agarose gel electrophoresis: (A) GM1 incubated with EtBr before running; (B) GM1 alone is running and then incubated with a solution of 5 μg% of EtBr during 30 min next to the run and then, (A) and (B) were exposed to UV light.

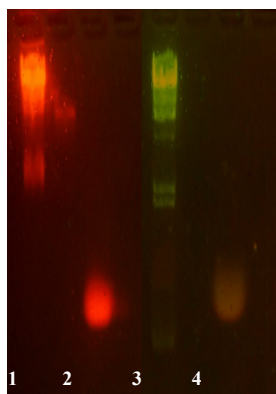


Figure 2: DNA and GM1 micelles stained with EtBr and SYBR Green I, Interaction between EtBr and SYBR Green I with GM1 micelles and DNA. Lines 1 and 3. DNA (5 μ g) stained with EtBr and SYBR Green I respectively; Line 2 and 4. GM1 micelles (50 μ g) stained with EtBr and with SYBR green I respectively.

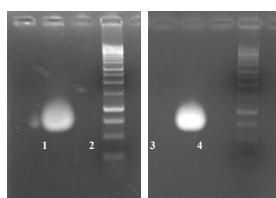


Figure 3: Effect of ionic strength on the interaction of DNA and GM1 micelles with EtBr, DNA (20 μ g) and GM1 micelles were run in 1.5% agarose gel. Lines 1 and 2 DNA and GM1 stained with EtBr (0.5 μ g %) without any treatment; lines 3 and 4. DNA and GM1 micelles stained with EtBr (0.5 μ g %) in 1M NaCl.

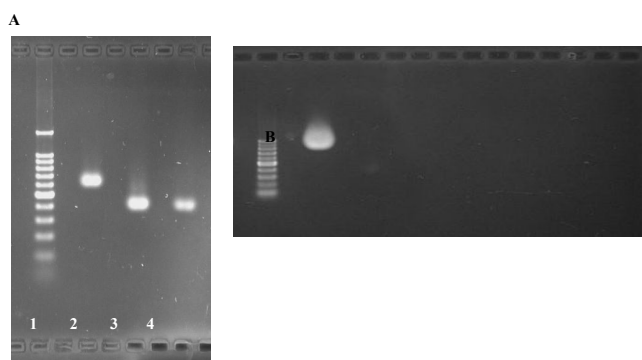


Figure 4: Interaction of EtBr with other micellar structures, Running of micelles with different compositions in 1.5% agarose gel and then stained with EtBr (A) 50 μ g of DNA Control (Line 1); Mono (GM1) (Lines 2), Di (mix of GD1a and GD1b) (Line 3) and Tri-sialogangliosides (GT) (Line 4). (B) 50 μ g of: DNA, GM1, SDS, Deoxicholate, Triton X100, Tween 20 and CPC, run in 1,5% agarose gel and then stained with EtBr.

with other gangliosides micelles with more polar head groups such as disialoganglioside GD1a and trisialoganglioside GT1, and even with other no lipidic micelles of different characteristics of surface charge, size or CMC. Within this group included anionic micelles as sodium dodecyl sulfate (SDS) and deoxicholate (CHO), nonionic micelles as Triton X100 (T-X100) and Nonidet P40 (N-P40) and cationic micelles as cetyl chloride pyridinium chloride (CPC). In the cases of gangliosides micelles with different content of sialic acid in their polar head, Figure 4A show that all of them, mono, di and tri sialoganglioside micelles

interact and fluoresce with EtBr. However, Figure 4B shows that none of the micelles from different detergent were able to interact with EtBr. Moreover, the addition of one of these ionic amphipathic molecules, such as SDS, to GM1 micelle in 1:1 GM1/SDS molar ratio inhibits the interaction with EtBr and the fluorescence disappears completely (data not shown).

Effect of the load of GM1 micelles on the interaction with EtBr

Previous reports of our laboratory showed that GM1 micelles are capable of spontaneously load hydrophobic and hydrophilic drugs such as paclitaxel (Ptx) and doxorubicin (Dox) to form GM1-Ptx and GM1-Doxo complex. Besides, as mentioned above, considering that the interaction of GM1 with EtBr appears to be of hydrophobic nature, the interaction of EtBr with GM1 micelle pre-loaded with Ptx was analyzed. The results show that both, GM1 alone as well as GM1-Ptx complex micelles interact with EtBr (Figure 5).

Discussion

EtBr has been used for many years for the visualization of nucleic acids in agarose gels and also to detect protein-DNA complexes in band shift assays. The irradiation with UV light (285 nm), next to the interaction of the dye with DNA, induce the emission of orange light at 605 nm. It was proposed that this property is due to the ability of EtBr to intercalate between adenine dA and thymine dT bases of double strand DNA. More recently, other dyes molecules with similar physicochemical properties but less toxic have been used in detection of nucleic acids. One of them, SYBR Green I, a component of the cyanines family has become increasingly important in a variety of analytical and diagnostic applications. Here we show that EtBr, a water soluble molecule with two amino groups, interact with anionic self assembled lipids micelles of GM1. However this interaction does not appear to be mediated by electrostatic interactions between amino groups of EtBr and carboxylic acid from lipids, but rather by hydrophobic interaction, because the presence of 1M NaCl cannot dissociate GM1-EtBr complex.

Moreover, the saline concentration induces a clear increase in the intensity of emission of fluorescence. This could be explained by the fact that high ionic strength induce a tight interaction between ceramide tails of GM1 that increase the hydrophobicity of the domain and enhance the intensity of fluorescence. These results are in agreement with that described for EtBr / DNA interaction, arising

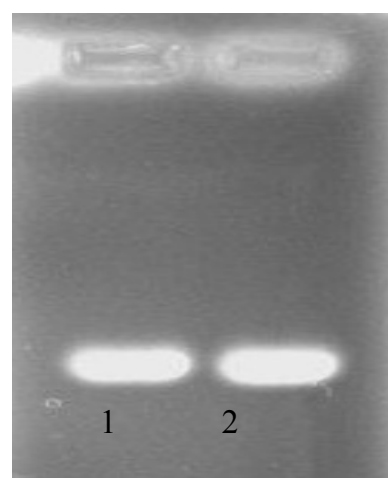


Figure 5: Interaction of EtBr with GM1 and GM1-Ptx micelles. GM1 (50 μ g) micelles alone (Line 1) and GM1-Ptx complex micelles (ratio GM1/Ptx 10/1 mol/mol) (Line 2) interact with EtBr (0.5 mg %).

between base pairs of hydrophobic molecules with water removal and dehydrogenation of EtBr that produces an increase in fluorescence of the ethidium. Additionally, we demonstrate that EtBr also interact and fluoresce with other ganglioside micelles with more electronegative superficial charge, with two or three sialic acid like GD1a and b and GT1.

However EtBr did not shows any interaction with other non lipidic micelles, like those from anionic, non-ionic or cationic detergent. When GM1 micelles are incubated together with these other micelles, a complete loss of fluorescence occurs, demonstrating that the interaction between GM1 molecules is necessary to ensure the proper environment where EtBr fluoresce. These results differ from previous studies which describe that EtBr interacts with micelles, for example SDS [15,16]. This discrepancy may be due to the different methodologies used. Many of these reports describe the phenomenon through spectroscopic analysis, while here we use a dynamic assessment process as electrophoresis. SYBR Green I, other DNA intercalating agent, was also able to interact with GM1 and fluoresce in a similar way that EtBr. However, its interesting to note that fluorescence of SYBR Green I in GM1 micelles do not occurs as was expected with emission at green light ($\lambda_{max}=520$ nm) like DNA, but a yellow-orange light.

This result could be explained by a change in the hydrophobicity of environment of SYBR Green I after interaction with GM1 than that observed for SYBR Green I with DNA. Finally exploiting the property of GM1 to interact with EtBr and the fluorescence emission resulting from this interaction, this could be used like a new method for the detection and characterization of the relative size of GM1 micelles by agarose gel with fluorescence detection after interaction EtBr, using standard dsADN as a reference.

Conclusion

This work shows that dyes commonly used for the detection of DNA, can also be used to reveal and identify different types of ganglioside micelles in electrophoresis runs. Moreover, the use of these dyes can detect concentrations of 0.8 μ g, while resorcinol technique detect from 20 μ g of GM1.

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