

1 *Proceedings*

2 **Use of lipid vesicles for revealing the potential contribution of** 3 **cytochrome c in the metabolism of environmental toxicants** †

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13 **Abstract:** Cytochrome *c* (*Cc*) is well-known as an electron carrier at mitochondria, but can also cat-
14alyze peroxidase-like reactions. Although the reported catalytic abilities are low, the role of *Cc* in
15the transformation of environmental toxicants requires additional studies. We employed cardiolipin
16vesicles to stimulate the enzymatic activity of *Cc* and tested the catalytic system towards the dye
17methyl orange and the polycyclic aromatic hydrocarbons (PAHs) benzo[b]fluoranthene and
18benzo[a]pyrene. The results showed that cardiolipin-containing vesicles promoted dye decoloriza-
19tion and the oxidation of both PAHs by *Cc*, indicating that lipid membranes can be useful to further
20investigate *Cc*-mediated metabolism of toxicants.

21 **Keywords:** Azo dye; pseudo-peroxidase; phospholipid; environmental toxicology; biocatalytic sys-
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23 **1. Introduction**

24 A recognized electron carrier at the mitochondrial respiratory chain, cytochrome *c*
25 (*Cc*) is a heme protein that can also catalyze peroxidase-like reactions [1–3].

26 *Cc* is able to catalyze different oxidation and hydroxylation reactions of aromatic hy-
27drocarbons and heterocyclic compounds, but with low catalytic efficiency [4]. For exam-
28ple the oxidation of polycyclic aromatic hydrocarbons (PAHs) was previously studied in
29systems containing 1 mM H₂O₂, conditions that cause fast auto-oxidation and inactivation
30of the enzyme [2,4].

31 On the other hand, the peroxidase activity of *Cc* can be enhanced by the protein
32chemical modification or interaction with amphiphilic structures [4,5]. Widely reported,
33vesicles containing anionic phospholipids such as cardiolipin (CL) increase the enzymatic
34activity of *Cc* in several folds [1,3]. The interaction of *Cc* with these vesicles induces con-
35formational changes that lead to a gain in peroxidase activity by enabling an easier access
36of H₂O₂ to the protein heme [2,3]. On this basis, we tested the hypothesis that CL vesicles
37(Figure 1) could be used to improve the study of *Cc* ability to metabolize environmental
38toxicants.

39 To test this hypothesis, in this work, we used methyl orange, an azo dye model, and
40the PAHs benzo[b]fluoranthene and benzo[a]pyrene. Most azo dyes are toxic and refrac-
41tory to conventional treatment processes [6]. PAHs are ubiquitous environmental pollu-
42tants with important toxic properties [7], and the metabolic transformation of benzo[a]py-
43rene is closely implicated in carcinogenicity [8].

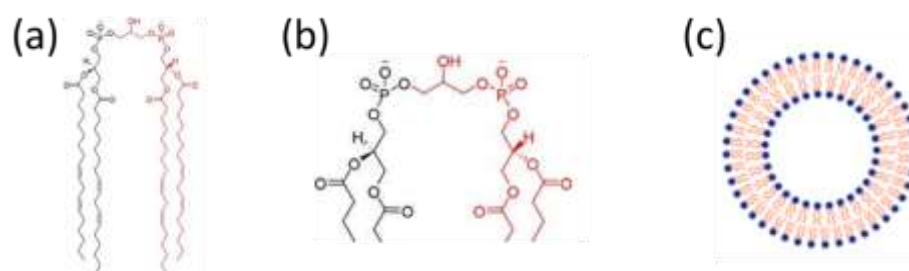


Figure 1. Representation of (a) the molecular structure of cardiolipin and (b) detail of the polar part, and (c) a unilamellar vesicle.

2. Materials and Methods

2.1. Materials and preparation of lipid vesicles

Benzo[b]fluoranthene was obtained from Tokyo Chemical Industry (cat. no. B2982) and benzo[a]pyrene from Sigma-Aldrich (B1760). *Cc* was from Sigma-Aldrich (equine heart, cat. no. C2506), phosphatidylcholine (PC) from Tokyo Chemical Industry (D4250) and CL from Avanti (710335P).

Small unilamellar vesicles composed of PC or mixtures of PC with CL at a 4:1 molar ratio were prepared as described in [1]. Chloroform stock solutions of the lipids were dried and resuspended in 20 mM phosphate buffer, pH 7.0. The small unilamellar vesicles were obtained by sonication of aliquots of the lipid suspension using a titanium-microtip-equipped Hielscher UP100H sonicator.

The lipid vesicles were added in a final concentration of 200 μM to the reaction media of the biotransformation assays described below.

2.2. Assays of methyl orange decolorization

The effect of the lipid vesicles in *Cc* transformation of methyl orange was assayed with 10 mg/L of the dye in 100 mM phosphate buffer, pH 7.0. *Cc* was added at a final concentration of 0.01 mg/mL and H_2O_2 at 100 μM . The transformation of the azo dye was monitored by UV-Vis spectrophotometry at 477 nm.

2.3. Assays of polycyclic aromatic hydrocarbons transformation

The effect of the lipid vesicles in *Cc* transformation of PAHs was studied with benzo[b]fluoranthene and benzo[a]pyrene in 24 h incubations. The initial concentration of the PAHs was 1 mg/L, *Cc* 0.01 mg/mL and H_2O_2 100 μM , in phosphate buffer pH 7.0.

At the end of the incubations, the remaining PAHs and the formed reaction products were extracted using hexane and analyzed by reverse-phase HPLC. The chromatographic analysis was carried out in an Agilent 1100 system equipped with a C18 column, using a mobile phase of acetonitrile and water (85:15) at a flux of 1 mL/min, and detection at 266 nm.

3. Results and Discussion

As shown in Figure 2, *Cc* in the presence of H_2O_2 , slowly transformed methyl orange. The presence of the PC vesicles in the reaction media did not significantly affect the transformation kinetics, but the PC/CL vesicles clearly increased the rate of methyl orange decolorization by *Cc*.

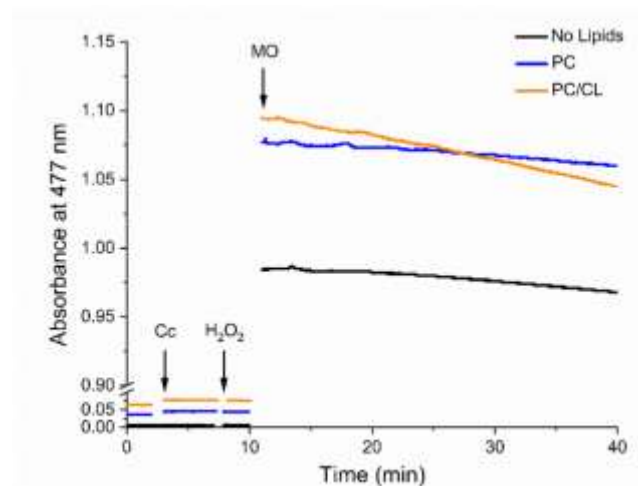


Figure 2. Representative assays of methyl orange decolorization catalyzed by cytochrome *c* in the absence of lipids and in the presence of phospholipid vesicles. Small unilamellar vesicles of phosphatidylcholine (PC) and of a mixture with cardiolipin (PC/CL) were tested. The concentration of cytochrome *c* (Cc) was 0.01 mg/mL, hydrogen peroxide 100 μ M, and methyl orange (MO) 10 mg/L. Other experimental conditions described in Methods.

In the case of the PAHs, the Cc-mediated oxidation was almost imperceptible (<15%), in spite of the high compounds' concentrations used in the assays (1 mg/L). In accordance with the results from the methyl orange assays, PC vesicles showed no effect in the Cc-catalyzed transformation of both PAHs.

Nevertheless, by including CL vesicles in the assay media, the transformation became evident from the decrease observed in the benzo[b]fluoranthene and benzo[a]pyrene chromatographic peaks and by the emergence of reaction products peaks in the chromatograms. Results from benzo[b]fluoranthene assays are shown in Figure 3.

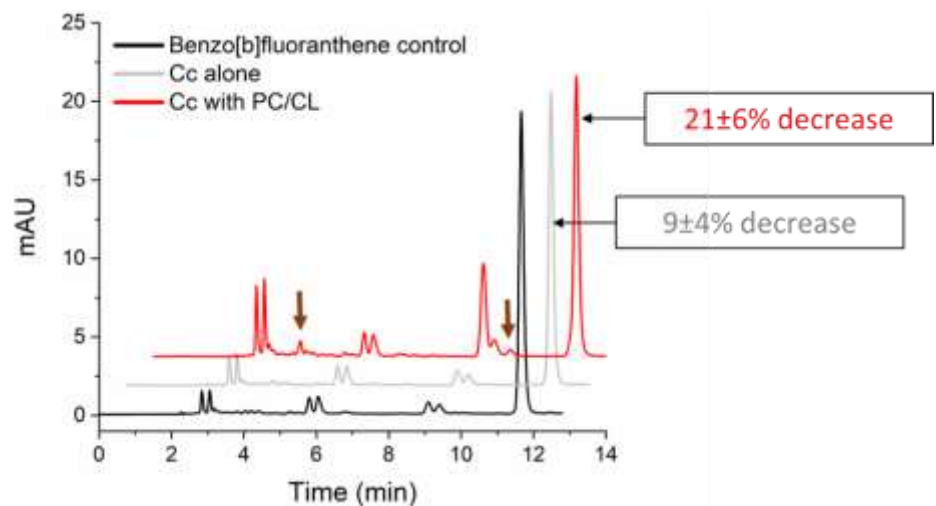


Figure 3. Representative chromatograms from assays of benzo[b]fluoranthene transformation catalyzed by cytochrome *c* (Cc) in the absence of lipids and in the presence of vesicles containing phosphatidylcholine and cardiolipin (PC/CL). A chromatogram from a control assay without cytochrome *c* is shown in the foreground. The decreases in the area of the benzo[b]fluoranthene peaks relative to the areas measured in the control assays are indicated in %. Chromatographic peaks detected only in assays with PC/CL vesicles are signaled with arrows. Experimental conditions of the assays described in Methods.

Concerning benzo[a]pyrene oxidation, it reached 71±2% in the presence of the CL-containing vesicles. Control assays were carried out with benzo[a]pyrene plus H₂O₂ and CL vesicles, in the absence of Cc, but the PAH concentration in the samples after incubation showed no significant difference relative to similar controls without CL. Overall, the results from control assays indicated that any Cc-independent oxidation of benzo[a]pyrene by H₂O₂ is negligible, even in the presence of CL.

4. Conclusion

Vesicles formed of a mixture of PC and CL consistently enhanced the Cc-mediated transformation of two different types of compounds with relevant toxicity, an azo dye and PAHs.

The results herein give support to the employment of lipid membranes as tools facilitating the future application of Cc in the generation of specific toxicants' metabolites. It also encourages studies of the potential participation of lipid vesicles to promote Cc activity in distinct contexts.

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