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Immobilization of chlorophyll by using layer-by-layer technique for controlled release systems and photodynamic inactivation

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ABSTRACT

The development of systems for the controlled release of drugs is important because they allow the control of drug absorption and tissue distribution and also can reduce local toxicity. This study aimed to assemble and characterize two types of release systems, consisting of layer-by-layer films obtained from poly(allylamine) hydrochloride with chlorophyll (PAH/CHL films) or chlorophyll incorporated into dipalmitoylphosphatidylcholine liposomes (PAH/Lip + CHL films). For these systems, the molecular aggregation, growth process, thermally stimulated desorption, wettability, and controlling release of CHL was studied by using UV–vis spectroscopy and wetting contact angle analysis. In addition, experiments of photodynamic inactivation using PAH/CHL or PAH/Lip + CHL films with a 633-nm laser light were performed and the susceptibility of *Candida albicans* (*C. albicans*) to this approach was examined. Fluorescence and atomic force microscopies were used to investigate the surface morphology after the application of the photoinactivation procedure. A redshift of the UV–vis spectrum associated to films when compared with the spectrum of the CHL solution indicated a molecular aggregation of CHL molecules in the films. The film growth process was determined by a nucleation and a growth of spheroids or rods for either PAH/Lip + CHL or PAH/CHL films, respectively. Thermally activated desorption experiments indicated that interactions between CHL and PAH (126 kJ/mol) in PAH/CHL or between Lip + CHL and PAH (140 kJ/mol) in PAH/Lip + CHL films may be governed by electrostatic interactions. The wettability of PAH/Lip + CHL films was larger than that for PAH/CHL films, which can be attributed to hydrophilic groups on the surface of the DPPC liposomes. Release experiments revealed that free CHL in PAH/CHL films was released more slowly than its partner incorporated into liposomes. After the photodynamic inactivation, results of survival fraction and fluorescence microscopy revealed that *C. albicans* presented similar susceptibility for the two kinds of films. AFM supported the fluorescence one suggesting that cell death of *C. albicans* may occur due to damages to its cell wall by *C. albicans*.

1. Introduction

Systems for controlled release of drugs or drug delivery systems (DDSs) have been developed because they are an alternative to conventional pharmacodynamic treatments [1]. DDSs have gained importance due to the control of absorption, tissue distribution, and the reduction of local toxicity systemic. Conventional treatments used to combat infectious processes, for example, in the form of pills or injectable administration, require a long period of administration to maintain therapeutic drug levels in the body, which often is not achieved. When compared with conventional methods, DDSs have the ability to potentiate the therapeutic effect by keeping the concentration of the drug constant for an extended period using a single dose. This does not happen with conventional systems, since most exhibit a drug concentration range that defines levels of security and efficiency, as well as concentrations above or below that level. This range can lead to inefficiencies in the treatment and/or serious toxic effects. In DDSs, the drug should be linked to biocompatible materials, chemically inert, free from impurities, and capable of performing delivery of a predetermined shape without suffering the effects of interaction with the environment. Low-density lipoproteins (LDL), biodegradable nanoparticles, and liposomes are among the most studied delivery systems to date [2].

Liposomes are a simple model for the study of biological membranes. They are not only biodegradable, but also biocompatible, and a suitable system to release drug in a controlled way [3]. The

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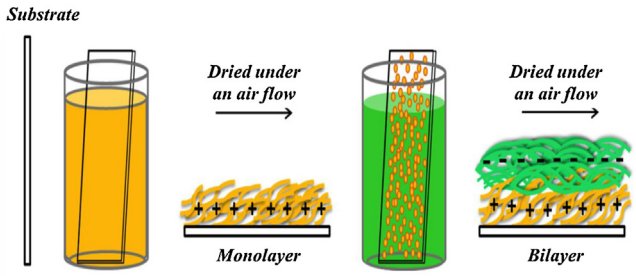


Scheme 1. Schematic representation of obtaining films using the LBL technique.

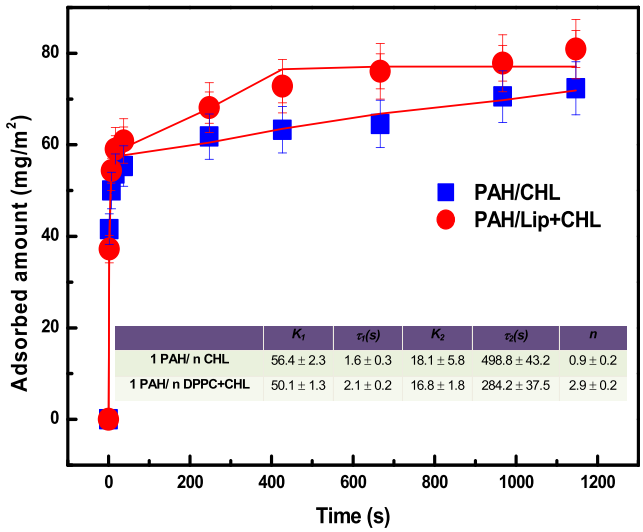
skin permeation, for example, is considerably increased when a drug is co-administered or incorporated into liposomes [4] due to compatibility of the human skin with lipid membrane [5]. The release of the content from liposomes can be performed in several ways, including photochemical activation (photoisomerization, photocleavage, and photopolymerization) as well as by photophysical activation, such as photothermal conversion [8]. In addition, the release rate of the drug incorporated into liposomes depends on the drug itself and the properties of the liposome such as composition, temperature, sensitivity to changes in pH, and osmotic gradient. Microwave radiation [6,7] and pulses of laser light can also lead to release of drugs from liposomes [8,9].

The possibility of the release of drugs incorporated into liposomes using a laser light can be exploited in combination with photodynamic therapy. Photodynamic therapy (PDT) is a medical procedure that intends to eliminate a disease through the combination of a photosensitizer (PS) excited by photons with a suitable wavelength. This approach can result in cell death through molecular oxygen or other free radicals present in biological tissues [10]. The products generated from the combination of molecular oxygen and PS can damage the membrane or cell wall of an organism, causing death. PDT has been a promising alternative for the inactivation of pathogenic microorganisms such as viruses, bacteria, and fungi [11,12]. Cell death after photosensitization of microorganisms is called photodynamic inactivation (PDI). To be considered a PS and be used in PDT or PDI, a compound must present low toxicity in the absence of radiation, among other features, and must absorb in the visible range of the electromagnetic spectrum. Chlorophyll (CHL) has been used as a photosensitizer for photodynamic therapy [13] by its high absorptivity in the visible light range and its low toxicity.

Candida albicans (*C. albicans*) is an opportunistic fungus present in the microbiota of skin, oral mucosa, gastrointestinal tract, and urogenital [14]. There are a few studies about PDT or PDI involving fungi when compared with bacteria, this is probably due to high resistance attributed to bacteria by their nuclear membranes, which can act as an additional cellular barrier protection, or by dimensional differences between the cells [15]. In this study, we have used LBL technique [16] to build and characterize films from PAH alternated with free CHL (PAH/CHL) or alternated with CHL incorporated into DPPC liposomes (PAH/Lip + CHL), which could be used as a model in studies of controlled drug release systems. In addition, a susceptibility analysis of *C. albicans* to a photodynamic inactivation approach employing the combination of laser light (633 nm) and free CHL or Lip + CHL is presented.

2. Materials and methods

Chlorophyll (CHL) was obtained from MP Biomedicals and poly(allylamine) hydrochloride (PAH) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma-Aldrich Co. and used as supplied. CHL and PAH stock solutions were prepared by dissolving CHL or PAH in purified water with a concentration of



	K_1	τ_1(s)	K_2	τ_2(s)	n
1 PAH/ n CHL	56.4 ± 2.3	1.6 ± 0.3	18.1 ± 5.8	498.8 ± 43.2	0.9 ± 0.2
1 PAH/ n DPPC+CHL	50.1 ± 1.3	2.1 ± 0.2	16.8 ± 1.8	284.2 ± 37.5	2.9 ± 0.2

Fig. 1. Absorbance at 410 nm versus immersion time for the PAH/CHL and PAH/Lip + CHL layers. The solid line represents the fitting with the JMA equation. The Table in the inset shows the parameters n, τ, and K employed in the JMA equation for fitting the data of growth kinetics of LBL films.

0.9 g L^{-1} and 0.5 g L^{-1}, respectively. The pH of the CHL dispersion was adjusted to 8 (close to physiologic pH) by adding appropriate amounts of ammonium hydroxide. CHL molecules were incorporated into DPPC liposomes (Lip) as described by Moraes et al. [17]. Preparation of films, using a layer-by-layer (LBL) technique, was essentially as described by Decher [16]. The films were adsorbed on quartz (36 mm × 14 mm × 1 mm). Since our experience shows that the rinsing step after the deposition of PAH layer leads to the formation of a surface with a low coverage ratio, the washing step was excluded. The films were assembled by alternating the immersion of the substrate into the PAH (3 min) and into the CHL or Lip + CHL solution. After each deposition step, the substrates were dried under an air flow, as shown in Scheme 1. The adsorbed amount, which is proportional to absorbance, was monitored by measuring the electronic absorption spectra using a double-beam UV–vis spectrophotometer (Thermo Scientific, Genesys 10, USA). The analysis of the surface morphology of the films was performed using a fluorescence microscopy (Nikon Eclipse Ci/L) and an atomic force microscope (Nanosurf EasyScan II) in intermittent mode (512 × 512 pixels). Images were acquired with scanning windows of 10 μm × 10 μm. The wetting contact angle analysis was carried out with a homemade instrument [18] in ambient conditions. Deionized water droplets (3.0 μL) were gently placed onto the film surfaces and the average values measured at six different locations of each sample were taken. To prepare *C. albicans* (ATCC 14053) dispersions, one isolated colony was seeded from the original culture onto a Sabouraud agar plate and incubated at 37 °C for 24 h. Subsequently, 9.0 mg of the resulting culture was dispersed in 10 mL of physiological solution to obtain stock dispersion. For viability and inactivation experiments *C. albicans* was used at a concentration of 10^7 CFU/mL. Dispersions were irradiated using a laser light beam at a wavelength of 633 nm provided by a continuous diode laser (MaxBrite Miniature OEC). Output power of the laser beam was 5.0 mW, spot size of ~3.0 mm in diameter, and irradiation time of 6 min resulting in a dose of about 25.5 J/cm^2. This dose was used to avoid hyperthermia [19]. The laser beam output was placed at 100.0 mm from the surface of each film or microtube with dispersion. At least five measures were taken and the errors were the sample standard deviations.