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Photodynamic Properties and Photoantimicrobial Action of Electrochemically Generated Porphyrin Polymeric Films

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Spectroscopic and photodynamic properties of polymeric films bearing porphyrin units have been studied in both solution containing photooxidizable substrates and in vitro on *Escherichia coli* and *Candida albicans* microorganisms. The films were formed by electrochemical polymerization of 5,10,15,20-tetra(4-*N*,*N*-diphenylaminophenyl)porphyrin (H_2P-film) and its complex with Pd(II) (PdP-film) on optically transparent indium tin oxide (ITO) electrodes. Absorption spectroscopic studies show the characteristic *Soret* and *Q* bands of the porphyrin in the visible region and a band at ∼350 nm corresponding to the tetraphenylbenzidine units. Upon excitation, the H_2P-film exhibits two bands of fluorescence emission from porphyrin, while it is not detected using PdP-film. The singlet molecular oxygen, $O_2(^1\Delta_g)$, productions of these surfaces were evaluated using 9,10-dimethylanthracene in *N*,*N*-dimethylformamide. Also, the photodynamic activity was compared in solutions of L-tryptophan. Under these conditions, oxidation of these substrates takes place indicating an efficient photodynamic action of both polymeric films. In vitro investigations show that these films produce photosensitized inactivation of microbial cells in aqueous suspensions. These films exhibit a photosensitizing activity causing a ∼3 log decrease of *E. coli* and ∼2.5 log of *C. albicans* cellular survival after 30 min of irradiation with visible light. The photodynamic effect of the surfaces was also tested by growth delay experiments. The results indicate that porphyrins immobilized on electropolymeric films are interesting and versatile photodynamic surfaces to inactivate microorganisms in liquid suspensions.

Introduction

The growing resistance against antibiotics and other chemotherapeutics has led to a search for novel antimicrobial treatments to which microorganisms will not easily develop resistance. In recent years, photodynamic inactivation (PDI) of microorganisms represents a potential alternative to inactivate microbes (*1*). This methodology is based on the administration in solution of a photosensitizer, which is preferentially accumulated in the microbial cells. Subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms (*2*−*4*). In this process, different oxidative mechanisms can occur after photoactivation of the photosensitizer. In the type I photochemical reaction, the photosensitizer interacts with a biomolecule to produce free radicals, while in the type II mechanism, singlet molecular oxygen, $O_2(^1\Delta_g)$, is produced as the main species responsible for cell inactivation (*5, 6*).

In most cases, Gram-positive and Gram-negative bacteria are susceptible to the photosensitizing action of a variety of sensitizers under certain conditions (*7*−*10*). In general, Gram-negative bacteria are damaged with more difficultly due to the presence of a highly organized outer membrane, which intercepts the photogenerated reactive species (*11*−*14*). Also, the cell wall provides structure to the cell and protects the cell from the environment. Recently, porphyrin and phthalocyanine derivatives have been investigated for PDI applications in the treatment and control of yeast (*15*−*17*). Thus, the interest in this field and the need for new molecules with improved characteristics are always high, owing to the large variety of therapeutic applications.

The majority of PDI experiments were carried out with the photosensitizer in solution. However, this approach can be inappropriate for applications where residual traces of photosensitizer in the medium are not acceptable, such as water disinfection. In this way, photosensitizers immobilized on polymeric supports have been proposed to avoid this problem (*18*−*21*). Films of regenerated cellulose impregnated by adsorption with 5,10,15,20-tetrakis(*N*-methylpyridinium)porphyrin tetratosylate have photomicrobicidal activity toward *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* (*19*). Porphyrinated cellulose laurate esters films containing protoporphyrin IX showed photobactericidal activity against *S. aureus* and *E. coli* bacteria (*20*). Photosensitizers have been incorporated into translucent chitosan membranes by different procedures. In particular, zinc(II) phthalocyanine tetrasulfonic acid covalently attached to chitosan was the most effective to photoinactivate *E. coli* cells in a model for a large-scale water-flow system (*21*). Sensitizing materials containing Ru(II) complexes immobilized on porous silicone were successfully tested for the inactivation of waterborne *E. coli* or *Enterococcus faecalis* bacteria (*22*). Alternatively, semiconductor systems, such as titanium dioxide, in the presence of ultraviolet or solar light represent a related approach to water disinfection (*23, 24*). In this way, porphyrin electropolymerization represents an interesting system to be evaluated because it can be used to form homogenic films on surfaces with intense absorptions in the visible region.

In previous studies, we have investigated the photodynamic activity of cationic porphyrin derivatives with different patterns of substitution in vitro as sensitizers to eradicate Gram-negative bacteria (*13, 25*). Porphyrin bearing three cationic charges and a highly lipophilic trifluoromethyl group were found to be active photosensitizers to inactivate *E. coli* cells in both liquid cellular suspension and cells immobilized on surfaces (*25, 26*).

In the present work, we examined the photodynamic action of surfaces bearing porphyrin units immobilized on electropolymeric films both in solution containing photooxidizable substrates and in cellular suspensions of microorganisms. The films were formed by electrochemical polymerization of 5,10,15,20-tetra(4-*N*,*N*-diphenylaminophenyl)porphyrin (H_2P-film) and its complex with Pd(II) (PdP-film) on optically transparent indium tin oxide (ITO) electrodes (Scheme 1). This metal forms stable complexes

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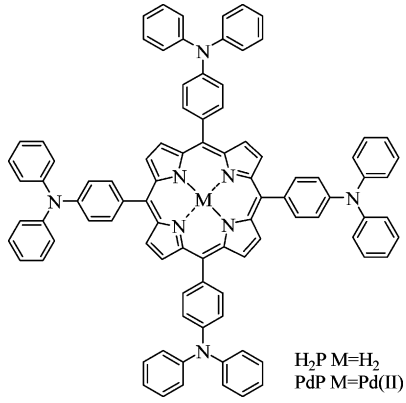
SCHEME 1. Molecular Structures of Porphyrins



with several porphyrins and it can be used to enhance the photodynamic action of the sensitizer (*13*). In vitro studies were performed on a Gram-negative bacterium, *Escherichia coli*, and a yeast, *Candida albicans*. The photoinactivation activity of these films shows interesting applications in the control and disinfection of the aqueous suspension of microorganisms.

Materials and Methods

General. Absorption and fluorescence spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length quartz cells on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. FAB mass spectra were taken with a ZAB-SEQ Micromass equipment. Proton nuclear magnetic resonance spectra were recorded on a FT-NMR Bruker spectrometer at 200 MHz. Irradiation experiments were performed using a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. A wavelength range between 350−800 nm was selected by optical filters. The light fluence rate at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA).

All the chemicals from Aldrich (Milwaukee, WI) were used without further purification. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labconco (Kansas, MO) equipment model 90901-01.

Synthesis of Porphyrins. 5,10,15,20-Tetrakis(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin *p*-tosylate (TMAP^{4+}) was purchased from Aldrich. 5,10,15,20-Tetrakis(4-*N*,*N*-diphenylaminophenyl)porphyrin (H_2P) was synthesized by the condensation of 4-(*N*,*N*-diphenylamino)benzaldehyde and pyrrole, using the Lindsey method (*27*). A solution of 4-(*N*,*N*-diphenylamino)benzaldehyde (1.09 g, 4.01 mmol) and pyrrole (300 μL, 4.32 mmol) in 400 mL of dichloromethane was purged with argon for 15 min. After that, trifluoroacetic acid (300 μL, 4.04 mmol) was added and the solution was stirred for 60 min at room temperature under argon atmosphere. Then, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1.08 g, 3.65 mmol) was added and the mixture was stirred for an additional 18 h, open to the atmosphere. The solvent was removed under reduced pressure and flash column chromatography (silica gel, *n*-heptane/dichloromethane 1:3) afforded 396 mg (31%) of pure H_2P. TLC (silica gel, dichloromethane) R_f 0.48. Spectroscopic data of H_2P coincide with those previously reported (*28*). Palladium(II) 5,10,15,20-tetrakis(4-*N*,*N*-diphenylaminophenyl) porphyrin (PdP) was prepared by dissolving H_2P (20 mg, 0.015 mmol) in 10 mL of *N*,*N*-dimethylformamide (DMF). Then, palladium (II) chloride (75 mg, 0.42 mmol) was added and the mixture was stirred for 5 h at 150 °C in atmosphere of argon. The solution was treated with water (30 mL) and the organic phase was extracted with three portions of chloroform (20 mL each). The solvents were evaporated under reduced pressure. The reaction afforded 20 mg (95%) of pure PdP. TLC (silica gel, dichloromethane) R_f 0.46. Absorption spectrum λ_{max} (dichloromethane) [nm] 306, 436 (ϵ = 120,000 M^{-1}cm^{-1}), 530, 568. ^{1}HNMR ($CDCl_3$, TMS) δ [ppm] 7.15 (d, 8H, J = 7.8 Hz, 5,10,15,20-Ar 3,5-H), 7.34−7.50 (m, 40H, *N*-phenyl), 8.02 (d, 8H, *J*=7.8 Hz, 5,10,15,20-Ar 2,6-H), 8.97 (s, 8H, pyrrole). MS [*m*/*z*] 1386 (M$^+$) (1386.4 calculated for $C_{92}H_{64}N_8Pd$, using Pd 105.9).

Electrochemical Experiments. All electrochemical studies were performed at room temperature using dichloromethane or 1,2-dichloroethane solutions containing 0.1 M tetra-*n*-butylammonium perchlorate (TBAP) as the supporting electrolyte. The solvents were dried over 3 Å molecular sieves for 48 h and used without further purification. TBAP was dried for 24 h under vacuum prior to use. The electrochemical porphyrin polymer formation was carried out using a homemade cell built from a commercial UV−visible quartz cuvette (45 × 10 × 10 mm, ~3.5 mL). An optically transparent indium tin oxide (ITO, Delta Technologies, Stillwater, MN, 7 × 50 × 0.9 mm cuvette slides, SiO_2 passivated and indium tim oxide coated one surface, antireflection coated opposite) electrode was used as working electrode, a platinum wire as the counter electrode, and a freshly prepared Ag/AgCl as quasi-reference electrode. The ITO electrode was carefully cleaned and dried prior to use. Cyclic voltammetry experiments were performed using an AUTOLAB PGSTAT30 potentiostat/galvanostat (general-purpose electrochemical system, GPES, Eco Chemie B.V., Utrecht, The Netherlands). Cyclic voltammogram of H_2P and PdP in dichloromethane and 1,2-dichloroethane, respectively, were performed using 1 mM of porphyrin and 0.1 M of tetrabutylammonium perchlorate (TBAP) on ITO electrodes (scan rate = 20 mV/s; continuous scan number 1−20).

Steady State Photolysis. Solutions in DMF of 9,10-dimethylanthracene (DMA, 35 μM) or L-tryptophan (Trp, 25 μM) were irradiated in 1 cm path length quartz cells (2 mL) containing the porphyrin TMAP^{4+} or the films (*Soret* band, absorbance 0.2) in different media. The samples were irradiated as described above but using a wavelength range between 370 and 800 nm. The kinetics of DMA and Trp photooxidation were studied following the decrease of the absorbance (A) at λ_{max} = 378 nm and the fluorescence intensity (F) at λ = 350 nm, respectively. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A or Ln F_0/F vs time. Photooxidation of DMA was used to determine $O_2(^1\Delta_g)$ production by the photosensitizer (*29*). TMAP^{4+} was used as a comparative reference, Φ_Δ = 0.65 in DMF (*25*). All the experiment were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 10%.

Microorganisms and Growth Conditions. The *E. coli* strain (EC7), recovered from clinical urogenital material, and the *C. albicans* strain (PC31), recovered from human skin lesion, were previously characterized and identified (*13, 17, 25*). The microorganisms were grown aerobically overnight in 4 mL tryptic soy (TS) broth for bacteria and in Sabouraud broth (Britania, Buenos Aires, Argentina) for yeast, at 37 °C. The cultures were treated as previously described (*13, 17*). Briefly, cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and resuspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH 7.0), corresponding to ~10^9 *E. coli* colony forming units (CFU)/mL and ~10^7 *C. albicans* CFU/mL. The cells were appropriately diluted to obtain ~10^6 CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used. Viable microbial cells were monitored and the number of CFU was determined on TS agar plates

(~24 h incubation at 37 °C) for *E. coli* or Sabouraud agar plates (~48 h incubation at 37 °C) for *C. albicans*.

Photosensitized Inactivation of Microbial Cells in PBS Suspension. The ITO electrodes with electropolymeric films were positioned in a Pyrex brand culture tubes (13 × 100 mm) containing the cellular suspensions of microorganisms (2 mL, ~10^6 CFU/mL) in PBS. After that, the cultures were exposed to visible light for different time intervals. Control experiments were carried out without illumination in the absence and in the presence of the electrodes. Also, irradiated controls were performed with cultures without electrodes and with ITO electrodes without the electropolymeric films. Cellular suspensions were serially diluted with PBS, each solution was plated in triplicate, as described above, and the number of colonies formed was counted. Each experiment was repeated separately three times.

Growth Delay of Microbial Cultures. A portion of overnight culture was transferred to 20 mL of fresh TS broth medium for *E. coli* and Sabouraud broth medium for *C. albicans* (*13, 17*). The suspension was homogenized and aliquots of 2 mL were incubated with the electrode at 37 °C. The culture grown was measured by turbidity at 660 nm using a Turner SP-830 spectrophotometer (Dubuque, IA). Then the flasks were irradiated with visible light at 37 °C, as described above. In all cases, control experiments were carried out without illumination in the absence and in the presence of electrodes. Each experiment was repeated separately three times.

All data were presented as the mean ± standard deviation of each group. Variation between groups was evaluated using the Students *t*-test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

Results and Discussion

Electropolymeric Film Formation. Palladium and free-base porphyrins usually show reversible two electron oxidation (*30*). On the other hand, upon oxidation, the triphenylamine (TPA) groups can undergo the well-known radical cation dimerization, to produce tetraphenylbenzidine (TPB) (*31*). In our case, both H_2P and PdP hold two electrochemical active centers: the porphyrin core and the TPA moieties (Scheme 1). As we already showed, polymers obtained from TPA oxidation produce electroactive films with good charge transport capability (*32, 33*). Therefore, the porphyrins here studied have the potential to generate polymer films over the electrode surface. Multiple CV scans of H_2P and PdP molecules on ITO electrodes are shown in Figure 1. In agreement with the outlined electrochemical properties, the current increments indicate the characteristic behavior of the formation of electroactive films. In Figure 1A the first wave (peak potential $E_p = 0.83$ V) corresponds to reversible porphyrin ring oxidation, while the wave at $E_p = 1.33$ V is due to TPA substituent oxidation, in agreement with the behavior previously reported (*28*). Successive continuous cycles shows the development of the typical formation of TPB units, which are oxidized at $E_p = 1.05$ V (*31*−*33*). On the other hand, it is know that when Pd(II) is introduced as the central metal in the tetrapyrrolic macrocycle, the ring oxidation potential moves to more anodic values (*30*). Thus, in the case of PdP the oxidation potential of both electrochemical active centers are close, and the voltammograms are not as clearly defined as with H_2P. Nevertheless the film formation is evidently observed, where the current increment of TPB redox processes indicates the electroactive polymer growing. Scheme 2 shows the linkage between porphyrin centers through conjugated tetraphenylbenzidine bridges, which confers highly branched characteristics to the polymers structure.

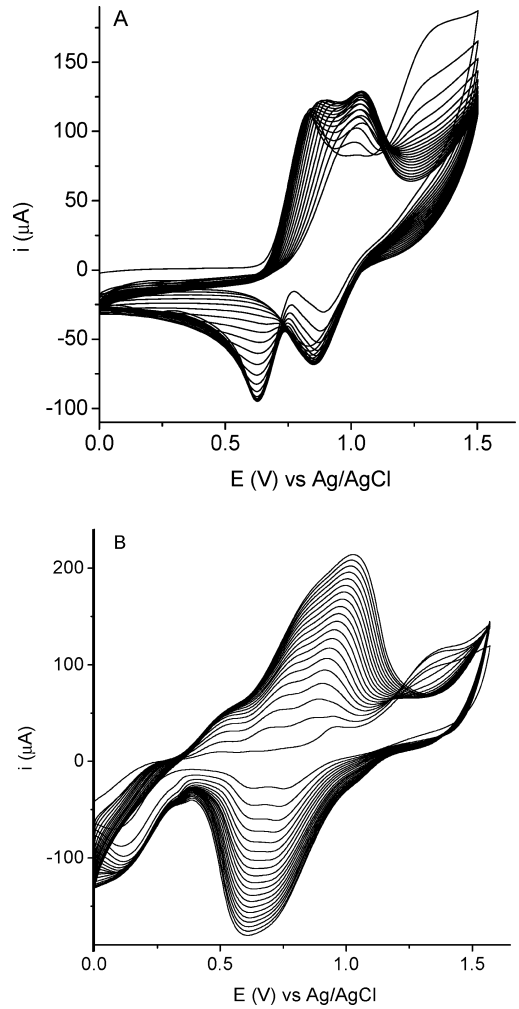
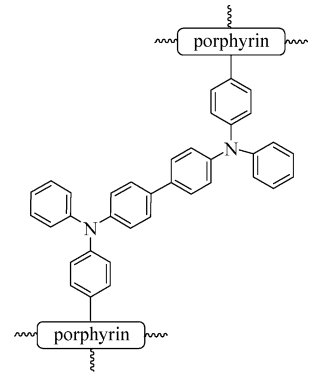


FIGURE 1. Cyclic voltammogram of (A) 5,10,15,20-tetrakis(4-[*N,N*-diphenylamino]phenyl) porphyrin in dichloromethane and (B) Pd(II) 5,10,15,20-tetrakis(4-[*N,N*-diphenylamino]phenyl)porphyrin in 1,2-dichloroethane (1 mM) containing 0.1 M of tetrabutylammonium perchlorate (TBAP) on indium tin oxide (ITO) electrodes; scan rate = 20 mV/s; continuous scan number 1−20.

SCHEME 2. Tetraphenylbenzidine Structure in the Electropolymeric Films



All the studies with the polymeric film were carried out with electrodes obtained by cycling the potential under the same conditions described in Figure 1.

Spectroscopic Studies. The absorption spectra of H_2P-film and PdP-film on ITO electrodes are shown in Figure 2A. The band absorption maxima of the films and the porphyrins in solution are summarized in Table 1. The H_2P and PdP porphyrins show the typical *Soret* and *Q*-bands, characteristic

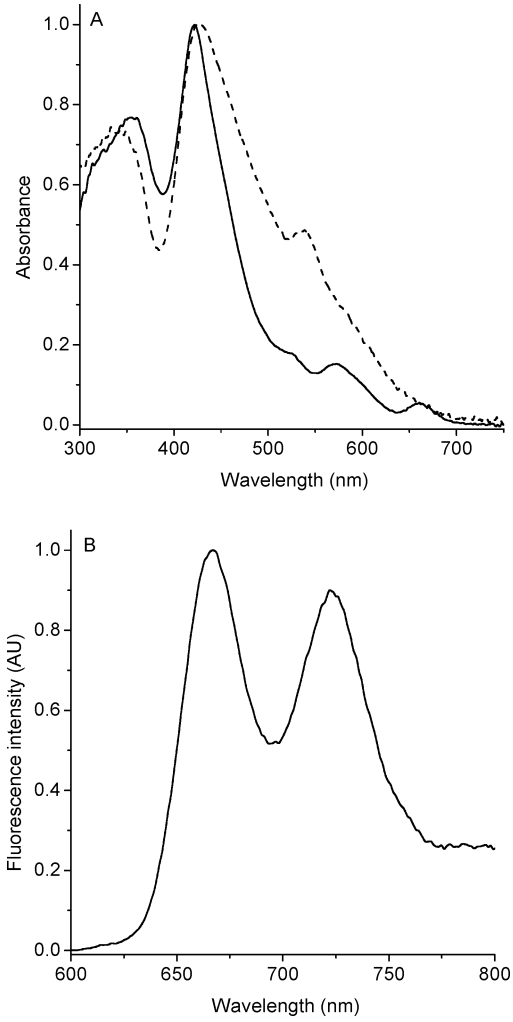


FIGURE 2. (A) Absorption spectra of H_2P-film (solid line) and PdP-film (dashed line); (B) fluorescence emission spectra of H_2P-fim (solid line, λ_{exc} = 430 nm) on indium tin oxide (ITO) electrodes.

TABLE 1. Absorption and Fluorescence Emission Maxima of Porphyrins in Solution and Electropolymeric Films (H_2P-Film and PdP-Film) on ITO Electrodes

compound	absorption λ_{max} (nm)						emission λ_{max} (nm)	
H_2P[a]	305	438	524	565	596	658	669	731
PdP[b]	306	436	530	568			578	620
H_2P-film	352	425	526	573		662	667	723
PdP-film	348	430	540					

[a] iln toluene. [b] In dichloromethane.

of a free-base H_2P porphyrin and the corresponding metalloporphyrin (*28, 30*). In the films, the *Soret* and *Q* bands show similar electronic transitions to those observed for the corresponding porphyrin in solution, although the bands of H_2P-film and PdP-film are broader and shifted in comparison with those of monomeric porphyrin in solution (Table 1). These facts indicate the presence of interaction between porphyrins in the hyperbranched film structure. Also, porphyrins in solution show an absorption band in the UV region (~305 nm) due to the TPA substituents, while in the films appears a broader band at about ~350 nm. This absorption is attributed to the tetraphenylbenzidine units formed in the electropolymerization process.

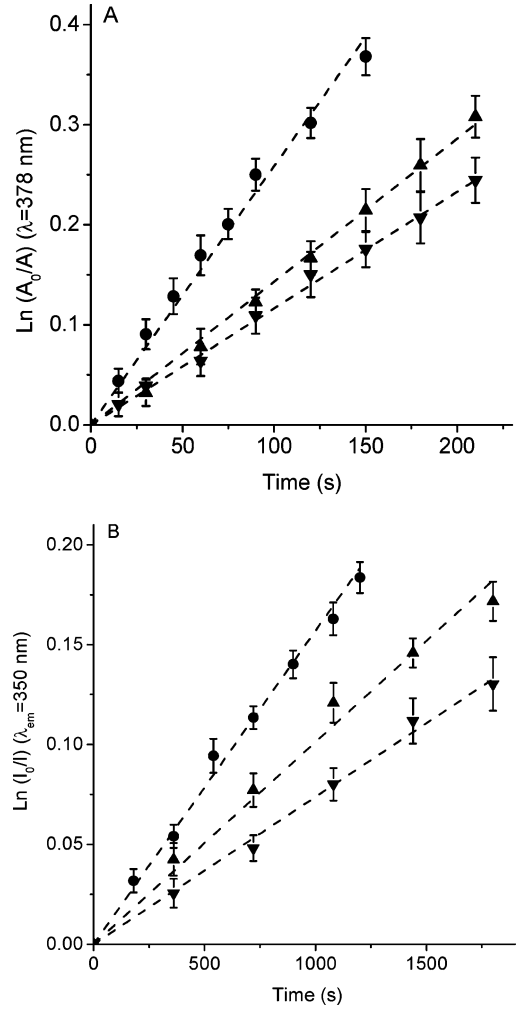


FIGURE 3. First-order plots for the photooxidation of (A) DMA (35 μM) in DMF and (B) Trp (25 μM) in water, photosensitized by H_2P-film (▼), PdP-film (▲) and $TMAP^{4+}$ (●), irradiated with light at λ = 370−800 nm. Values represent mean ± standard deviation of three separate experiments.

The steady-state fluorescence emission spectrum of the H_2P-films is shown in Figure 2B. The two bands are characteristic for free-base porphyrins in solution (Table 1) and they have been assigned to $Q(0-0)$ and $Q(0-1)$ transitions (*34*). From the absorption and fluorescence spectra, the Stokes shift was calculated ~5 nm for H_2P-film. A small Stokes shift is expected for tetraphenylporphyrin derivatives indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. Thus, the photophysical properties of the units of H_2P in the electropolymeric film are similar to those in solution.

On the other hand, even though a very low emission intensity was observed from solution of complexes of porphyrins with Pd(II) (*34*), the fluorescence of PdP-film was not detected (Table 1) probably because its partial aggregation in the electropolymeric films on the surface.

Photosensitized Decomposition of Substrates. Taking into account that DMA quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction, it is used as a method to evaluate the ability of the sensitizers to produce $O_2(^1\Delta_g)$ in solution (*35*). Thus, photooxidation of DMA sensitized by the porphyrin films was studied in DMF under aerobic conditions (Figure 3A). The sensitizing activity of the surfaces was compared with that obtained for a tetracationic porphyrin, $TMAP^{4+}$, which represent an active sensitizer to produce $O_2(^1\Delta_g)$ and also it is effective to eradicate microorganisms (*25*). From first-order kinetic plots of the DMA absorption at 378 nm

TABLE 2. Kinetic Parameters for the Photooxidation of DMA and Trp Sensitized by the Electropolymeric Films (Sup-H_2P and Sup-PdP) and $TMAP^{4+}$ in different media

porphyrin	k_{obs}^{DMA} $(s^{-1})^a$	k_{obs}^{Trp} $(s^{-1})^b$
sup-H_2P	$(1.1 \pm 0.2) \times 10^{-3}$	$(0.7 \pm 0.3) \times 10^{-4}$
sup-PdP	$(14. \pm 0.2) \times 10^{-3}$	$(1.0 \pm 0.3) \times 10^{-4}$
$TMAP^{4+}$	$(2.5 \pm 0.2) \times 10^{-3}$	$(1.6 \pm 0.1) \times 10^{-4}$

[a] In DMF. [b] In water.

with time (Figure 3A) the values of the observed rate constant (k_{obs}) were calculated (Table 2). As can be observed, the PdP-film photodecomposes DMA with a slightly higher rate than that of the H_2P-film. Under these conditions, a higher value of k_{obs} for DMA was obtained for the reaction sensitized in solution by $TMAP^{4+}$. This result is expected since in the polymeric films the $O_2(^1\Delta_g)$ is only formed on the surface, while using $TMAP^{4+}$ the photodynamic effect takes place in the solution bulk. Formation of aggregates leads to a decrease in the lifetime of the triplet excited state, diminishing the $O_2(^1\Delta_g)$ production (*36*).

The amino acid Trp was used as a substrate models for the compounds of biological interest that would be potential targets of porphyrin photosensitization. This substrate can be efficiently photooxidized by both type I and type II reaction mechanism (*25*). The photoprocess follows first-order kinetics with respect to Trp concentration, as shown in Figure 3B for [Trp] = 25 μM in water. From the plots in Figure 3B, the values of the k_{obs}^{Trp} were calculated for the films and $TMAP^{4+}$. As can be seen in Table 2, photosensitized decomposition of Try follows a similar tendency to that found with DMA photooxidation. This behavior observed with both substrates indicates that these electropolymeric films appear to perform their photosensitizing actions via the intermediacy of $O_2(^1\Delta_g)$. Although an absolute comparison between the photosensitization action produced by the films and $TMAP^{4+}$ is not possible, the results show that these polymers are efficient photoactive surfaces.

Photosensitized Inactivation of Microorganisms. The photomicrobicidal activities of the electropolymeric films were initially examined against suspensions of *E. coli* and *C. albicans* cells. These microorganisms were chosen as a representatives of a gram-negative bacterium and a yeast, which are important human and animal pathogens. In particular, the opportunistic pathogen *C. albicans* has the ability to colonize and cause disease within a diverse range of mammalian host sites (*37*).

PDI of microorganisms produced by the immobilized porphyrin films was evaluated in PBS cellular suspensions. Thus, the polymeric surface ($0.7 \times 3.0 \times 2.1$ cm^2) was placed inside of the cell suspension in culture tube containing $\sim 10^6$ CFU/mL and irradiated with visible light for different times. Typical results of cellular survival and growth delay curves of *E. coli* and *C. albicans* cells are shown in Figure 4 and Figure 5. Control experiments showed that the viability of *E. coli* or *C. albicans* was unaffected by illumination alone or by dark incubation with the films. Also, the cell viability was not changed by irradiation of the cultures in the presence of an ITO electrode without the electropolymeric films. This indicates that the cell mortality obtained after irradiation of the cultures containing the polymeric surface is due to the photosensitization effect of the immobilized porphyrin, produced by visible light. After treatment, spectroscopic analyses showed that the cultures were not contaminated with porphyrin and the absorption spectra of the films were not changed under these experimental conditions.

The survival curves for *E. coli* and *C. albicans* using different irradiation periods are shown in Figures 4A and 5A, respectively. The viability of microbial cells irradiated with

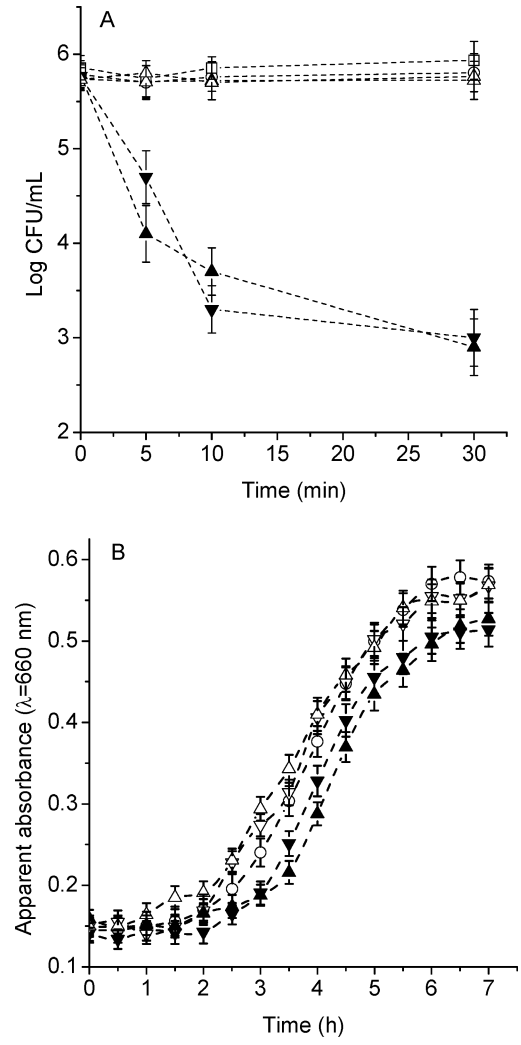


FIGURE 4. (A) Survival curves and (B) growth delay curves of *E. coli* cells photosensitized by H_2P-film (▼) and PdP-film (▲), after different times of irradiation with visible light (90 mW/cm^2). Control cultures in the presence of H_2P-film (▽) and PdP-film (△) in the dark, control cultures irradiated in the presence of an indium tin oxide (ITO) electrode (□) and untreated (○). Values represent mean ± standard deviation of three separate experiments.

visible light depended on the light exposure. As can be observed, the microorganisms are rapidly photoinactivated when the cultures in the presence of the polymeric films are exposed to visible light. Both H_2P- and PdP-films exhibit a photosensitizing activity causing a ~3 log decrease of *E. coli* survival after 30 min of irradiation. These results represent a value greater than 99.9% of cellular inactivation. A shorter irradiation time of 10 min is sufficient to produce a significant photoinactivation of ~2 log. In contrast to bacteria, eukaryotic *C. albicans* cells are more difficult to photoinactivate (*38*). In spite of that, the photodynamic action of both polymeric films produces a ~2 log (99.7%) decrease in the cell viability of yeast, when the cultures are irradiated for 60 min (Figure 5A). Also, it can be noted that no significant difference in the microbial photoinactivation is found between both polymeric films. Repetitive results were obtained using the same polymeric film for at least three times.

Photodynamic experiments were also performed under condition of microbial growth to ensure that PDI of cells is still possible when the cultures are not under starvation conditions or the potential damaging effects of PBS washing. Growth delay activity caused by the polymeric films was studied on *E. coli* and *C. albicans* in growth media. Thus, the

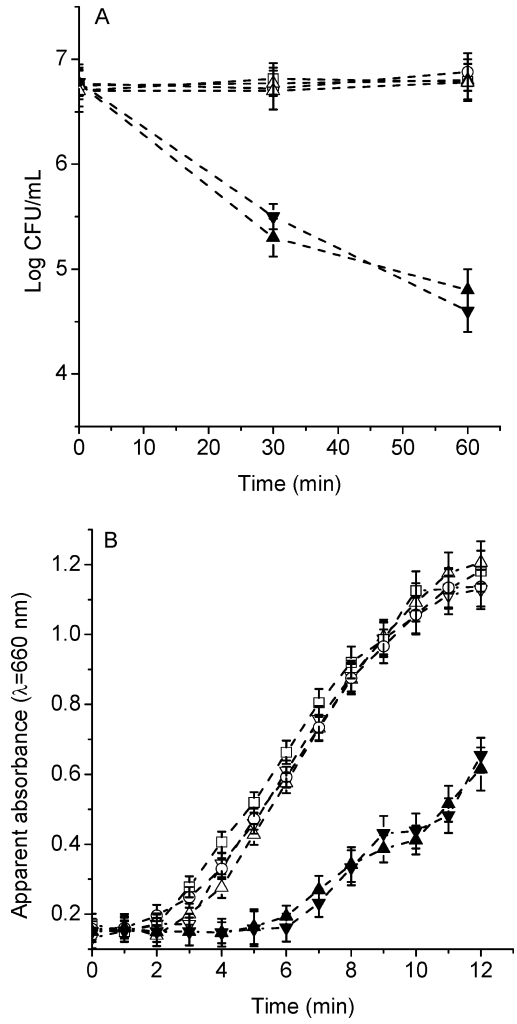


FIGURE 5. (A) Survival curves and (B) growth delay curves of *C. albicans* cells photosensitized by H_2P-film (▼) and PdP-film (▲), after different times of irradiation with visible light (90 mW/cm^2). Control cultures in the presence of H_2P-film (▽) and PdP-film (△) in the dark, control cultures irradiated in the presence of an indium tin oxide (ITO) electrode (□) and untreated (○). Values represent mean ± standard deviation of three separate experiments.

films were placed into fresh cultures of cells reaching the log phase and the flasks were irradiated with visible light at 37 °C. Figure 4B and Figure 5B show the growth curves of *E. coli* and *C. albicans* cells, respectively. Microbial cells exposed to porphyrinated films in the dark or not treated with the films and illuminated showed no growth delay compared with controls. Changes of pH were not observed for cell growth and survival. Therefore, the data illustrate that the observed growth delay is due to the photoinactivation effect of the films on the cells. However, as can be observed in Figure 4B, only minor effects on the growth delay was found for *E. coli* cells treated with H_2P- and PdP-films. Under these conditions, the photosensitized inactivation of *E. coli* competes with the cellular growth. Therefore, the growth of bacteria is faster than the films action to eradicate *E. coli* cells. However, cell viability evaluations post treatments indicate that ~90% of *E. coli* cells were killed. In contrast, growth was suppressed when *C. albicans* cultures were treated with the films and illuminated (Figure 5B). The lag phase in the typical yeast growth curve was considerably enlarged due to the photodynamic activity of the antimicrobial films. After 6 h of incubation, the *C. albicans* cells appeared to be slowly growing as measured by turbidity at 660 nm. The slower growth of *C. albicans* cells, in comparison with the *E. coli*, suggests that this culture can be more efficiently eradicated by the antimicrobial action of the porphyrinated films.

In previous investigations, porphyrinated cellulose laurate esters films were tested as active plastic surfaces to inactivate bacteria on nutrient agar when irradiated with visible light (*20*). Also, porphyrins were incorporated into translucent chitosan membranes by adsorption, 5,10,15,20-tetrakis(*p*-hydroxyphenyl)porphyrin (*p*-THPP), and by dissolution and casting, 5,10,15,20-tetrakis(*p*-aminophenyl)porphyrin (*p*-TAPP) (*21*). These polymeric membranes were tested to inactivate lower microbial levels in water. Evaluation of photomicrobicidal action against *E. coli* (300 cell/mL) revealed some activity in each case, and showed that the cell viability is reduced ~30% and ~80% for membrane impregnated with *p*-THPP and *p*-TAPP, respectively, after 40 min of irradiation. However, no viable cells could be detected for *E. coli* (at 3500 cell/mL) using a chitosan film chemically bonded to zinc(II) phthalocyanine tetrasulfonic acid after 30 min irradiation in a static system (*21*). In the present case, PDI experiments were performed using a considerable higher cellular density (~10^6 CFU/mL) and the electropolymeric films were effective to photoinactivate microorganisms. Thus, PDI treatment with H_2P- and PdP-films produces about 9.99 × 10^5 cell/mL of *E. coli* inactivation after 30 min of irradiation.

Conclusions

In the present work, two porphyrinated films were formed by electrochemical polymerization of free-base H_2P and its complex with Pd(II) on ITO electrodes. These electrodes represent an appropriated surface to obtain mechanically stable electropolymeric films and they are optically transparent to visible light. Thus, irradiation of these surfaces with visible light induces efficient photooxidation of substrates (DMA and Trp) in solutions, indicating an efficient photodynamic action of both polymeric films.

In vitro investigations show that these films produce photosensitized inactivation of *E. coli* and *C. albicans* microorganisms in aqueous suspensions. After 30 min of irradiation, the films exhibit a photosensitizing activity causing a ~3 log decrease of *E. coli* and ~2.5 log decrease of *C. albicans* cellular survival. The photodynamic effect of the surfaces was also evidenced by growth delay experiments.

Under these conditions, two mechanisms can be considered, which include a direct cell damage by $O_2(^1\Delta_g)$ produced at the film surface and diffusing to the closely associated microorganism and the slow release of the photosensitizer into the suspension of the microbial cells. However, the last case is not possible using H_2P and PdP-films, since the porphyrin structures are immobilized by irreversible covalent bonding. Also, the cultures were not contaminated with porphyrin (spectroscopic examination) and the absorption spectra of the films were not changed after treatment. Therefore, $O_2(^1\Delta_g)$ or other reactive oxygen species appear to be mainly responsible for the cell inactivation.

The results indicate that porphyrins immobilized on electropolymeric films are interesting and versatile photodynamic surfaces to inactivate microorganisms in liquid suspensions. Thus, the main advantage of these new antimicrobial surfaces to heterogenic eradication of microorganisms is that they can be easily and quickly removed from the media after cell inactivation, avoiding permanent photodynamic effects. These films could be used to eliminate microorganisms growing in a liquid media, such as disinfection of water, and to form permanent antimicrobial surfaces activated by visible light.

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