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Development of Chitosan Membranes Containing Photosensitizer for Water Disinfection

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Keywords: *chitosan; membrane; photosensitizer; disinfection.*

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Development of Chitosan Membranes Containing Photosensitizer for Water Disinfection

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Keywords: chitosan; membrane; photosensitizer; disinfection.

I. Introduction

Chemical processes are the most widely used methods of drinking water disinfection; however, modern analytical methods for water analysis have revealed by-products that are toxic and potentially carcinogenic[1, 2]. Photodynamic inactivation (PDI) seems to be a very promising method to inactivate microorganisms in water without the formation of hazardous compounds[3-5]. PDI utilizes photosensitizers and light to promote a rapid phototoxic effect, normally oxidative, which is capable of damaging biomolecules and cellular structures and thus killing microorganisms[6-8]. However, the photosensitizer must not persist as a contaminant. One way to solve this problem is to immobilize the photosensitizer in a polymeric support. Interest in polymers containing photoactive groups results from their broad applications, as drug carriers, sensors, sensitizers[9-11] and (in the environmental field) for water purification[9, 12]. Due to environmental concerns, there is interest in developing such a system using natural polymers[13].

In this context, chitosan, a non-toxic, biocompatible and biodegradable polysaccharide obtained by N-deacetylation of chitin, appears to offer a number of distinct advantages. One of the most abundant polysaccharides in the biosphere, chitin is a low-cost source material, obtained from crustaceans, molluscs, insects, mites, fungi and algae[14]. However, chitin is insoluble in water and organic solvents, which makes it difficult to use. Chitosan, however, is more suitable for biological applications[15]. Chitosan is insoluble in water, concentrated acids, alcohol and acetone; however, it is freely soluble in solutions of weak organic acids (acetic, formic and citric acid) and diluted inorganic acids (hydrochloric, nitric, perchloric or phosphoric acid)[16, 17]. The main characteristics that make chitosan of great interest for a large number of applications are the potential for chemical modification, or for being prepared in different forms such as solutions, sponges, films, membranes and gels[17]. Furthermore, the antimicrobial activity of chitosan and its derivatives have been widely studied, with good inhibitory activity against bacteria, fungi and yeasts[18, 19]. These effects are influenced by the physical characteristics of the preparations (such as the degree of deacetylation and molecular weight), as well as by concentration, exposure time, viscosity and pH[20, 21].

Applications for biomaterials obtained from chitosan have evolved over the last decade. Properties such as biocompatibility and biodegradability allow several applications for these biomaterials. The versatility of chitosan enables its use in agriculture, food processing, tissue engineering, and the pharmaceutical, medical and dental areas[17, 22, 23]. Chitosan can capture metals and flocculants to purify and clarify water[17, 24-26]. For water disinfection, Bonnett et al, 2006[27] proposed the use of chitosan as a polymeric support for PDI because chitosan is water-insoluble, promoting contact between the membrane-bound photosensitizer and the aqueous suspension of microorganisms[28].

Therefore, the goal of this study was the incorporation of photosensitizers into chitosan

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membranes, for use in the microbiological disinfection of water.

II. Materials and Methods

a) Chemicals and preliminary characterization of the chitosan sample

Chitosan was obtained by deacetylation of β-chitin, from pens of Loligo sp[29]. The degree of acetylation of chitosan was determined by conductimetric measurements. Molar mass was determined by viscometric measurements[30]. The commercially available photosensitizers methylene blue (MB, Sigma Aldrich- USA), rose bengal (RB, Vetec Química-Brazil), meso-tetrakis(4-N-methylpyridyl)-porphyrin (TMPyP, Midcentury Chemicals, USA) and 5,10,15,20-tetrakis(p-aminophenyl)-porphyrin (p-TAPP, Sigma Aldrich, USA) were used without further purification. The chemical structures of the photosensitizers are shown in Fig. 1. All other reagents used in this study were of analytical grade.

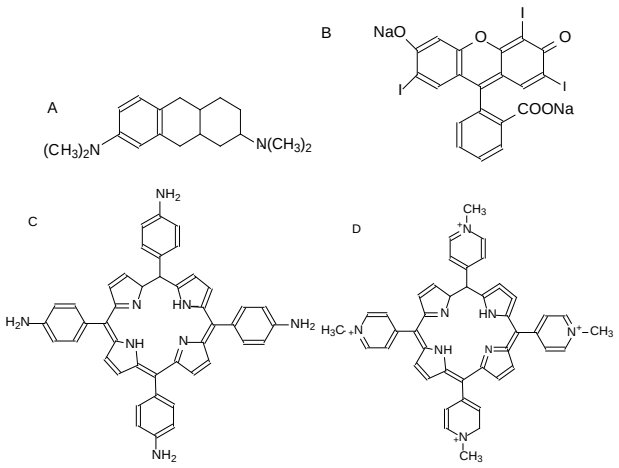

Fig. 1: Chemical structure of MB (A), RB (B), p-TAPP (C) and TMPyP (D).

b) Preparation of the chitosan membranes

The stock of chitosan was prepared at a concentration of 1% (w/w) in acetic acid. Ten grams of chitosan gel was placed in a Teflon® tray4.7 × 4.7 × 0.7 cm. The trays were kept in a chamber with air flow for three days. When submerged in water (Milli-Q), chitosan membranes with photosensitizer swelled and began to fragment. Sodium tripolyphosphate (TPP) is a crosslinking agent that interacts with chitosan via electrostatic forces, forming a network of ionic crosslinks that inhibit its dissolution[31, 32]. Therefore chitosan membranes with and without photosensitizer were treated with 0.5% (w/w) of TPP in 2 mol L^{-1} NaOH for 30 minutes in order to stabilize the material against fragmentation and swelling. The membranes were then washed, dried and stored at room temperature (25°C).

c) Preparation of chitosan membranes with incorporated photosensitizer

A stock solution of each photosensitizer at a concentration of 1 mg mL^{-1} in ethanol was prepared and stored in the dark at 4°C for up to seven days. Each photosensitizer solution (1 mL) was added to 15 g of chitosan gel (1% (w/w) in acetic acid). The mixture was homogenized and then treated as described under "preparation of the chitosan membranes". After drying, the photosensitizer not immobilized in the membranes was released. Membranes (22 cm^2) containing photosensitizer were placed in 200 mL of distilled water under mechanical agitation for three days or until no more photosensitizer was observed by spectroscopy (600, 565, 416 or 425 nm, for AM, RB, p-TAPP or TMPyP, respectively). The membranes were then air-dried. The concentration of photosensitizer in the chitosan membrane was estimated by UV-Visible Spectroscopy at the above wavelengths[27].

d) Fourier transform infrared spectroscopy (FT-IR)

To obtain FT-IR spectra, samples of chitosan (1% w/w) with and without photosensitizer were prepared in 1% acetic acid, transferred to a silicon support and oven dried under vacuum. IR spectra were obtained in a Bomen MB-102 at 400 to 4000 cm-1 with 32 scans.

e) Microorganisms and preparation of cell suspension

Escherichia coli (ATCC 25922) was kindly provided by Prof. José Francisco Hoffling, Department of Oral Diagnosis, UNICAMP. A suspension of *E. coli* containing 1×10^9 cells mL^{-1} was prepared after growing in Brain Heart Infusion Agar (BHI, Oxoid, São Paulo, Brazil) in an incubator (002 CB Model - Fanem, São Paulo, Brazil) for 48 h at 37°C. Then, the bacterial suspension was inoculated in Luria-Bertani Broth (LB, Oxoid, São Paulo, Brazil) and maintained at 37°C for 18 h in an orbital shaker (Marconi MA 410, Piracicaba, Brazil) at 100 rpm. After the incubation period, a pellet was obtained by centrifugation (Excelsa II - Fanem, São Paulo) at 1300 rpm for 10 minutes and suspended in 10 mL of sterile saline. This procedure was repeated two

more times. Counting of cells in the suspension was performed using a spectrophotometer at 590 nm (Hitachi U2800, Japan).

f) Light source and photoinactivation setup

Photoinactivation of the bacteria was evaluated by exposing the membranes to a series of LEDs (BioTable).Blue LEDs (452 ± 30 nm) were used to irradiate the porphyrins only, yellow (590 ± 30 nm) to irradiate rose bengal and porphyrins, and red (630 ± 30 nm) to irradiate methylene blue, based upon the maximum wavelengthof each photosensitizer. The fluence rate of the blue LED was 14 mW cm^{-2}, yellow 10 mW cm^{-2}, and red 18 mW cm^{-2}.

The photocytotoxicity of the four immobilized photosensitizers towards *E. coli* in water was assessed by incubating the chitosan membranes with the bacterial suspension, followed by irradiation with the appropriate LED for different time intervals (20, 40, 60, 80, 100, 120, 140, 160 and 180 min). Chitosan membranes with MB were irradiated with red LEDs; those with RB, p-TAPP or TMPyP were irradiated with yellow LEDs. The porphyrins were also irradiated with blue LEDs because these photosensitizers also absorb these wavelengths.

Membranes (1 cm^2) were placed in a 24-well polystyrene plate (Corning Costar) and submerged in bacterial suspension (1.2 mL, 1×10^9 cells mL^{-1}). Aliquots of 0.1 mL were removed, and the number of colony-forming units per millilitre (CFU mL-1) determined. Control experiments were performed on chitosan membranes both without photosensitizer under irradiation and with photosensitizer in the dark. For each membrane in each experimental condition, three independent experiments were performed and the results presented as the average of the three assays.

III. Results and Discussion

a) Chitosan membranes

The degree of acetylation of chitosan was determined by conductimetric measurements to be 9%. Viscometric measurements were used to estimate the molar mass as 1.248 x 10^5 g mol^{-1}.

Four photosensitizers from different classes (two porphyrins, one phenothiazine and one halogenated xanthene) were incorporated into chitosan membranes. Chitosan membranes prepared without photosensitizer (CH) were translucent, with a thickness of ~60.0 μm. Membranes with MB (CHMB), RB (CHRB), p-TAPP (CHpTAPP) and TMPyP (CHTMPyP) were blue, pink, light brown and light yellow, respectively.

Chitosan gel with and without photosensitizer was characterized by FT-IR. Spectra obtained with or without photosensitizer are shown in fig. 2. We assign the absorption bands as follows: 1550 cm^{-1} - angular deformation of N–H (amide II); 1150 cm^{-1} - axial deformation of O–H in the hydrogen bond; 1600-1670 cm^{-1} - C=O stretch of amide I, because chitosan is not completely deacetylated; 800-1200 cm^{-1} - pyranoside ring[33-36]. FT-IR spectra were similar with or without photosensitizer, but the amino group peaks (amide I) of chitosan with photosensitizer (MB, RB, p-TAPP and TMPyP) were shifted to lower wavenumbers, suggesting an interaction with the photosensitizers.

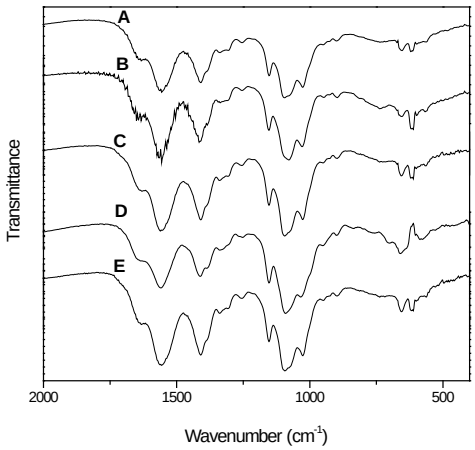

Fig. 2: Infrared spectra of chitosan membranes with no photosensitizer (A), AM (B), RB (C), p-TAPP (D) or TMPyP (E).

To ensure that photosensitizer would not be released from the membranes during PDI experiments, liberation of the photosensitizer not immobilized in chitosan membranes was performed. After these procedures, part of the membrane was used in photoinactivation assays, and part was used to determine the final concentration of photosensitizer incorporated into the membrane.

Because the photosensitizers are attached to chitosan membranes, which are insoluble in water, one cannot directly compare their spectroscopic properties with free photosensitizer molecules. For this purpose, the absorption spectra of the chitosan membranes with photosensitizer were obtained by dissolving in 1% (w/w) acetic acid and compared to spectra of the photosensitizer alone in 1% acetic acid.

The spectrum of CHMB showed maximum absorbance at approximately 600 nm, compared to 665 nm for MB in acetic acid (Fig. 3a). This indicates aggregation of AM-forming dimers[37, 38]. The formation of aggregates may reduce the efficiency of the photosensitizer because no radioactive decay occurs by internal conversion, making it difficult to transfer energy to ground-state oxygen[39, 40]. From the absorbance at 600 nm, a rough estimate may be given that the MB content in the membrane was approximately 5 μmol L^{-1} MB per gram of chitosan membrane. The estimate of the concentration of photosensitizer in the membranes is rough because the molar extinction coefficient is dependent on solvent composition[12]. It is not possible to compare this result with values from the literature because the applications for which the membranes were developed were different. Previous studies in our group incorporating MB into polymeric supports such as collagen-based membranes have estimated MB content from the concentration released versus time of incubation in artificial saliva. This allowed us to evaluate the photodynamic effect against *Candida albicans*[41].

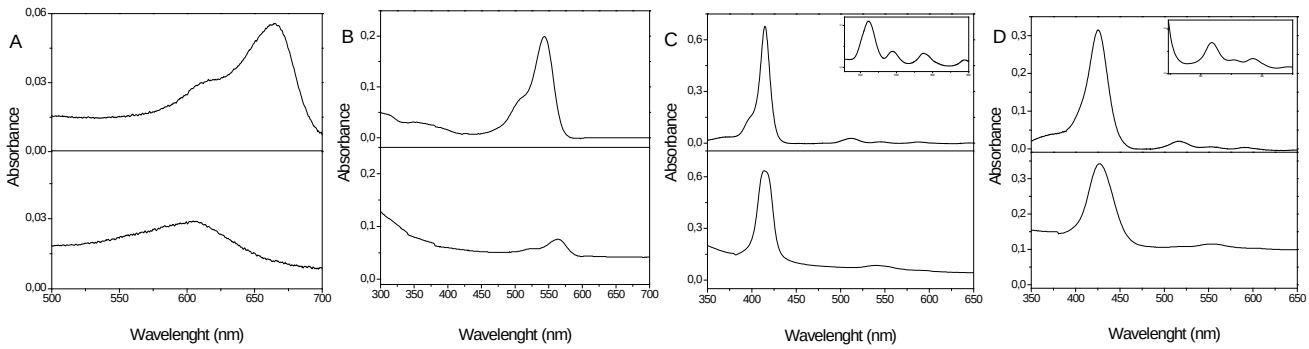

Fig. 3: Absorption spectra of MB (A), RB (B), p-TAPP (C) and TMPyP (D) photosensitizer solutions in 1% acetic acid (upper graph) and dissolution of chitosan membranes with photosensitizers in 1% acetic acid (bottom graph). The inserts in graphs C and D show an expansion of the Q-band of porphyrin in ethanol

Fig. 3b shows that the spectra obtained from the dissolution of the CHRB membrane and those of the solution of RB in 1% (w/w) acetic acid exhibited maximum absorbances at approximately 554 and 565 nm, respectively. This indicates a small red-shift due to a change in environment (bathochromic shift)[27]. A rough estimate of RB concentration in chitosan membranes from 565 nm was approximately 3.5 μmol L^{-1} RB per gram of chitosan membrane. Again, these results cannot be compared with those reported in the literature. In studies using chitosan and RB, chitosan chains were modified by covalent attachment of RB. Thus RB was attached to the polymer chain and not adsorbed, as in this study[12, 42]. Other studies that use RB employ polystyrene as the polymeric support. The mechanism of action using this support is not acceptable for the disinfection of water because the photodynamic effect is mediated by the slow release of RB into the cell suspension[43].

As shown in Fig. 3c, a Soret band (at 416 nm) was observed in both spectra of the p-TAPP (dissolved CHpTAPP membranes and p-TAPP solution). In the dissolved CHpTAPP spectrum, a small red shift of the Q-band (500-600 nm) was observed. From the absorbance at 416 nm it was estimated that approximately 1 μmol L^{-1} p-TAPP per gram of chitosan membrane remained embedded. The results in the literature show that approximately 5 mg cm^{-2} of p-TAPP was incorporated into chitosan membranes when 7.5 mg cm^{-2} was added to the chitosan gel before drying. Therefore the incorporation efficiency was 67%[27], much higher than that obtained in this study (1.5%).

TMPyP spectra (Fig. 3d), obtained by CHTMPyP dissolution and in solution, showed a Soret band at 425 nm. Again a small red shift of the Q-bands (500-600 nm) is seen in the TMPyP spectrum of dissolved membranes. From the absorbance at 425 nm, it was estimated that 2 μmol L^{-1} TMPyP remained incorporated per gram of chitosan membrane. To our knowledge there is no prior study in the literature using TMPyP incorporated into a polymeric support.

Quantification of the four photosensitizers in chitosan membranes suggest that a small proportion of the photosensitizers was retained on the polymeric support. Retention of photosensitizers in polymeric membranes depends on the concentration and chemical structure of photosensitizer, and on the nature of the polymer[27, 41, 44].

Furthermore, our results suggest that in developing membranes for use in water disinfection, prior release of the photosensitizer not immobilized in the membranes is important to ensure the absence of the photosensitizer from the treated water[45].

b) Photoinactivation of Escherichia coli

Control membranes, kept either in the dark with photosensitizer or exposed to light without photosensitizer, did not cause a significant decrease in *E. coli* count (Fig. 4, 5 and 6). This indicates that the reduction in cell survival after irradiation was due to photoinactivation.

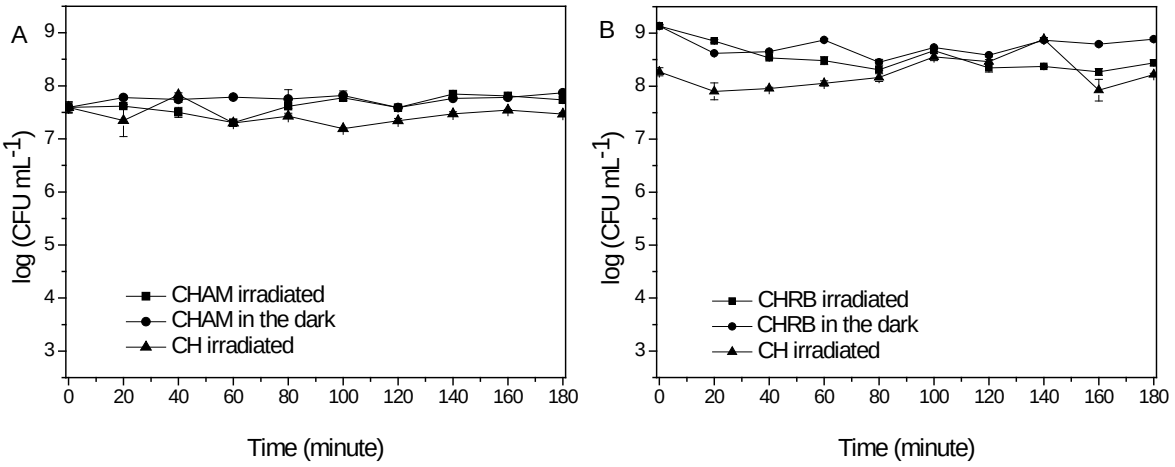

Fig. 4: Viability of *Escherichia coli* in the presence of chitosan membranes incorporating MB (A) or RB (B) exposed to red LED and yellow LED, respectively, and control membranes (irradiated without photosensitizer, and with photosensitizer in the dark). Points represent the average of two independent experiments

The results obtained in the PDI experiments using *E. coli* are summarized in Figs. 4-6. CHRB and CHMB did not cause a significant decrease in bacterial cells after irradiation (Fig. 4). There are some reports that MB (3.65 μmol L^{-1}) and RB (3.0-10.0 μmol L^{-1}) solutions significantly reduce *E. Coli* count[46-48]. This indicates that photodynamic inactivation was not effective using CHMB (5 μmol L^{-1} per gram of chitosan membrane) or CHRB (3.5 μmol L^{-1} per gram of chitosan membrane). This may be due to the low rate of singlet oxygen production by photosensitizers immobilized on polymeric supports. This rate is about one hundred times lower than that of photosensitizers in solution[49, 50]. Moreover, the formation of aggregates in the CHMB membranes may also have contributed because the formation of aggregates decreases the photodynamic efficiency of the photosensitizer[39].

However, chitosan membranes with porphyrins were effective against the bacteria. CHpTAPP membranes caused an approximately 2 log reduction in cell survival after 140 min of exposure to yellow or blue LEDs (Fig. 5 *a* and *b*, respectively). CHTMPyP membranes caused a 4 log reduction after 140 and 120 min irradiation with yellow (Fig. 6a) and blue (Fig. 6b) LEDs, respectively. These results suggest that CHpTAPP and CHTMPyP membranes have significant photodynamic activity even against concentrated bacterial suspensions (1×10^9 cells mL^{-1}). Literature results[27] indicate a significant reduction in cell count when using higher concentrations of p-TAPP incorporated in chitosan and an initial bacterial concentration of 3.5×10^3 cells mL^{-1}. Thus, the present results suggest that chitosan membranes containing p-TAPP have photodynamic activity even against concentrated bacterial suspensions.

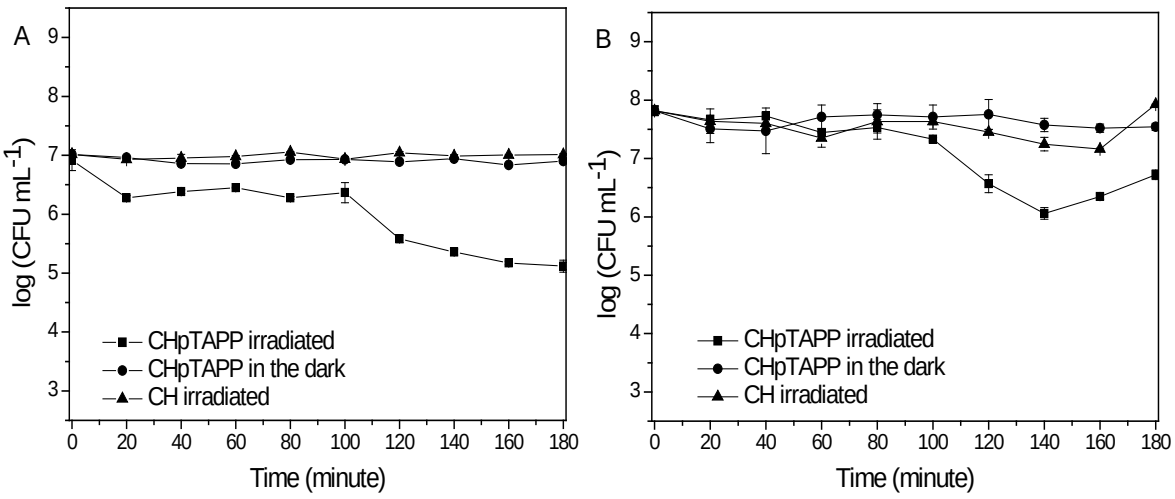

Fig. 5: Viability of *Escherichia coli* in the presence of chitosan membranes incorporating p-TAPP exposed to yellow (A) and blue (B) LEDs and control membranes(irradiated without photosensitizer, and with photosensitizer in the dark). Points represent the average of two independent experiments

In irradiating CHTMPyP with blue LEDs, we observed the same cell count reduction (4 log) at 120 minutes as with yellow LEDs. As shown in Fig. 3d, the TMyP molar extinction coefficient is greater in the blue region than in the yellow region, leading to higher absorption of light. This in turn leads to an increased production of oxidative species, giving the porphyrin better photodynamic activity when irradiated in the blue region[7].

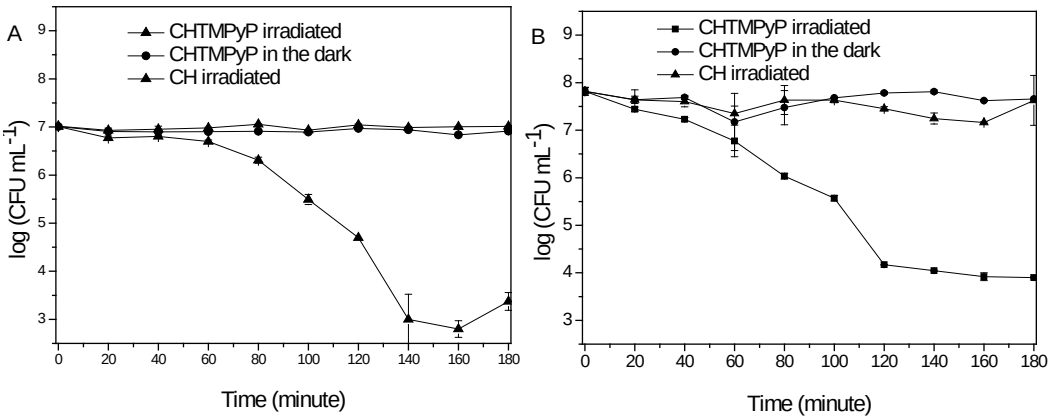

Fig. 6: Viability of *Escherichia coli* in the presence of chitosan membranes incorporating TMPyP exposed to yellow (A) and blue (B) LEDs and control membranes (irradiated without photosensitizer, and with photosensitizer in the dark). Points represent the averages of two independent experiments.

It can be observed in Table 1 that the concentration of photosensitizer remaining embedded in the polymeric carrier is not a limiting factor for the photodynamic process because the p-TAPP incorporated in lower concentrations (i.e., 1 μmol L^{-1} p-TAPP per gram of chitosan membrane) produces a photodynamic effect, while MB at 5 μmol L^{-1} per gram of chitosan was not able to inactivate the bacteria. Furthermore, the ionic charge of the molecule and the quantum yield of singlet oxygen are factors that contribute to photoinactivation of *E. coli*. For example, TMPyP is tetra-cationic, and has a quantum yield slightly lower than RB, which is di-anionic. TMPyP showed greater photodynamic activity on the Gram-negative bacteria employed (Table 1). These results agree with other studies using photosensitizer in solution[46, 47, 51, 52], which suggests that photoinactivation depends on concentration, quantum yield of singlet oxygen and the ionic charge of photosensitizers. Furthermore, these results also suggest that Gram-negative bacteria (such as E. coli) are more easily photoinactivated by cationic photosensitizers. A paper of our group in which the efficacy of the developed collagen membranes with these porphyrins incorporated was tested for photoinactivation of microorganisms in circulating water has been published elsewhere[53].

Table 1: Summary of results and properties of photosensitizers

Photosensitizer	TMPyP	p-TAPP	RB	MB
Immobilized photosensitizer concentration	2	1	3	5
(μmol L^{-1}/ gram of chitosan membrane)				
Produces photodynamic effect	Yes	Yes	No	No
Ionic charge	4+	0	2-	1+
Quantum yield of singlet oxygen	0.74[53]	0.53[54]	0.76[55]	0.39[56]

IV. Conclusions

The development of chitosan membranes incorporating photosensitizers was investigated for the photoinactivation of the bacterium *Escherichia coli*. Only a small proportion of the photosensitizers was immobilized on the chitosan membranes. Furthermore, it is important to remove non-immobilized photosensitizers from the polymeric support, because residual traces of photosensitizer are unacceptable in water disinfection. It was observed that the photodynamic inactivation process depends on the ionic charge as well as the spectroscopic and photophysical properties of the photosensitizer. TMPyP incorporated in chitosan membranes was the most effective in inactivating *E. coli*, and thus shows the one with better potential to inactivate bacterial water contaminants. This is of great importance because water disinfection using immobilized photosensitizers may have significant practical applications. These

include the purification of water tanks in hospitals, dental offices, schools and homes, being a promising approach to avoid the eventual recontamination of water seen after traditional methods of disinfection.

V. Acknowledgements

We are grateful to CAPES and FAPESP for financial support. We thank Prof. José Francisco Hoffling from the Department of Oral Diagnosis, UNICAMP, for kindly providing microorganisms, Prof. Vanderlei S. Bagnato (CEPOF, IFSC/USP) for developing the BioTables used, and to Wanessa C. M. A. Melo for her able technical assistance.

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