Latex membranes with methylene blue dye for antimicrobial photodynamic therapy

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Abstract

The search for new materials that can be applied in the treatment of injured human tissues has led to the development of new dressings. Membranes have potential as dressing materials because they can be fitted to and interact with the tissue surface. In this study, we analyze the morphological properties and wettability of latex membranes, along with the incorporation of the photosensitizer methylene blue, in the context of the utility of the membranes in curative applications involving photodynamic therapy (PDT). It was observed that deposition of the photosensitizer into latex membranes increased both the surface roughness and wettability. Antifungal testing indicated that antimicrobial PDT assisted by the latex membranes incorporating methylene blue effectively inactivated *Candida albicans*.

Keywords Natural rubber · Burn · Microscopy · Wettability

1 Introduction

According to the World Health Organization, it is estimated that 300,000 deaths are caused by burns annually [1]. Burns are traumatic wounds that can cause partial or total destruction of the skin. In these situations, the skin is no longer able to maintain its function as a protective barrier for the body [2]. Treatments are designed to alleviate the systemic changes that result from the breakdown of this protective barrier [3]. Dressings that are used during recovery from burns must be able to maintain high humidity since the hydration of the wound facilitates the exchange of the dressing, thus aiding in the cicatrization process [4].

Among the candidate materials being investigated for the manufacture of dressings for burns, those based on natural rubber (NR) latex stand out for being highly flexible, translucent, and non-adhesive. These membranes have been investigated for regenerative medicine applications because of their angiogenic properties, high flexibility, and capacity for incorporating drugs into their structures [5–7], allowing the incorporation of photosensitizing dyes (PS) which are used

in photodynamic therapy (PDT). Antimicrobial PDT is an alternative treatment for microbial infections [8] which uses a PS excited by light in the presence of molecular oxygen. After being excited, the PS can produce reactive oxygen species, resulting in the destruction of specific cells of a microorganism without damaging the surrounding tissues [9, 10].

According to Moore [11], septicemia due to *Candida* is a rare but important cause of death in burn patients. In burn injuries, colonization by *Candida* is a common cause of persistent infection. As the time and cost of the treatment of burn patients increases due to fungal infections, infected burns are a target of interest for antimicrobial PDT [8, 12].

The objective of this work was to analyze the morphological and wettability properties of latex membranes and to evaluate the effectiveness of antimicrobial PDT assisted by latex membranes incorporating methylene blue (MB) photosensitizer, thus investigating the potential of the membranes for use as curative membranes in PDT.

2 Materials and methods

The membranes were prepared as described by Silva [6]. Latex was extracted from a *Hevea brasiliensis* clone RRIM600 and stabilized with ammonium hydroxide. The NR membrane was obtained by drying the mixture in petri

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dishes at 50 °C for 96 h. MB (Sigma Aldrich) was used as supplied. The NR membrane was immersed in the MB solution (0.1 gL^{-1}) for 4 min and then dried under ambient conditions (named NR+MB). Some membranes were stored in petri dishes wrapped with plastic film to avoid exposure to air, for 1 year. The packaged plates containing the membranes were protected from light during the storage period.

The membranes were monitored by a double-beam UV–vis spectrophotometer (Genesys 10, Thermo Scientific, Waltham, MA, USA). Surface morphology was analyzed by an atomic force microscope (AFM; EasyScan II, Nanosurf, Switzerland) in the intermittent mode (512×512 pixels) with scanning windows of $5 \times 5 \mu\text{m}$. The contact angle was measured with a homemade instrument under ambient conditions. The wetting contact angle analyzer is a compound of a monocular microscope and a positioner attachment to the microscope by clamps as described by Gomes [13]. Droplets of deionized water ($3 \mu\text{L}$) were placed onto the membrane, and average values were taken from measurements at six different locations on each sample.

Inactivation tests were performed by spreading the dispersion containing *Candida albicans* (ATCC 14,053 at 10^7 CFU/mL) over the entire surface of the culture medium, and after 1 h, membrane discs (diameter 0.6 cm) were deposited on the medium. Discs were irradiated using a continuous diode laser (HLM1845) with a wavelength of 655 nm. The output power of the laser beam was set to 100 mW, using a spot size diameter of ~ 0.6 cm and irradiation time of 85 s, resulting in a dose of 30 J/cm^2 . The laser beam output was positioned 15 cm from the top of a Sabouraud agar plate [14]. The Petri dishes were incubated at 37 °C for 24 h. MB has already been acknowledged for the inactivation of *C. albicans* [15, 16]; hence, instead of classical tests, we used a variant of inhibition zone formation [6, 17] with the aim of verifying whether NR+MB avoided colonization of the fungus on the agar surface, which served as a proxy for the surface of burned skin.

For a quantitative analysis of the antifungal effect, we used the inactivation tests with adaptations. $80 \mu\text{L}$ stock suspension of *C. albicans* (10^7 CFU/mL) and membrane discs (diameter 0.6 cm) were placed in a microtube and after 5 min of contact, the material was irradiated and prepared for viability tests. This work used a viability assay based on the exclusion of the methylene blue dye [18–22], which acts as an indicator of cell viability. The counting in the Neubauer chamber was done with the help of the optical microscope, which considered the cells that were stained blue to be dead and the transparent ones to be viable [23]. For morphological analysis, films with the dispersion of fungi were prepared by the casting technique [24], which involves the spreading of the material on a substrate. The entire procedure was performed in a laminar flow hood. The susceptibility of fungi was analyzed by fluorescence microscopy

(Nikon Eclipse Ci/L, Tokyo, Japan) using the acridine orange dye as marker, which fluoresces green when the cells are viable and red–orange when damaged [23, 25–29]. The microscope uses a mercury vapor lamp and for this experiment, the images were taken using a blue excitation filter (with dichroic barrier filter) which results in green, yellow or red emission. In this study at least three measurements for each sample were taken, and errors were determined as standard deviations. The groups were compared by ANOVA tests followed by a Tukey–Kramer multiple comparison test. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Incorporation of MB

The absorption at 670 nm for MB (Fig. 1) corresponded to monomers, and the shoulder at 610 nm was related to a vibrational transition. The MB dye was effectively deposited onto the latex membranes, as can be seen in the inset of Fig. 1. The NR membrane did not absorb in the region of the spectrum investigated.

3.2 Wettability

The contact angles, θ_c , of the latex membrane decreased from 53° to 19° after MB deposition; i.e., there was an increase in wettability (Table 1) [13]. Two factors determine variations in the θ_c : chemical composition and surface morphology. The observed variations in θ_c may thus have been caused by changes to both the morphology and the composition [13] upon incorporation of MB into the NR.

Fig. 1 UV–Vis spectra of MB solution and NR and NR+MB membranes. The inset shows the membranes based on NR and NR+MB

Table 1 Values of contact angle (θ_c), roughness (W), fractal dimension (DF), and roughness exponent (α)

Membrane	θ_c	W (nm)	df	α
NR	$53.4^\circ \pm 5.6$	10.2	2.19	0.81
NR+MB	$18.7^\circ \pm 3.0$	84.7	2.25	0.75

3.3 Morphological analysis

Figure 2 shows images of the membranes along with their height profiles. NR+MB was composed of aggregates, as indicated by the results of UV–Vis spectroscopy (Fig. 1).

Studies suggest a strong correlation between the roughness and the wettability of a surface [30]. To establish a relationship between the morphology and the wettability of the membranes, the fractal dimension and roughness were analyzed together with the contact angle (Table 1). The roughness values were obtained using the microscope software NanoReport from Nanosurf (Switzerland). The roughness of the latex membrane increased upon deposition of MB.

3.4 Antifungal susceptibility

Tests for the inactivation of *C. albicans* were performed using the different membranes with and without laser irradiation. It can be seen that the NR membrane did not inhibit cell growth (Fig. 3a) even after irradiation (Fig. 3b). For the NR+MB membranes without irradiation (Fig. 3c), no fungal inactivation was observed. However, in the presence

of laser irradiation (Fig. 3d), the NR+MB membranes exhibited zones of inhibition. The results were similar for freshly prepared membranes and those that had been stored for 12 months.

Figure 4a shows the percentage of *C. albicans* that were viable as a function of different membranes with and without laser irradiation. In the control groups, we see that *C. albicans* is not susceptible to irradiation with a laser (30 J/cm^2) without the presence of MB. Although inactivation was not fully achieved, the cell viability of the fungus was significantly reduced in the presence of the NR + MB + laser irradiation (30 J/cm^2). Fluorescence microscopy (Fig. 4b) associated with appropriate markers can be used to evaluate cell viability. The vital dye, acridine orange, enters cells, mixes with intact DNA, and fluoresces green; if damaged, the cells appear orange-red [24]. The images confirm the susceptibility results presented in Fig. 4a. With the filters used, autofluorescence of *Candida albicans* and methylene blue is not verified. The corresponding images are presented in the supplementary material.

4 Discussion

When molecules from the dye solution are transferred to a membrane surface, aggregation can be observed through an analysis of displacements or the appearance of new peaks in the UV/Vis spectra [31]. For the NR + MB membrane, it was verified that the monomer-related peak was not displaced, but a new peak appeared at 600 nm which

Fig. 2 AFM images of NR (left) and NR+MB (right) membranes. Height profiles were obtained from AFM images

Fig. 3 **a** NR membrane and **b** NR membrane with 30 J/cm² irradiation, **c** NR+MB membrane and **d** NR+MB membrane with 30 J/cm² irradiation

Fig. 4 **a** Cell viability of *C. albicans* dispersion. The control group is composed of an analysis of the dispersion of *C. albicans*+NR; irradiated *C. albicans*+NR (30 J/cm²); *C. albicans*+NR+MB (nonirradiated). The other results relate to the mixing of the *C. albicans*+NR+MB irradiated (30 J/cm²). Error bars represent the standard deviation. For each group 3 samples were analyzed and at least three measurements were taken. **b** Fluorescence microscopy of *C. albicans* dispersion. The dimension of the scale bar is 50 μ m

was related to the formation of dimers during aggregation [31, 32]. Aggregation can occur due to the high concentration of PS or the porosity of the membrane, which allows diffusion of the dye into the matrix volume [6, 33]. We recognize that the difference in energy excitation and geometry of monomers and aggregates must be considered when assessing the efficiency of the PS. However, studies have shown that MB dimers contribute to the type I and type II mechanisms of the photosensitization and

photodestruction process of microorganisms [34, 35]. From our results, it is possible to assume that the presence of dimers should not be responsible for the decrease in PS efficiency. This question could be better answered, if the effect of different irradiation wavelengths were analyzed on the susceptibility of microorganisms. But these experiments were not conducted in this work.

Wettability is an important property when a surface is to be used for biomedical applications such as implants, sensors, or dressings, because the affinity of hydrophilic surfaces for the medium is generally stronger than that of hydrophobic surfaces [36, 37]. For example, higher wettability facilitates a stronger interaction between the surface of the dressing and the injured tissue. Increased wettability is an advantageous characteristic for dressings since these surfaces exhibit poor cellular adhesion to the new skin that is being formed during the cicatrization process; thus, using materials with higher wettability can minimize the pain and discomfort experienced during removal of the dressing [6, 38].

The relation between α and df of a surface can be obtained by: $df = D - \alpha$, where D is the dimension of the space observed [39]. The interface-forming process that determines the surface morphology can be investigated using fractal concepts and dynamic-scale laws [39, 40], which can be done by analyzing the log–log dependency of W as a function of the scan window length (L). In the linear region, wherein the laws of scale are satisfied, the value of the angular coefficient gives α [39]. df values of ~ 2 are consistent with quasi-Euclidean surfaces associated with relatively flat interfaces [39]. It is reasonable to assume that a rearrangement of the molecules may occur in the NR+MB membranes which minimizes the irregularities resulting from the random formation process of the surface, creating a less fractal structure.

W is determined by differences in height between the different points of the surface, whereas fractality is related to the way the roughness changes when the scale of observation changes [39]. Therefore, although the NR + MB membrane surface was rougher, the fractality was similar to that of the NR membranes. This can be clarified by analyzing the height profiles (Fig. 2). Since the height scale is the same, it is noted that at the same scanning windows ($L = 5 \mu\text{m}$), the cross section of the NR + MB membrane was equally as fractal as the NR membrane, but the height variation between the peaks and valleys corresponding to the roughness was much larger for the NR + MB membrane.

Antifungal tests indicated that even with aging (Fig. 3), the NR + MB membrane did not lose its ability to inhibit cell growth. The result of irradiation (30 J/cm^2) of *C. albicans* dispersion mixed with NR + MB showed that cell viability was reduced ($\sim 70\%$), confirming that membranes with the incorporation of the photosensitizer MB is effective for the photoinactivation of *C. albicans* (Fig. 4). The fluorescence images confirm the susceptibility results and indicate that the irradiation process had a fungicidal effect on *C. albicans*, although inactivation was not fully achieved with the parameters used. This result indicates that latex membranes incorporating MB can be used as antimicrobial dressings for the treatment of burns. These membranes are promising candidate materials for burn dressings since NR promotes angiogenesis and the combination of MB and laser irradiation exerts antimicrobial activity. As far as we know, this is the first time that an antimicrobial PDT is proposed using NR membranes incorporating a photosensitizer dye.

5 Conclusion

The incorporation of MB in latex membranes and the resultant changes were evaluated, opening opportunities to study the use of such materials as curative membranes with antimicrobial properties which are activated by light.

The deposition of MB in the latex membranes was responsible for increasing both surface roughness and wettability. It is suggested that for membranes with quasi-Euclidean surfaces, the adhesion between human skin and the dressing should be limited such that the membrane interacts with the injured tissue without excessive adhesion, thus minimizing the pain experienced during removal of the dressing. Antibacterial PDT tests indicated that the latex membrane incorporating MB was effective in inactivating *C. albicans*, which was used as a model microorganism. These results provide a starting point for research into curative membranes employing antimicrobial PDT.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s43630-021-00077-z>.

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Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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