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RESEARCH ARTICLE

Ozone as a method for decontamination of dissolving microneedles for clinical use

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Abstract

Dissolving microneedles (DMNs) is a promising technology for transdermal and intradermal drug delivery. However, effective decontamination protocols are necessary to ensure safety and efficacy in clinical applications. The challenge is to use a technique that preserves mechanical properties, does not introduce chemicals, and can decontaminate DMNs without affecting the drug. With its potent antimicrobial properties and minimal residual effects, ozone presents a novel and safe method for decontaminating DMNs. Specifically, the present study assesses ozone's efficacy in decontaminating DMNs loaded with aminolevulic acid, intended for photodynamic therapy in skin cancer treatment. The results showed that it effectively decontaminates *E. coli* and *S. aureus* without compromising the polymer properties or promoting drug degradation. Overall, ozone represents an approach that can be adopted to decontaminate DMNs, offering a safer and effective strategy that enhances their potential to translate to clinical application.

KEYWORDS

Aminolevulinic acid, decontamination, dissolving microneedles, ozone, polymer

Abbreviations: ACM, acetaminophen; ALA, aminolevulinic acid; BHI, brain heart infusion; DMNs, dissolving microneedles; FDA, Food and Drug Administration; GMP, Good Manufacturing Practice; ITO, indium-tin oxide; LNNano, Brazilian Nanotechnology National Laboratory; MNs, microneedles; O_3, ozone; PDMS, poly(dimethylsiloxane); PDT, photodynamic therapy; PLGA, poly(lactic-co-glycolic acid); PS, photosensitizer; TSB, tryptic soy broth; USP, University of São Paulo.

Michelle B. Requena and Thaila Q. Corrêa contributed equally to this study.

INTRODUCTION

Microneedles (MNs) are minimally invasive devices widely explored for drug delivery. They can penetrate the skin by crossing the stratum corneum without causing pain or bleeding since they do not reach nerve termination and blood vessels. Even though relying on established methodologies, MNs are constantly explored in pharmaceutical and biomedical fields because their applications are continuously developing.[1] There are different types of polymeric MNs, such as solid, hollow, hydrogel, or dissolving, that can also be coated with the drug. The delivery method differs depending on the type.[2] For dissolving microneedles (DMNs) expressly, the drug is incorporated in the polymeric formulation, allowing the dissolution after piercing the skin. For the DMNs, non-toxicity criteria must be fulfilled.

It is well established that MNs offer low risk to patients concerning microbial contamination levels; however, considerations must be made in the sterile manufacture or use of sterilization methods to guarantee a safe application following regulatory agencies' recommendations. The gamma radiation method is more commonly used; nevertheless, it can promote degradation effects on polymers,[3] decreasing purity and increasing degradation products in some drugs.[4]

It is possible to sterilize the molds and implement Good Manufacturing Practice (GMP) standards in the processes; however, it is challenging to guarantee a sterile manipulation process and characterize the desired quality. Due to their high aqueous solubility, DMNs cannot be decontaminated after fabrication by most chemical agents, and some drugs cannot withstand high temperatures, which makes autoclaves not a viable option. Chemicals that are suitable agents for microbial control may be incorporated into the polymer with subsequent reactions after application in the patient. Also, a terminal sterilization method is preferable in the pharmaceutics industry rather than relying on aseptic procedures in manufacturing.[4] Decontamination with immediate packaging certainly is the most desired situation.

The decontamination process must be carefully designed to minimize any potential impact on the drug stability within the DMNs. Degradation of the drug could render it ineffective or even harmful to patients. Regulatory agencies, such as the Food and Drug Administration (FDA) require that medical devices, including DMNs, meet stringent safety, efficacy, and quality standards. All decontamination methods must comply with these regulatory requirements and be validated to demonstrate their effectiveness without compromising the drug. Therefore, it is essential to establish a straightforward and effective decontamination protocol that minimally interferes with DMNs strength, allowing safe translation to clinical studies.

With all the considered requirements, ozone (O_3) is widely recognized as one of the fastest and most effective microbicidal agents for bacteria and viruses. Its oxidative properties primarily target lipids, proteins, and amino acids while displaying aggression for the genetic material. The use of ozone for decontamination has recently been explored for medical and nonmedical devices.[5] Rediguieri et al. demonstrated ozone application as a safe sterilization method for poly(lactic-co-glycolic acid) (PLGA) nanofiber scaffolds, successfully preserving all the inherent properties of non-sterilized PLGA nanofibers.[6]

Our traditional studies focus on developing photodynamic therapy (PDT) applications for treating superficial skin cancer lesions using topical drugs. Topical PDT is based on the interaction of a photosensitizer (PS) molecule (accumulated by using a cream containing PS precursors) activated by light at a precise wavelength within the absorption spectrum of the PS in the molecular oxygen presence, which will induce the target cells to death.[7] PDT is an alternative technique for non-melanoma skin cancer treatment explored around the world.[8] However, one of the main PDT limitations is poor cream penetration into lesions, causing prolonged incubation time and poor in-depth drug delivery that can lead to increased lesion recurrence. The use of DMNs allowed the achievement of deeper layers in tumors, overcoming limitations in topical application.[9]

As a proof of concept, DMNs containing aminolevulinic acid (ALA) and Gantrez AN-139 as the polymer were submitted to the ozone decontamination process. Gantrez is a copolymer of methyl-vinyl-ether and maleic anhydride and its derivative forms, whose molecules are biodegradable with low toxicity, high biocompatibility, and bioadhesive properties.[10,11] ALA molecule is described as sensitive to pH, concentration, temperature, and oxygenation level in aqueous solution,[12] limiting the option of sterilization methods. This study evaluated the potential of ozone as a decontamination method for DMNs and its effects on the polymer and drug properties. In what follows, we describe the methodology employed and the results obtained.

MATERIALS AND METHODS

Dissolving microneedles

Gantrez AN-139 (Ashland, UK) at 30% w/w stock was prepared as previously described[13,14] and used at 20% w/w concentration to fabricate the DMNs. ALA (EmiPharma, Embrapii Program, Brazil) powder was used at 5% w/w in

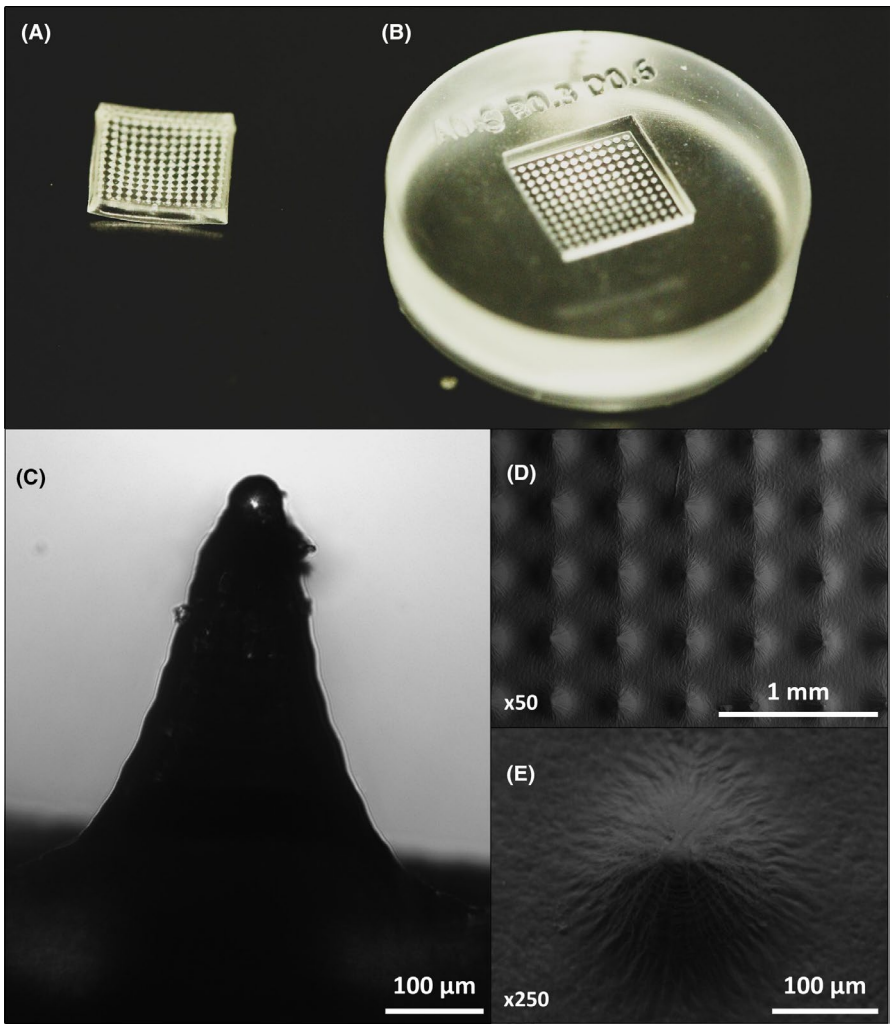

FIGURE 1 (A) DMNs array with a conical profile, (B) PDMS mold made, (C) microscopic image from one tip in the array, (D, E) scanning electron microscopy (SEM) images of a DMNs with a magnification of 50x and 250x, respectively.

the DMNs or film forms.[9] The fabrication process did not follow any sterile procedures.

The molds used for MNs were fabricated with the Brazilian Nanotechnology National Laboratory (LNNano) in the Brazilian Center for Research in Energy and Materials (CNPEM). The master template was printed using 3D (Form 2 model, FormLabs, USA) stereolithography. The molds (Figure 1A) were made of poly(dimethylsiloxane) (PDMS), which is a silicone composed of a mixture of polymers. Approximately, 0.1 g of the formulation was deposited in the molds and placed in a pressurized chamber (VCR Equipamentos, Brazil) to reduce bubble formation and fill the holes. The pressure was increased to 4 bar and maintained for 5 min, repeated after slow depressurization. The molds containing only polymer were dried at room temperature for 48 h. DMNs containing 5% ALA were dried in a refrigerated incubator for 72 h at 25°C. Then, the arrays were removed from the molds and stored in the fridge, protected from humidity and light, in sealed aluminum packs until the experiments.

The films were produced using the same protocol as DMNs. Drops of each formulation were placed on a silicon plate. After drying, the films (2–3 mm diameter) were removed and sealed in Petri dishes. These films were used for disk diffusion microbiologic tests and MALDI-TOF measurements.

Ozone decontamination

An ozone chamber prototype was employed for decontamination. This device was developed at the São Carlos Institute of Physics at the University of São Paulo (USP) and has been used for multiple purposes. It comprises an ozone generator with a 10 g/h production capacity. For the experiments, two sequence cycles were carried out; each cycle was composed of 5 min of low vacuum, 1.5 min of ozone release into the samples, 1.5 min of ozone release out of the chamber, and 3 min to depressurize with purified air.

Microbiological assays

For microbiological tests, brain heart infusion (BHI) media (KASVI, Spain), and tryptic soy broth (TSB) (KASVI, Italy) were prepared according to the manufacturer's protocol. The tests were carried out with the microorganisms *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) as examples of Gram-positive and Gram-negative strains, respectively. Bacteria from a frozen stock were reactivated in a Petri dish containing BHI as a culture medium for 48 h at 37°C. After growth, eight colonies of each bacterium were transferred to Falcon tubes containing 10 mL of TSB to obtain the pre-inoculums, which were incubated for 18 h at 37°C. After incubation, the inocula were prepared by transferring 0.5 mL of the pre-inocula to Falcon tubes containing 9.5 mL of fresh TSB medium. Then, *S. aureus* and *E. coli* inoculums were incubated for 4 h at 37°C for the bacteria to reach the mid-log growth phase. The bacterial inocula were adjusted between 10^7 and 10^8 colony-forming units per milliliter (CFU/mL) in an absorption spectrophotometer (UV-M51 UV–Visible Spectrophotometer, Bel Engineering, Italy) at 600 nm wavelength. For the nebulization, 10 mL of inoculum solutions were placed in a commercial nebulizer (Omron, Brazil) that was nebulized for 5 min into the plates, depositing a good quantity of microorganisms at the surface.

Decontamination test

Considering the contamination experiments, a DMN array was placed in the center of each plate before nebulization with bacteria. The control condition did not receive O_3, while the treatment condition was subjected to the decontamination protocol. Then, the plates were placed in an incubator to evaluate the bacteria growth in 24 h. This condition was tested in triplicate (Figure 2A,B and A′B′). This test was conducted to assess the ability of ozone to decontaminate the array after receiving a large concentration of bacteria.

Disk diffusion test

The disk diffusion test, also known as the Kirby-Bauer test, was utilized to determine whether the presence of the polymer influenced bacterial growth. Three small

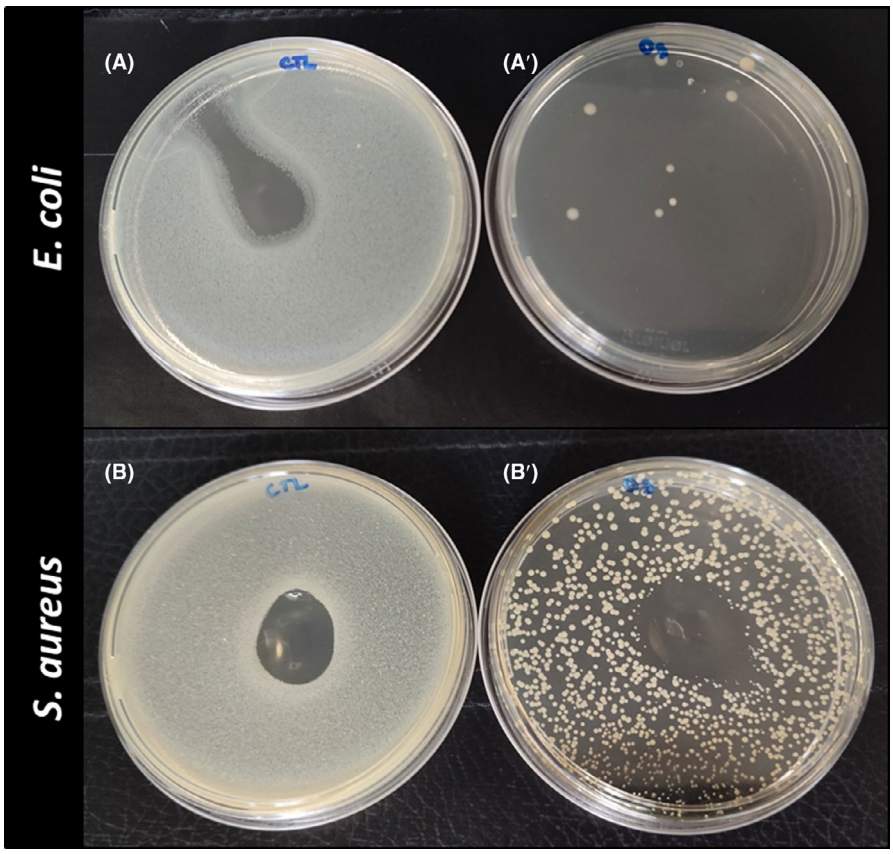

FIGURE 2 Representative images from 24 h after experiments using DMNs arrays were placed on the center of Petri dishes before nebulization with two types of bacteria, *S. aureus and E. coli*. Plates (A) and (B) served as controls and did not receive ozone treatment, whereas plates (A′) and (B′) were exposed to ozone. All conditions were tested in triplicate in different Petri dishes.

films (to avoid flowing) containing only Gantrez AN-139 at a concentration of 20% w/w were placed on plates previously contaminated with bacteria via nebulization. To assess the effect at lower polymer concentrations, 10 μL of a solution containing a single DMN array dissolved in 1 mL of PBS was applied. The plates were then incubated for 24 h for analysis.

Swab test

Since the materials and manufacturing process are not sterile, three DMNs arrays were subjected to a 30-s rubbing action with a dry swab or a wet PBS swab to detect the presence of intrinsic contamination. Each swab was then applied to a culture plate and incubated for 24 h. This experiment was performed in triplicate.

Insertion capability

This experiment was performed to assess the influence of the decontamination method on the mechanical properties and insertion characteristics of the DMNs. A texture analyzer (Stable Micro Systems Ltd., UK) was used to press the DMNs arrays into parafilm layers, following the artificial membrane model for insertion described by Larraneta et al.,[15] in which a force of 32 N is applied in the array for 30 s. For this test, a 20 mm diameter acrylic probe (TA-520) was used to attach the arrays using double-sided adhesive tape. Then, the parafilm layers underwent visualization after applying compression load utilizing a light microscope. A comparison between samples exposed or not to ozone was performed.

MALDI-TOF

MALDI mass measurements were acquired in positive-ion reflectron mode with an AutoFlex-Max (TOF/TOF) instrument (Bruker Daltonics, Bremen, Germany) equipped with a 355 nm and 2 kHz Nd: YAG laser source. Spectra were generated by laser shooting at random positions with continuous accumulations summing 2000 single spectra over 40–500 Da mass. To avoid analyte fragmentation, the laser power was adjusted slightly above the desorption/

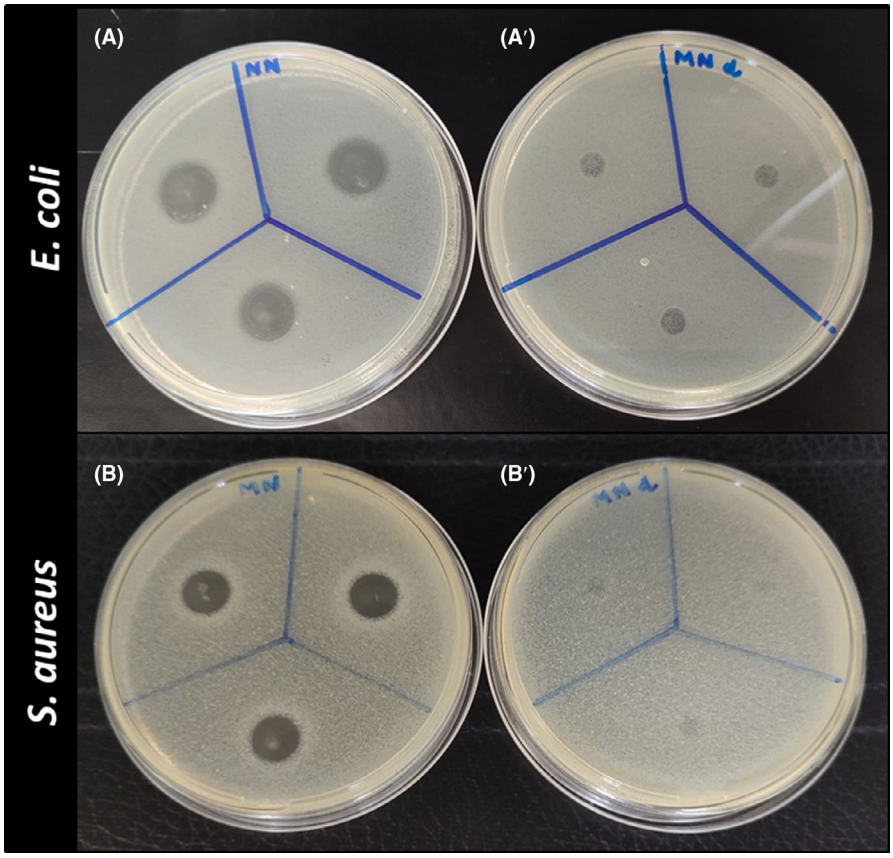

FIGURE 3 The plates were previously contaminated with bacteria by nebulization with *S. aureus* and *E. coli*. Images (A, B) and (A′, B′) show the results 24 h after the film was placed or 10 μL of a DMN dissolved in 1 mL of PBS was applied after nebulization, respectively. The lines limited the triplicate of sample positioning.

ionization threshold, and the mass spectra were typically recorded at accelerating and reflectron voltage of 19 kV and 21 kV, respectively. The mass spectra were processed by Flex Analysis software (Bruker Daltonics, Bremen, Germany), where external calibration was conducted using an antibiotic standard mixture (MBT STAR-ACS, Bruker).

The ALA sample solution was mixed 1:1 (v/v) with 10 mg/mL HCCA matrix (α-a-Cyano-4-hydroxycinnamic acid, Bruker) and prepared in acetone for MALDI-TOF analysis. The mix of sample solution and matrix (1 μL) was spotted onto the MALDI target plate and air-dried for mass spectrometry (MS) analysis.

The concentration of ALA was determined by internal standard calibration using acetaminophen (ACM) as internal standard at different concentrations 5, 10, 25, 30, and 40 mg/mL. For MS imaging, the films were placed in ITO slide glass sample plates (glass slides coated with indium-tin oxide to provide a conductive surface) and marked for MALDI imaging (Figure 6A). The key parameters and calibration used for ImagePrep were the same as described above. The MS imaging (MSI) was operated in the positive mode, and MSI spatial resolution was set to 50 μm. Each pixel was accumulated of 200 laser shots. The imaging data were acquired and processed using FlexImaging 3.0 (Bruker Daltonics, Bremen, Germany).

These experiments were replicated in 2 days. Films not exposed to the decontamination process were used as a control.

RESULTS AND DISCUSSION

Figure 1 contains representative images of a DMNs array (13 × 13), the mold used, microscopic images illustrating the conical profile, 500 μm high, and 300 μm of spacing.

According to the results observed in Figure 2, the plates submitted to the decontamination process (A′, B′) presented a visual reduction in the number of colonies compared with the control condition (A, B). Considering that the nebulization was performed using the inoculum in high concentration (between 10^7 and 10^8 UFC/mL), it is expected to have remaining bacteria. Nevertheless, in both scenarios, the area where the DMNs arrays were positioned and contaminated showed an absence of bacteria, including *S. aureus*. Besides that even in the control condition, no bacteria were observed in the position of the DMNs, which may be related to antimicrobial properties from Gantrez AN-139[16] or associated with bacteria due to not having appropriate conditions to survive in the polymer. The inhibition observed also can be related to the acidic nature of the polymer due to the leaching of Gantrez AN or H^+ ions. This effect was reported for *S. aureus* by Caló et al. (2016), considering that it interferes with its gene expression, creating a hostile environment for its growth.[17]

After 24 h, bacterial growth was also inhibited, mainly under conditions in which polymeric films were positioned directly on the contaminated plate (Figure 3A,B). In the case of applying the solution with the DMN dissolved in PBS (Figure 3A'B'), the lower concentration of the polymer in the dilution probably reduced this effect. For this experiment, three samples were used per dish.

Specifically, for the experiment to verify intrinsic contamination from the manufacturer, only four colonies were observed across the six plates: three from the dry swab and one from the wet swab (no images presented). This outcome aligns with existing literature, suggesting minimal contamination in polymeric microneedles.[18] It may be attributed to the polymer's inherent inhospitality to bacterial growth, even without an explicit antibacterial effect.

Although our experiments demonstrated the potential of using bacteria for decontamination, literature provides substantial evidence of ozone's effectiveness in inactivating various viruses. This highlights its potential application in sanitizing medical facilities to enhance infection control and patient safety.[19] Additionally, evidence supports that ozonation is an effective method for reducing microbial load in the air, suggesting its utility in improving air quality and reducing the risk of airborne infections, considering both bacteria and fungus.[20]

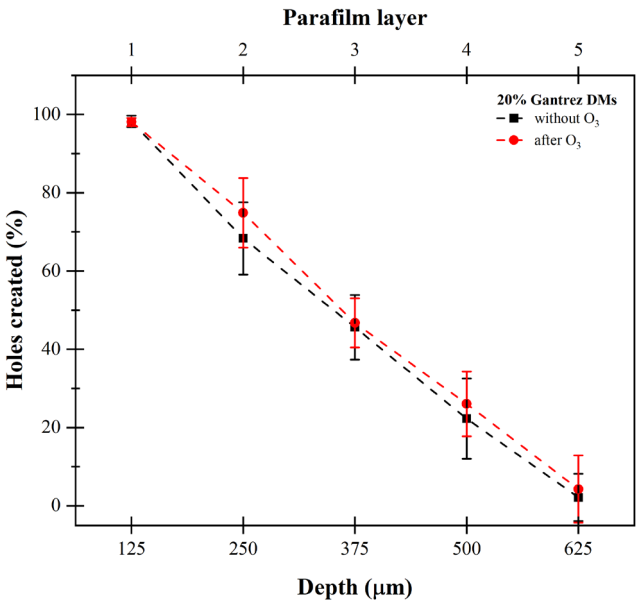

FIGURE 4 Percentage of holes created with the DMNs using the parafilm as an artificial membrane to estimate the insertion capability.

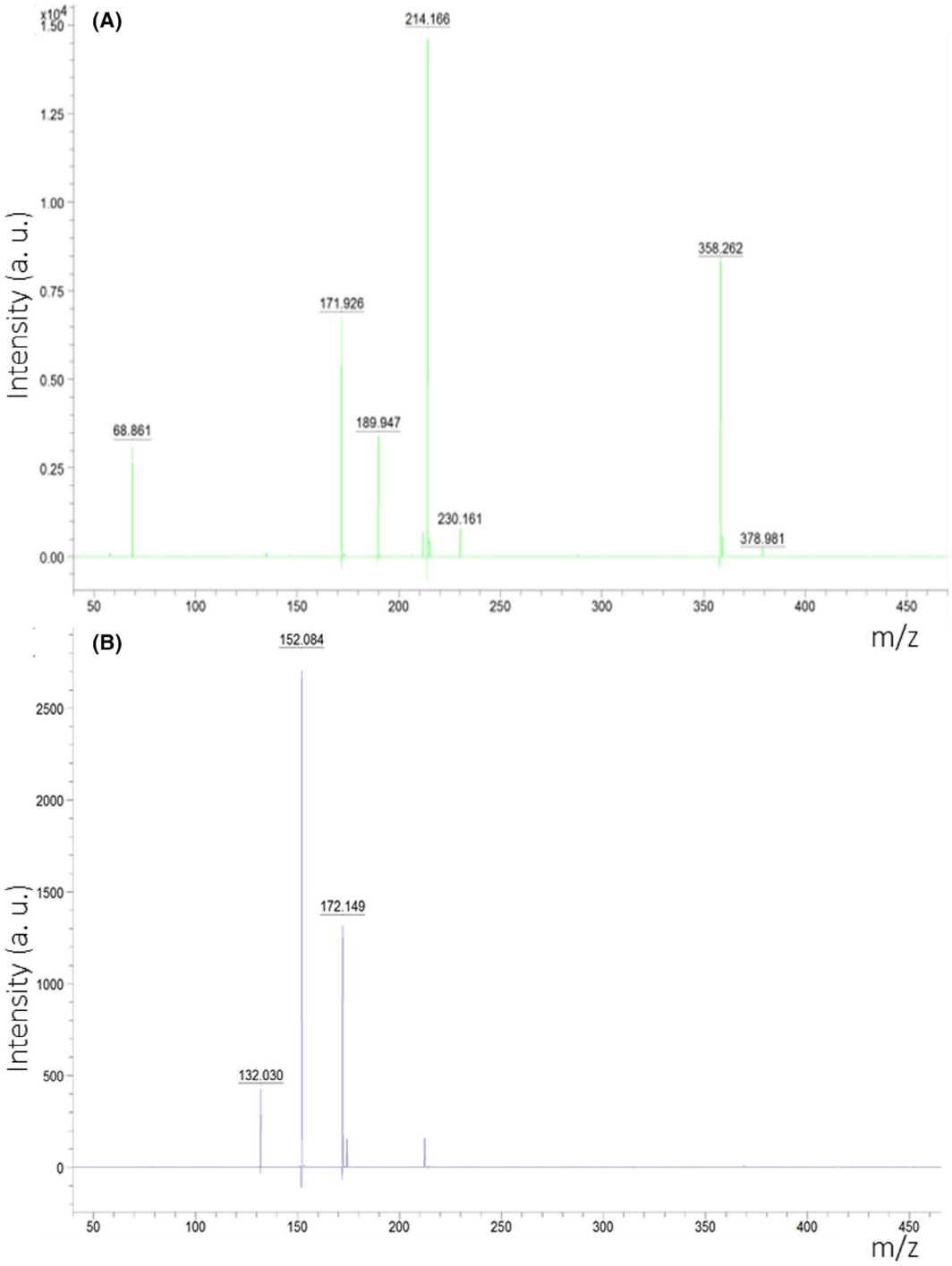

FIGURE 5 Mass spectra for (A) Gantrez AN-139 and (B) ALA ($m/z = 132$) with Gantrez AN-139.

Regarding mechanical properties, the polymer may be most affected by exposure to O_3. The experiments (Figure 4) showed that the same penetration characteristics were observed for the DMNs samples, exposed, and non-exposed. These results demonstrate that the polymer is not intrinsically affected by mechanical properties.

Even though ALA is a molecule that can be combined with other polymers,[21,22] their interaction results in differences in the drug's stability, biocompatibility, and controlled release kinetics. Considering the MALDI-TOF mass spectra from films containing only the polymer (Figure 5A) and polymer with ALA (Figure 5B), ALA ($m/z = 132.030$) can be detected even when dissolved in the polymeric formulation, indicating that the molecule did not form aggregates with Gantrez AN-139, so it can be identified using this technique. Maintaining a drug that does not aggregate within a polymer matrix helps achieve

uniform distribution and enables precise control over drug delivery, preserving chemical integrity.

Films containing ALA were exposed to ozone to verify eventual variations of chemical content. A calibration curve determined the ALA concentration before and after decontamination. ACM ($m/z = 152.084$) was used as an internal standard at 20 μg/mL concentration. The ratio between the respective mass peaks (ALA/ACM) was considered (132/152). The calibration curve was linear in the concentration range with mean correlation coefficients ($R^2 = 0.99697$). ALA concentration in the films submitted or not to ozone does not present significant differences, indicating that the method did not cause drug degradation. Figure 6 contains the ALA concentration estimated after the ratio comparison for each sample. There was no difference between ALA concentration before and after decontamination with ozone.

Figure 7 shows the positioning of films containing ALA and its distribution acquired by MALDI-TOF imaging. The colored images were performed by selecting the ALA mass peak ($m/z = 132.030$). According to the color scale bar, the pinkest color in the image reflects regions that contain closer to 100% of ALA. This means that even when using ozone in decontamination (Figure 7C), it is possible to observe a homogeneous composition of ALA in films with slight intensity variation.

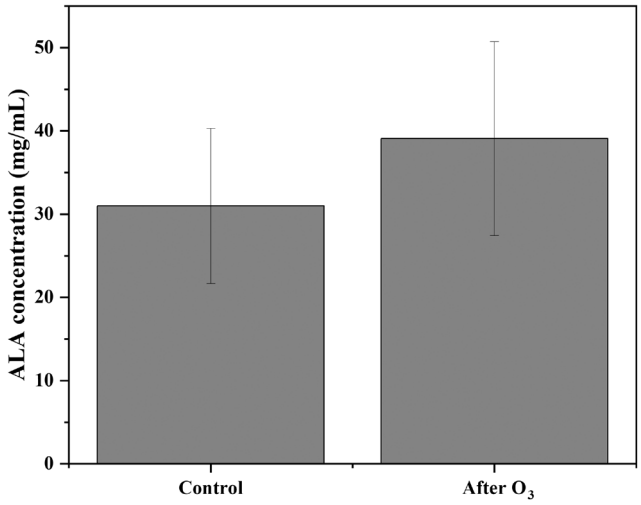

FIGURE 6 ALA concentration was estimated using MALDI-TOF spectra following internal standard calibration with acetaminophen. The mean concentration was calculated based on two measurements per film, with three films analyzed per day. The error bars represent the standard deviation of these measurements.

The regulatory requirements for microneedle fabrication remain ambiguous. They are positioned between transdermal patches produced in low-bioburden environments and intradermal injection technologies requiring aseptic conditions or terminal sterilization, which make manufacturers consider aseptic processes, low-bioburden with terminal sterilization, or low-bioburden-only approaches. Challenges arise from most active pharmaceutical ingredients that may not tolerate terminal sterilization, necessitating costly aseptic production unless developers can justify a lower-cost, low-bioburden method with sufficient safety assurances to regulators. Aseptic manufacturing introduces complexities such as stringent sterility requirements for materials and increased monitoring; it likely increases manufacturing expenses and requires substantial investment for scale-up and production readiness. Additionally, the drying process for DMNs presents specific challenges under aseptic conditions since they usually require longer drying times.[23] Irie et al. also reported that drying was a significant design obstacle, underscoring the need for continued research and development efforts.[23]

In this context, ozone's antimicrobial properties have been recognized for decades; its primary use has been removing or breaking down pollutants in waste and drinking water. Its application for decontaminating medical and nonmedical devices is relatively recent and remains underexplored. Moreover, ozone production costs have decreased substantially over the past two decades, facilitating new developments in laboratory and industrial settings. Despite these advancements, most developments have been at a small scale, and there are significant knowledge gaps regarding implementing ozone decontamination systems on a larger scale.[5]

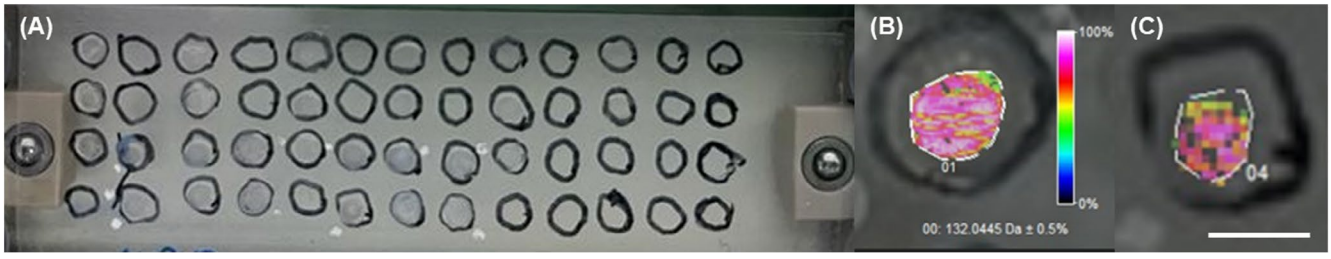

FIGURE 7 (A) Films positioned on ITO slide glass sample plates (A); MALDI-TOF imaging showing the distribution of ALA ($m/z = 132.030$) in the films (B) without ozone and (C) after ozone decontamination. The white scale bar at the bottom right represents 2 mm.

Although we have demonstrated the validity of DMN decontamination using the ozone method, exploring the different possibilities for parameter variations is necessary. Excessive and prolonged exposure to ozone leads to the gradual degradation of the polymer, resulting in the deterioration of its properties, which can affect the integrity of the ALA molecules. These extreme situations need to be elucidated and studied to have a safe window of parameters involving the relationship between ozone time exposure and the partial pressure used. In such studies, we can optimize the process, preserving desired characteristics. The method can be more widely usable by obtaining an adequate balance between decontamination and preserving mechanical–chemical properties. Furthermore, different polymers must be tested.

CONCLUSION

In conclusion, while decontamination protocols are essential for maintaining the safety and integrity of dissolving microneedles, ensuring that these protocols do not degrade the polymers or drugs they deliver is equally vital. In this work, a proof of principle was carried out using ozone. While the results appear promising and create real possibilities for large-scale use, it is important to explore conditions where this can be used. Insufficient exposure fails to guarantee effective decontamination, while excessive exposure risks compromising material properties. The exploration of optimization and compromise between desired characteristics is now our action plan.

AUTHOR CONTRIBUTIONS

Michelle B. Requena and Thaila Q. Corrêa participated equally in the paper's conception, design, experiments, analysis, data interpretation, and drafting. Dianeth Sara L. Bejar participated in the article's conception, experiments, data interpretation, and drafting. Juliana C. Barreiro and Kelly T. Cristina contributed to experiments and data discussion obtained from MALDI-TOF tests and SEM, respectively. Vanderlei S. Bagnato participated in the conception, analysis, data interpretation, and discussion. All authors have revised it critically for intellectual content and approved publishing the final version.

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CONFLICT OF INTEREST STATEMENT

The authors report that there are no competing interests to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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