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Superficial Distribution and Identification of Antifungal/Antimicrobial Agents on a Modified Tissue Conditioner by SEM-EDS Microanalysis: A Preliminary Study

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Abstract

Purpose: This study evaluated the incorporation pattern of antifungal/antimicrobial agents added to a tissue conditioner by scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDS) analysis.

Materials and Methods: The nystatin dosages incorporated into the tissue conditioner (Softone, Bosworth Co., Skokie, IL) powder were 500,000 U (G1) and 1,000,000 U (G2). The addition of miconazole was at 125 mg (G3) and 250 mg (G4), and ketoconazole was at 100 mg (G5) and 200 mg (G6). Chlorhexidine diacetate was blended at levels of 5% (G7) and 10% (G8) w/w of the total amount (6.35 g) of the tissue conditioner. The drug powder concentrations were blended with the tissue conditioner powder at different concentrations before the addition of the tissue conditioner liquid (5 mL) to the mixture. One group (G0) without any drug incorporation was used as control. Specimens ($n = 5$) ($36 \times 7 \times 6$ mm^3) were plasticized at room temperature for 10 minutes and carbon sputter coated. All specimens were submitted to SEM-EDS analysis.

Results: Nystatin and miconazole specimens exhibited particles with irregular shapes and sizes uniformly distributed. Ketoconazole specimens showed small spherical particles with a slight distribution throughout the matrix. Chlorhexidine specimens exhibited irregular particles up to approximately 50 μm in size randomly dispersed within the matrix.

Conclusions: Within the limitations of this in vitro study, the modified tissue conditioner showed differences in the particle distribution and size of the antifungal/antimicrobial agent added to the plasticized matrix. Further studies would discriminate the most important particle features that may influence the drug leaching from the plasticized matrix.

The usual treatment of denture stomatitis comprises the elimination of the infectious agent and its sources from the oral tissues by antifungal therapy, denture cleaning, and disinfection.[1,2] Three main groups of antifungal drugs are used to reduce the acute overgrowth of *Candida* species: the polyenes (amphotericin B and nystatin), the azoles (miconazole, ketoconazole, fluconazole, and itraconazole), and the antiseptics, such as chlorhexidine gluconate; however, *Candida*-induced denture stomatitis is most commonly treated with polyene antifungal agents, which are applied to the infected region and may be applied to the intaglio denture surface before insertion in the mouth.[3] Chlorhexidine is used as an adjunctive therapeutic supplement due to its antimicrobial activity against a broad spectrum of microorganisms, in which *Candida* ssp. is included.[4-6]

In addition, tissue conditioners are commonly used to ensure the recovery of denture-bearing tissues from trauma usually caused by ill-fitting dentures; however, tissue conditioners are easily degradable and susceptible to colonization by microorganisms,[7] which may cause denture-induced

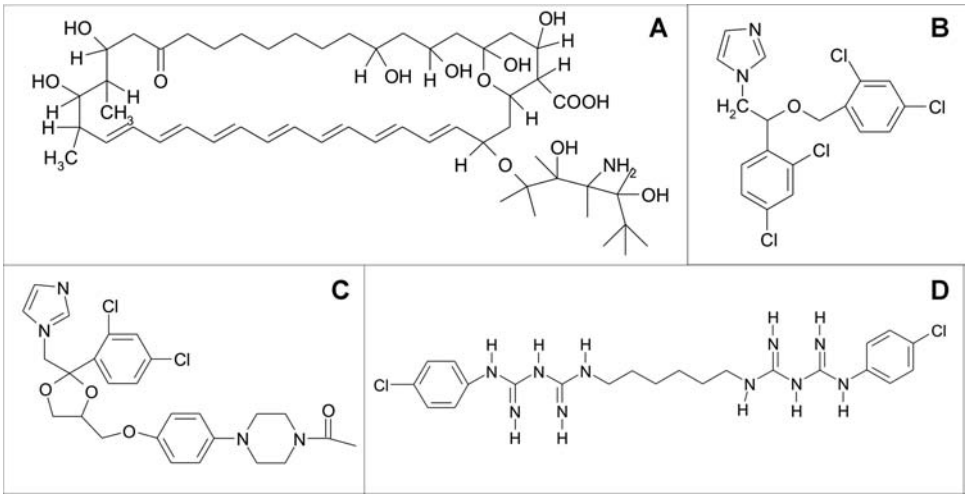

Figure 1 Chemical structure of antifungal/antimicrobial agents. (A) nystatin, (B) miconazole, (C) ketoconazole, (D) chlorhexidine.

stomatitis.[8-11] Therefore, some authors have proposed methods such as the incorporation of antifungal or antimicrobial agents into the material to prevent such colonization on soft liners.[12-15]

The major advantages of adding antifungal agents to tissue conditioners as a method of drug delivery are (1) reduced cost for the patient considering that only a fraction of the antifungal agent is used when compared to conventional therapy, (2) no need for patient compliance,[16] (3) simultaneous treatment of injured denture-bearing tissues and candidal infection,[17] and (4) reduced application frequency.[12] In addition, conventional therapies are difficult to add to in institutionalized and palliative care settings, as many patients have memory loss as well as reduced cognitive control and motor dexterity, which may lead patients to depend on caregivers to follow drug administration instructions.

Despite these therapeutic advantages, drug incorporation can affect the structural properties,[18] increase tensile strength,[12] and compromise the hardness and elastic modulus[18,19] of several polymeric/plasticized materials, including tissue conditioners. Furthermore, changes in surface roughness caused by drug incorporation may allow bacterial and yeast colonization on the tissue conditioner surface.

Although the effects of drug incorporation on the physical and mechanical properties of tissue conditioners have been studied,[12,18,19] further information regarding drug dispersion into the tissue conditioner is required. Brook and van Noort[20] reported that drug loading, particle size, and dispersion are important factors affecting drug release. Therefore, the aim of this study was to evaluate the incorporation pattern of some antifungal/antimicrobial agents into a tissue conditioner using scanning electron microscopy (SEM). Energy dispersive X-ray spectroscopy (EDS) was also used to identify the antifungal, antimicrobial, and tissue conditioner components. The working hypothesis was that the incorporation of antifungal agents at different concentrations might promote changes in morphological features of tissue conditioner surfaces.

Materials and methods

The tissue conditioner selected for this study was Softone (Bosworth Co., Skokie, IL, batch number, powder: 0401-031; liquid: 0401-048-X), which is presented as powder and liquid. The polymer powder consists of polyethyl methacrylate, and the liquid has an ester-based plasticizer and ethyl alcohol, which acts as a penetrant.

One group (G0 = drug-free specimens) without any drug incorporation was used as control group. Three antifungal agents (Fig 1): nystatin (Purifarma, Sao Paulo, Brazil, 05520304), miconazole (SP-Farma, Sao Paulo, Brazil, MCN/062/2003-1), and ketoconazole (Galena, Campinas, Brazil, KT-16703); and one antimicrobial agent (chlorhexidine diacetate; Acros Organics, Morris Plains, NJ, A012105001) were added to the tissue conditioner in two concentrations.

The antifungal/antimicrobial powders (Table 1) were uniformly blended into 6.35 g of tissue conditioner powder by hand in a vessel, and 5 mL of the tissue conditioner liquid was added to the blended powder according to the manufacturer's instructions. The mixed material was packed in Teflon molds

Table 1 Drug dosages incorporated into the tissue conditioner powder in all experimental groups

Group	Antifungal/antimicrobial agents	Amount of drugs incorporated
G0	None (control)	None
G1	Nystatin	500,000 U
G2	Nystatin	1,000,000 U
G3	Miconazole	125 mg
G4	Miconazole	250 mg
G5	Ketoconazole	100 mg
G6	Ketoconazole	200 mg
G7	Chlorexidine diacetate	5%
G8	Chlorexidine diacetate	10%

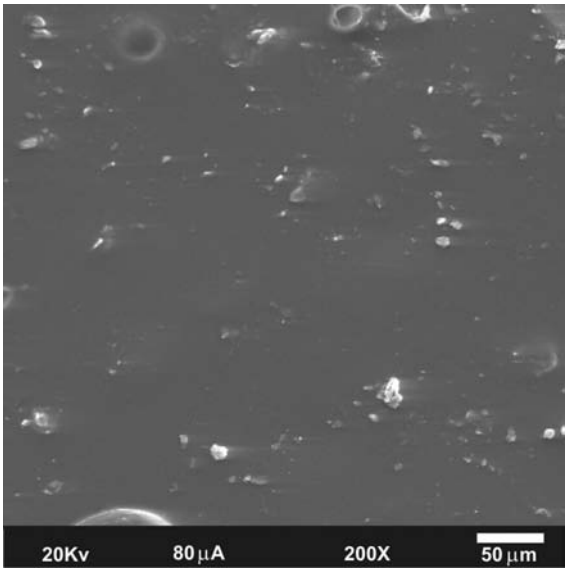

Figure 2 SEM of a drug-free resin surface from the control group, G0 (original magnification 200×).

with dimensions of 36 × 7 × 6 mm^3 and was sandwiched between two glass slides, and the specimen was left covered under pressure of 1 kg at room temperature (25°C) for 10 minutes. Afterward, the specimen was removed from the mold, and the excess was removed with a scalpel. Five specimens were used for each experimental group (n = 5).

The specimens were mounted on metal stubs and sputter coated with a thin carbon layer (MED 010, Balzers, Liechtenstein). All specimens were observed using SEM (DSM 940-A, Zeiss, Oberkochen, Germany), and three regions of each specimen were randomly selected for analysis. The selected regions were submitted to energy-dispersed X-ray analysis (Link ISIS, Oxford, Oxon, UK) attached to SEM to determine and map the main elements into the tissue conditioner matrix. Secondary electron images were obtained from the specimen surfaces at an accelerating voltage of 20 kV, and spot-EDS analyses were performed on the areas using a 25 μm^2 window under 100-second scans.

Results

Figure 2 is a representative SEM micrograph of drug-free specimens (G0). X-ray analysis indicated weak evidence of S^{2-} (sulfur) and Ca^{2+} (calcium) ions (Fig 3A).

The representative SEM image of 1,000,000-U nystatin specimen (G2) exhibited uniform distribution of particles with irregular shapes and sizes ranging from 10 to 50 μm (Fig 4). The spot-EDS spectrum obtained from those specimens exhibited traces of the elements S^{2-} and Ca^{2+} (Fig 3B).

Figure 5A is a representative SEM appearance of G4 specimen surface (250-mg miconazole). The irregular particles ranged in size from 25 to 50 μm. The EDS map showed Cl^{1-} (chlorine) ion well distributed within the tissue conditioner matrix (Fig 5B). The elements S^{2-}, Ca^{2+}, and Cl^{1-} were detected by X-ray analysis (Fig 3C).

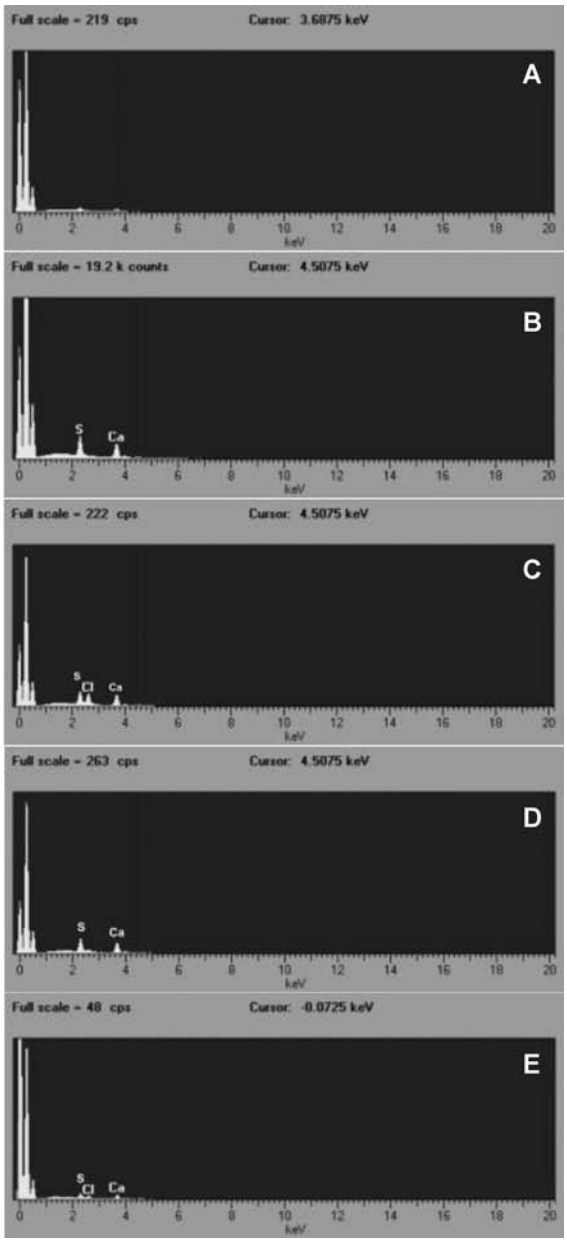

Figure 3 Surface scan of tissue conditioner modified specimens. Elements identified by energy dispersive X-ray spectroscopy microanalysis of (A) drug-free specimens, (B) a 1,000,000-U nystatin specimen, (C) a 250-mg miconazole specimen, (D) a 100-mg ketoconazole specimen, and (E) a 5% chlorhexidine specimen.

Figure 6 is a representative SEM micrograph of a tissue conditioner specimen containing ketoconazole (G5). The antifungal particles had a uniform spherical shape with sizes ranging from 10 to 25 μm. Particles slightly distributed throughout the tissue conditioner matrix were also observed. No Cl^{1-} was detected by EDS analysis on the surface of the specimen containing ketoconazole, so only S^{2-} and Ca^{2+} ions were detected (Fig 3D).

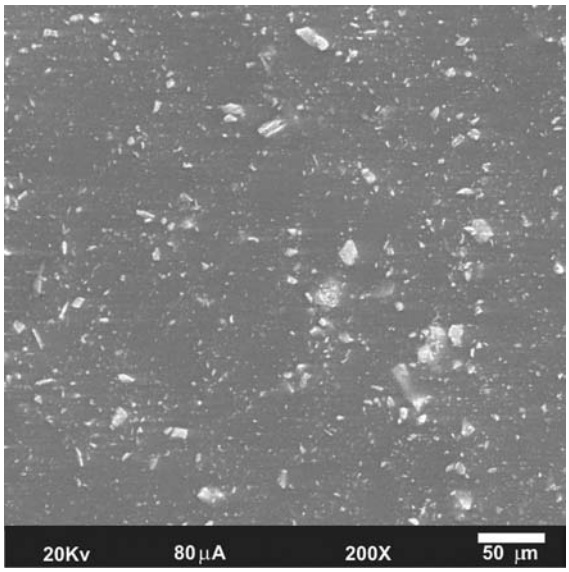

Figure 4 SEM appearance of the resin surface of a 1,000,000-U nystatin specimen, G2 (original magnification 200×).

Figure 7A shows one representative SEM image of a specimen modified by the addition of chlorhexidine 5% (w/w) (G7). The SEM analysis exhibited irregular particles of chlorhexidine diacetate with particles up to approximately 50 μm in size. EDS mapping analysis of Cl^{1-} element allowed the identification of chlorhexidine particles, which were randomly dispersed within the soft material (Fig 7B). The spot-EDS spectrum demonstrated the presence of S^{2-}, Ca^{2+}, and Cl^{1-} ions (Fig 3E). A higher SEM magnification (1000×) exhibited a 70-μm-long chlorhexidine diacetate particle exhibiting an irregular shape (Fig 8A) detected by EDS map due to the presence of the Cl^{1-} element (Fig 8B). No significant difference in the amount of antifungal agents spread on the tissue conditioner surfaces was noted when different concentrations were added to the tissue conditioner (Figs 4, 5A, and 6).

Discussion

In the current study, SEM images demonstrated that all drug-containing specimens showed changes at the surface in comparison to those of drug-free specimens (Figs 2 and 4 to 8). Some surfaces showed not only microscopic, but also macroscopic changes, as observed in nystatin-added specimens, which exhibited change in color from white to yellow because of the nystatin particles. In addition, visual changes in roughness related to the presence of bigger particles were noted in chlorhexidine-added specimens. Therefore, the working hypothesis that there are differences in morphological features of tissue conditioner surfaces when antifungal agents are added at different concentrations was accepted.

SEM and X-ray analyses were performed simultaneously to identify and measure the sizes of drug particles, as well as to determine their distribution within the tissue conditioner matrix. The detection of $S^{2-}$and Ca^{2+} ions in the Softone powder and the presence of only Cl^{1-} ion in the chemical structure of both miconazole (Figs 1B and 3C) and chlorhexidine (Figs 1D and 3E) were used to confirm the location of these drug particles within the plasticized matrix (Figs 5B, 7B and 8B). Despite the presence of Cl^{1-} ion in the chemical formula of ketoconazole (Fig 1C), this element was not found in EDS mapping analysis (Figs 3D and 6). Therefore, analyses of the presence and features of nystatin (Fig 4) and ketoconazole (Fig 6) added to the specimens were only based on comparisons among SEM images from drug-containing and drug-free specimens (Fig 2).

The desirable drug activity of a polymeric or plasticized system containing antifungal or antimicrobial agents is strongly related to its ability to release drug particles.[12,14,16,17,21] Drug

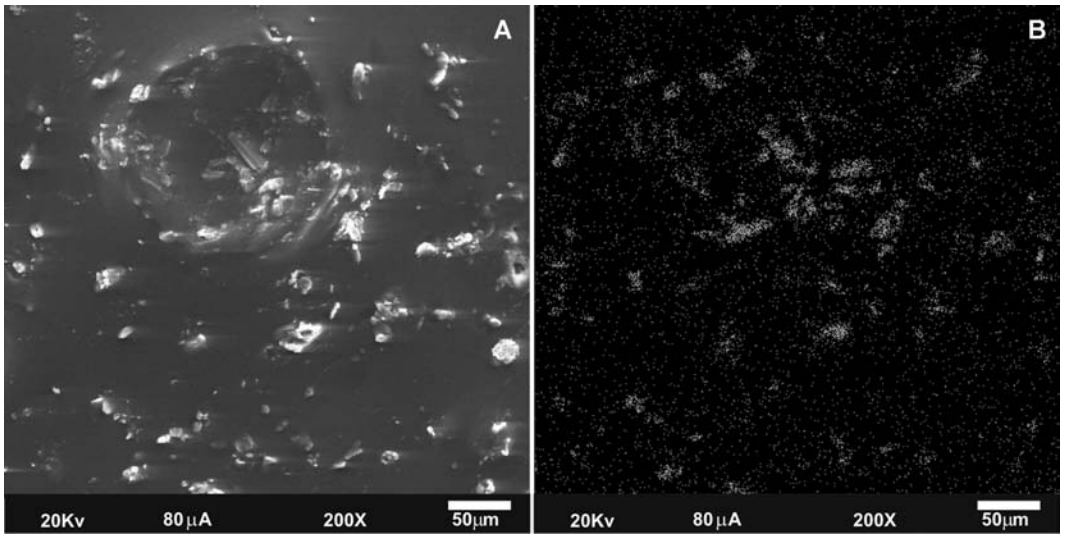

Figure 5 (A) SEM appearance of the surface of a 250-mg miconazole specimen, G4 (original magnification 200×). (B) Elemental dot-map showing distribution of chlorine within the particles of miconazole shown in (A).

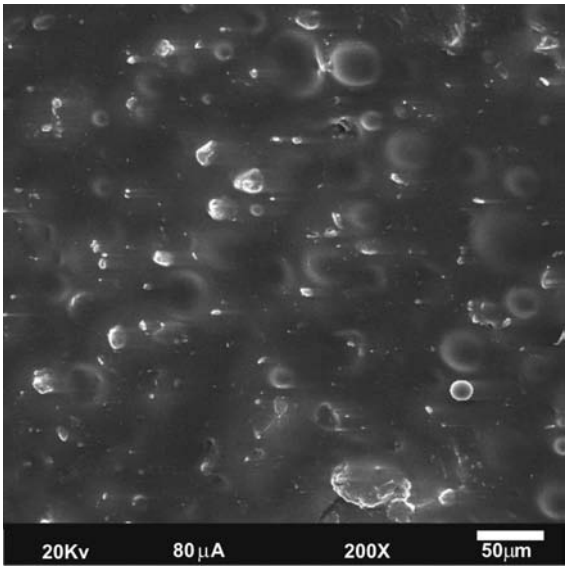

Figure 6 SEM appearance of the surface of a 100-mg ketoconazole specimen, G5 (original magnification 200×).

release rates may be associated with some factors, such as particle size, average molecular weight, distribution within the matrix, and concentration profile inside the matrix and dissolution-diffusion properties. In addition, features related to the matrix properties, such as micromorphology, permeability, porosity, and drug-matrix interaction, may also affect the drug release rates.

Particle size

Ketoconazole exhibited the smallest average size among all drugs added to the tissue conditioner (Fig 6). Although drug-release profiles were not evaluated in this study, it is possible to speculate that drugs with low average sizes may improve the releasing ability of such components, because the drug release rates can be controlled by manipulating the particle size.[22] In other words, when compared to bigger particles, smaller particles can be more quickly and easily released from the matrix surface due to the increased surface area/volume ratio.[22] Furthermore, the releasing period of smaller drug particles from a polymeric/plasticized matrix may be longer, because particles located at deeper layers are continuously released whereas bigger particles are effectively entrapped within the deeper matrix layers.[23]

Average molecular weight

Molecular weight is another factor affecting drug-releasing rates. Nystatin has the highest average molecular weight (926.11), while miconazole has the lowest (416.13). Ketoconazole (531.44) and chlorhexidine (625.56) have intermediary average molecular weights (Fig 1). The diffusion rate of drug molecules through the polymeric or plasticized matrix is increased by decreasing either the drug particle size or the average molecular weight of the drug.[20,22] Therefore, antifungal agents with high molecular weight are trapped within the matrix and only a small amount of antifungal agent is released from the surface.[23] The same concept is also applied to plasticizers and residual monomers.[24] Ferracane[24] observed that a higher amount of small monomers than large monomers might be eluted within the polymer matrix due to enhanced mobility. Moreover, Jones et al[25] demonstrated that higher diffusibility of plasticizers into polymer is related to their low molecular weight. The authors also observed that the solvation rates of plasticizers decrease when their molecular weight is increased. It may be difficult to determine the molecular weight limits of an antifungal agent that still allow an effective drug release; however, even nystatin, which has the highest molecular

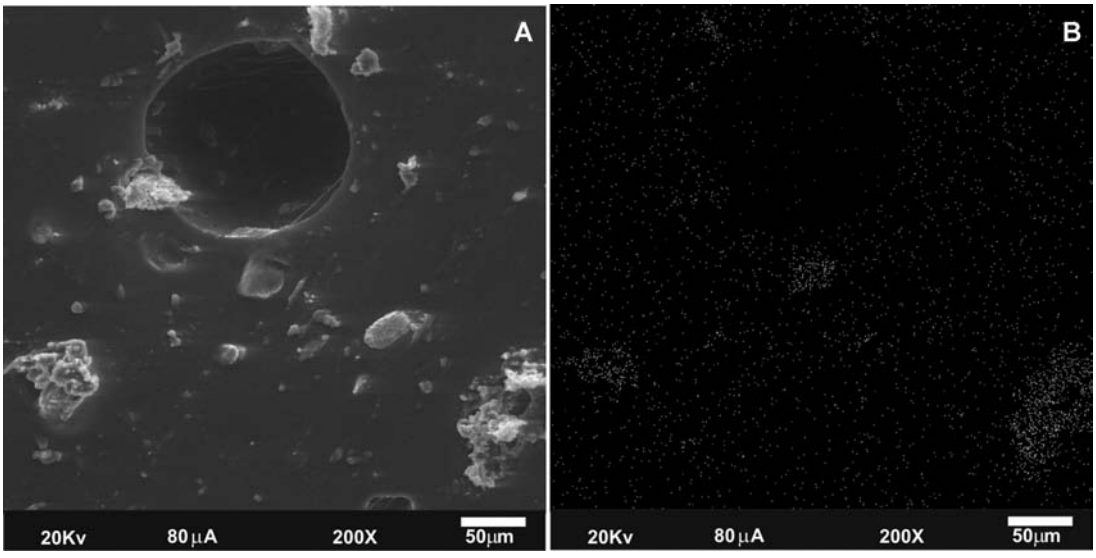

Figure 7 (A) SEM of the surface of a 5% chlorhexidine specimen, G7 (original magnification 200×), (B) Elemental dot-map showing distribution of chlorine within the particles of chlorhexidine shown in (A).

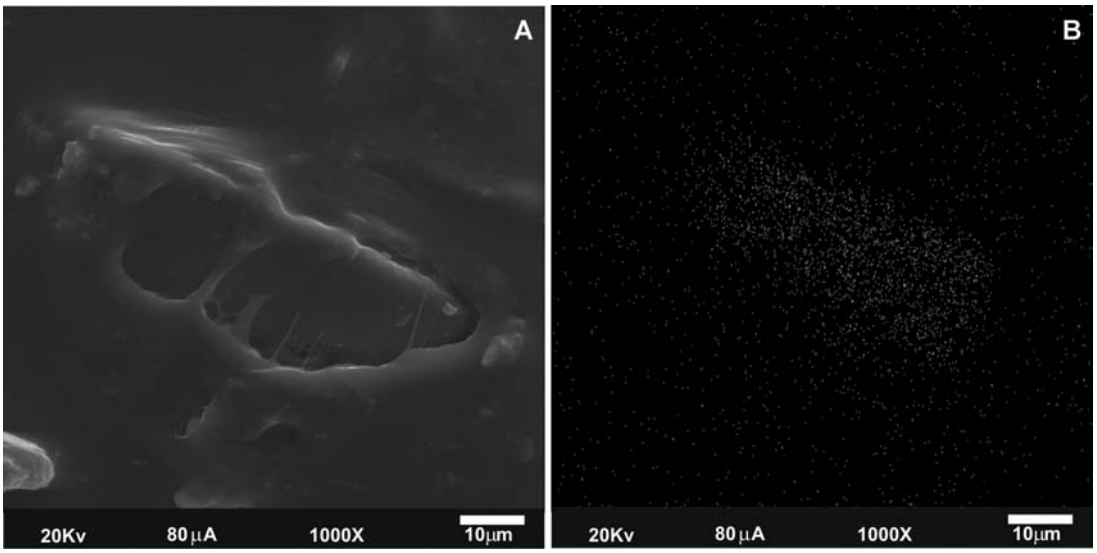

Figure 8 (A) SEM appearance of the surface of a 10% chlorhexidine specimen at a higher magnification, G8 (original magnification 1000×). (B) Elemental dot-map showing distribution of chlorine within the particles of chlorhexidine shown in (A).

weight among all other drugs, is able to leach out from the tissue conditioner and inhibit *Candida albicans* growth.[12,16,17,21] Such findings demonstrate that tissue conditioners are capable of releasing antifungal agents with an average molecular weight of around 1000. Lin et al[26] observed that more energy would be required for larger size molecules to penetrate and diffuse (translocate) through the matrix. Therefore, as the nystatin particle size is bigger than that of ketoconazole, higher energy of activation is required for nystatin to penetrate and diffuse through the tissue conditioner matrix; however, further studies evaluating the diffusion ability of drugs with different molecular weights throughout the Softone matrix are required to establish a correlation between molecular weight and energy of activation.

Distribution within the matrix

Drug distribution within the tissue conditioner matrix may also affect the drug-release profile.[20] Drug release begins at the outer matrix layers followed by its release from the inner layers.[27] Among all antifungal agents evaluated in this study, nystatin and miconazole showed particles uniformly distributed within the tissue conditioner matrix (Figs 4 and 5). As drug distribution is related to drug release,[20,27] it may be expected that this pattern of particle distribution within the tissue conditioner could result in constant and effective release rates for sustained therapies. Based on this aspect, further evaluation of drug release may demonstrate that nystatin and miconazole have a more favorable releasing pattern than ketoconazole and chlorhexidine.

Concentration profile inside the matrix

Another important factor affecting the drug release is the concentration profile of the drug inside the polymeric/plasticized matrix.[28,29] The low and high concentrations of nystatin were determined based on Truhlar et al's[16] study, which demonstrated 100% inhibition of *C. albicans* growth when 500,000 and 1,000,000 U of nystatin were used over a 14-day period in a nonaqueous environment. Quinn[21] also demonstrated that five combinations of tissue conditioners with 500,000 U of nystatin inhibited yeast growth in 2 weeks. The high concentrations of miconazole and ketoconazole were determined from the results of yeast growth inhibition observed by Quinn[21] and corresponded to 250 and 200 mg. The low concentrations were equivalent to half of the high concentrations (125 and 100 mg, respectively). The high and low concentrations of chlorhexidine diacetate were determined based on other studies, which evaluated the release of chlorhexidine diacetate from a plasticized acrylic.[30,31] Such studies also evaluated the incorporation pattern of the drug into this material,[30] the ability of chlorhexidine diacetate to diffuse out from an autopolymerizing acrylic resin[30,31] as well as the potential of the soft material with chlorhexidine diacetate to treat palatal candidosis in rats.[31]

When drug concentration falls below the expected range, drug efficacy is reduced. No significant differences were noted between SEM images from specimens with low and high concentrations of the same antifungal components. It has been demonstrated that a sustained antifungal/antimicrobial agent release and antifungal activity are proportionally related to drug concentration.[16] Based on this evidence, it is possible to speculate that differences in the release duration of antifungal/antimicrobial agents and antifungal activity may rely more on drug release from deep matrix regions than on drug release from the surface. Hence, further studies are needed to evaluate the releasing profiles and antifungal activity of such agents with different concentrations.

Dissolution-diffusion properties

Drug release from a matrix is primarily directed by diffusion, which is closely related to drug diffusivity and solubility,

among other factors.[32] Several drugs have poor solubility, poor permeability, or both. Some of them have less-than-optimal performance specifically due to their poor solubility, low bioavailability, incomplete absorption, and slow onset of action. Among the antifungal/antimicrobial agents, chlorhexidine has the highest water solubility (19 mg/mL at 20°C), followed by nystatin (4 mg/mL), miconazole (1 mg/mL), and ketoconazole (0.017 mg/mL at 25°C). The administration of a polymeric/plasticized delivering system containing a poorly soluble drug requires dosage maximization to obtain the required drug efficacy.

Matrix properties and the drug-matrix interaction

Other factors related to the matrix properties and drug-polymer matrix interactions also affect the drug release rates. Structural properties of the matrix, its micromorphology, permeability, and porosity[23,33] are important factors regarding mass transport (of water/drug) through the polymer.[34] Tissue conditioners exhibit an increased porosity, which is created during material mixture.[12,35] In the current study, all drug-free specimens and drug-containing specimens also showed high porous surfaces, which might enhance the antifungal/antimicrobial particle diffusion.

Drug mode of action

The drug mode of action is a very important factor when antifungal agents are used. Nystatin is a polyene antibiotic (Fig 1A) that binds to sterols within the fungal membrane, thereby disrupting its integrity. On the other hand, miconazole (Fig 1B) and ketoconazole (Fig 1C) are antifungal agents from the class of imidazoles, which act by inhibiting synthesis of ergosterol, a component of fungal membranes. Chlorhexidine is an antiseptic agent that has a broad spectrum of antimicrobial activity including *C. albicans* and other common non-*albicans* yeast species.[36] Chlorhexidine is a highly cationic chlorophenyl bisbiguanide (Fig 1D) and binds avidly to negatively charged surfaces, including epithelial cells.[37] The structural characteristics of chlorhexidine are responsible for its potent antimicrobial activity as well as its effectiveness at low concentrations and substantivity in the oral cavity.[36]

The fact that only one tissue conditioner brand available was evaluated may be considered one of the limitations of this study; the features of drug-particle distribution may not be applicable to other materials. In addition, SEM analyses were made just after specimens' preparation instead of after storage in distilled water. Nevertheless, based on the results of this preliminary study, it is essential to evaluate and specify the most important characteristics related to drug leaching from a polymeric/plasticized matrix. Further research is needed to evaluate the effects of the incorporation of antifungal agents and the long-term effects of water storage on some mechanical properties, such as hardness, roughness, tensile strength, porosity of the tissue conditioner, as well as the drug release, and antifungal effects of those antifungal/antimicrobial agents against *C. albicans* growth. Such parameters are crucial to establish if the tissue-conditioner-containing antifungal/antimicrobial agents can be recommended for candidiasis treatment without detrimental effects on tissue conditioner properties.

Conclusion

Within the limitations of this in vitro study, the modified tissue conditioner showed differences in distribution and size of the antifungal/antimicrobial particles added to the plasticized matrix. Nystatin and miconazole specimens exhibited particles with irregular shapes and sizes uniformly distributed within the tissue-conditioner matrix. Ketoconazole specimens showed small spherical particles with slight distribution throughout the matrix. Chlorhexidine specimens exhibited irregular particles up to approximately 50 μm in size randomly dispersed within the tissue conditioner matrix.

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