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Beneficial Effects of Ethyl-Cyanoacrylate Coating Against *Candida Albicans* Biofilm Formation

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The aim of this study was to verify whether modifications made in a hard chairside reline resin by an ethyl-cyanoacrylate adhesive, ECA (Super Bonder®, Loctite, Itapevi, SP, Brazil) would be able to inhibit or reduce *Candida albicans* biofilm formation on its surface, comparing to a commercial surface sealant (BisCover®, Bisco, Schaumburg, USA). Reline resin specimens were fabricated and randomly divided into 6 groups (n=8): CG (control group), no surface treatment; ECA1, ECA coating on the surface before sterilization; ECA2, ECA coating after sterilization; ECA3, ECA incorporated in the resin bulk; DPE1, BisCover® coating before sterilization; DPE2, BisCover® coating after sterilization. Specimens were inoculated with *C. albicans* SC5314 ($1x10^7$ cells/mL) and incubated for 24 h. Then, the biofilm were stained with LIVE/DEAD® BaclightTM L7007 Kit and analyzed by Confocal Laser Scanning Microscopy. The images were evaluated by bioImageL® v.2.0 software and total biovolume (μm^3), viable cells (%), and covered area (%) were calculated. Data were statistically analyzed by Kruskal-Wallis and Dunn tests ($p<0.05$). Results showed that ECA-coated groups presented better results, reducing *C. albicans* biofilm formation. Acquired images revealed that these groups (ECA1 and ECA2) presented a reduced number of cells, mostly in yeast form (less pathogenic), while the other groups presented higher number of cells, mostly in hyphae form (more pathogenic). Based on these findings, a beneficial effect of Super Bonder® coating reline resins surface could be demonstrated, suggesting a promising way to prevent fungal biofilm formation on dentures.

Key Words: denture liners, *candida albicans*, confocal microscopy, cyanoacrylates, biofilm formation.

Introduction

Denture stomatitis (DS) is one of the most common oral diseases, which has prevalence between 15 to 70% in denture wearers, especially in elderly and immune-compromised patients (1). The greatest challenge regarding the management of this condition is related to the high rates of clinical relapse and recurrence in up to two weeks post-treatment with topical and systemic antifungal drugs (2). DS is associated with poor denture hygiene, poor denture quality, nocturnal denture use, residual monomer allergy, impaired salivary flow, systemic diseases and the presence of *Candida albicans* in form of highly resistant microbial biofilm, which is adhered and retained on the denture´s inner surface. The development of a biofilm is a complex mechanism, mostly influenced by the environment, the pathogen virulence and the material´s surface characteristics that it colonizes (3). The latter is mainly attributed to high surface roughness (2-3 µm), which serves as a reservoir for these microorganisms and to hydrophobicity and surface free energy that favors the fungal initial adhesion (4-7).

Normally, these characteristics mentioned above are inherent to dentures´ most common material, heat-polymerazing polymethyl-methacrylate (PMMA). Often, over time, PMMA resins require repairs due to mucosal modifications and/or material wear. In such cases, it is common to clinicians to use a hard chairside reline resin as a reparation material, which are faster, easier and cheaper option compared to relining dentures using laboratory procedures (8,9). Hard chairside reline resins are generally self-cured polyethyl-methacrylate (PEMA) resins. Self-cured PEMA also have features that promote biofilm adhesion and development, with the aggravation of being made directly in the mouth, in contrast to a plaster model and not being polished in laboratory, leading to higher surface roughness (7,10).

In order to overcome DS prevalence and recurrence issues, much attention has been given to the recognition and development of biocompatible materials that can decrease microbial colonization on denture surface by changing its physicochemical surface characteristics. To address this matter, researchers developed a number of techniques and coating materials such as parylene (11), silver nanoparticles (12), silica nanoparticles (13),

titanium dioxide (14), among many others (15-18). However, most of these coatings have some limitations, such as the decline of denture´s mechanical and/or aesthetic properties, questionable long-term stability, complex production techniques and high final costs (11). Considering that dentures are relatively low-cost products, it is not reasonable that its coating process exceed the denture cost itself. Thus, the search for other materials that could be used as coating materials, acting in the control of microbial biofilm is still current.

With the evolution of composites, surfaces seals seem to be one of the potential alternatives to accomplish this function. These materials are resinous low viscosity compounds that reduce surface roughness, eliminating irregularities and the oxygen inhibition layer after polymerization. Among these materials stand out the Biscover (Bisco) that with anti-adherent properties, demonstrated his effectiveness in inhibiting microbial biofilm when applied on PMMA (17,19).

Authors have reported the antimicrobial and antiadhesive activity of cyanoacrylate-based adhesives when used as biocompatible materials (20). Ali et al. (18) investigated *C. albicans* growth on resin plates coated with octyl-cyanoacrylate, a long side chain cyanoacrylate, and verified complete inhibition of fungal adhesion. Among cyanoacrylate adhesives, there is, also, ethyl cyanoacrylate (ECA), an ester of cyanoacrylic acid with a smaller side chain, with only two carbon atoms. ECA is a commercially available instant adhesive, with relatively low costs, indicated by manufacturers for bonding leather, rubber, porcelain, metal, wood, cardboard and plastics. ECA is presented as a low-viscosity transparent liquid that polymerizes after contact with the atmospheric moisture or any fluid (21). Despite usually not being marketed for medical purposes, ECA has been used in the medical and dentistry area for several years. Results of recent studies show a satisfactory outcome when using ECA as tissue adhesive (22-24). For that reason, ECA could be suitable as a coating material for dentures.

In this context, the aim of this study was to verify whether modifications made in a hard chairside reline resin by an ethyl-cyanoacryolate adhesive (Super Bonder®, Loctite, Itapevi, SP, Brazil) would be able to inhibit or reduce *C. albicans* biofilm formation on its surface, comparing to a commercial surface sealant (BisCover®, Bisco, Schaumburg, USA).

Material and Methods

Specimens Preparation

Forty eight self-cured hard chairside denture reline resin (New Truliner®, Bosworth Co., Skokie, IL, USA) specimens were manufactured and randomly divided into 6 groups (n=8) according to the surface treatment. The codes of groups, compositions, manufacturers and surface treatment of each group are listed in Table 1.

Specimens of the hard chairside reline resins were fabricated using a rectangular metal matrix placed on a glass slab previously isolated with vaseline. The reline resins were mixed following manufacturer's instructions and placed into the mold spaces (30x5x5 mm). Then, a second glass slab was placed on the matrix and light pressure was applied (5kg for 15 min). After polymerization, materials´ excess were removed and specimens were trimmed using a metal bur (Maxi-cut; Dentsply-Maillefer, Ballaigues, Switzerland). In order to establish an average roughness between 1 to 2 µm, one specimen surface was polished for 30 s using a 120-grained sandpaper (Abrasives Norton, São Paulo,

Table 1. Group codes, product used and surface treatment for in each group in this study

Codes	Composition	Manufacturer	Surface Treatment
CG	No product was used	-	No products were applied or incorporated in the manufacturing of these specimens
ECA1	Ethyl-cyanoacrilate adhesive	Super Bonder® (Loctite, Itapevi, SP, Brazil)	Super Bonder® layer was applied on the surface before sterilization
ECA2	Ethyl-cyanoacrilate adhesive	Super Bonder® (Loctite, Itapevi, SP, Brazil)	Super Bonder® layer was applied on the surface after sterilization
ECA3	Ethyl-cyanoacrilate adhesive	Super Bonder® (Loctite, Itapevi, SP, Brazil)	Four drops of Super Bonder® were incorporated in the resin bulk
DPE1	Dipentaerythritol pentaarylate ester	Biscover® (Bisco, Schaumburg, IL, USA)	Biscover® layer was applied on the surface before sterilization
DPE2	Dipentaerythritol pentaarylate ester	Biscover® (Bisco, Schaumburg, IL, USA)	Biscover® layer was applied on the surface after sterilization

Brazil) at low speed, under refrigeration. Afterwards, specimens were cut in half by diamond wheel. These specimens (15x5x5 mm) were subjected to an additional round of polymerization at 100 °C for 2 h in water bath (Solab, Piracicaba, SP, Brasil) to residual monomer removal according to ISO 1567:1988 standards (International Organization for Standarization Specification 1567, 1988). Specimens were further divided into 6 groups (Table 1). All groups were sterilized with ethylene oxide (EO) before *C. albicans* biofilm inoculation, following each group specification (Table 1).

To ECA-coated groups, specimens received two-layers of Super Bonder®. Each layer had one drop of the adhesive uniformly distributed using a brush tip. After its complete polymerization, the second layer was applied. DPE surface sealant was applied following manufacturer´s instructions and light-cured for 15 s.

Candida albicans Biofilm Growth

C. albicans SC314 frozen culture stocks (-80°C) were incubated in trypic soy broth (TSB) (Accumedia Manufactures, Inc. Lansing, USA) for 36 h at 30°C in aerobic conditions. Afterward, cells were harvested, washed with phosphate buffered saline (PBS) and standardized to 1 x 10^7 cells/mL in PBS (25). Then, reline resin specimens were placed in a 24-well plate (Cell Culture Plate, Nest Biotech Co., Ltd., China) containing 3 mL of the standardized cell suspension to be incubated for 90 min at 37°C under 75 rpm (25,26). Next, non-adherent organisms were removed by washing specimens with 3 mL of PBS. Subsequently, for biofilm growth, inoculated specimens were immersed in 3 mL of Roswell Park Memorial Institute solution (RPMI-1640, Gibco®, Grandlsland, NY) and remained in orbital shaking (75 rpm) for 24 h at 37 °C (2).

Confocal Laser Scanning Microscopy

After 24 h of incubation, all of the specimens were transferred to a new 24-well plate and washed individually in PBS. After that, specimens were stained with LIVE/DEAD® BaclightTM L7007 Kit (Molecular Probes, Invitrogen Brazil Ltd., São Paulo, SP, Brazil) at 1% for 20 min in the absence of light at 37°C. The LIVE/DEAD® solution includes two dyes, the green dye (SYTO-9) for staining both live and dead cells and the red dye (Propidium Iodide) for non-viable cells. Specimens were then analyzed by Confocal Laser Scanning Microscopy (CLSM) (TCS-SPE, Leica Mycrosystems, Mannhein, Germany) (26).

Images were obtained from six different fields in a standardized manner, so that there was no possibility of recording the same field repeatedly. Each field was horizontally sectioned with 1 µm range throughout the depth of the biofilm. All sections were grouped together to create a final image. The images were evaluated by bioImageL® v.2.0 Software (Dr. Luis Chavez de Paz, Malmö University, Sweden) and total biovolume (viable and non-viable cells - µm3), cell viability (percentage of green cells in each specimen field - %), and covered area (area covered by biofilm in each specimen field- %) were calculated. Statistical analysis was performed using software Prisma 5.0 (GraphPad Software Inc, La Jolla, CA, USA) and data were analyzed by Kruskal-Wallis and Dunn's tests, considering a significance level of 5% ($p<0.05$).

Results

Total biovolume, cell viability and covered area of *C. albicans* biofilms are summarized in Table 2. Specimens coated with ECA (ECA1 and ECA2) presented lower *C. albicans* biofilm formation, less viable cells and reduced covered area when compared to the control group (CG) ($p<0.05$). When ECA was incorporated on resin´ bulk (ECA3) instead of coated on its surface no difference were noticed on biofilm´ development, compared to CG ($p>0.05$). Cell viability were statistically different between groups. However, all groups showed viability greater than 89%. As for Biscover ® coated groups (DPE1 and DPE2), results showed greater amounts of biofilms when compared to ECA coated groups, but similar to CG, except for covered area values. EO sterilization did not affect the biofilm quantification for ECA or DPE coated groups.

Additionally, as shown by fluorescence microscopy ECA coated surfaces (ECA1 and ECA2 groups) presented biofilms with scarce cells, mainly blastopores, while the other groups presented multilayered viable (green) biofilms (Fig. 1).

Table 2. *C. albians* total biovolume, cell viability and covered area for each tested group analysed by CLSM

	Total biovolume (µm³)	Cell viability (%)	Covered area (%)
CG	517 x $10^{3\ ab}$	96.64 ^{a}	4.3 ^{a}
ECA1	19 x $10^{3\ c}$	89.10 ^{b}	0.4 ^{b}
ECA2	35 x $10^{3\ c}$	89.40 ^{b}	0.8 ^{b}
ECA3	324 x $10^{3\ a}$	95.27 ^{ab}	3 ^{a}
DPE1	1196 x $10^{3\ b}$	93.69 ^{ab}	9.4 ^{c}
DPE2	1094 x $10^{3\ b}$	96.77 ^{a}	9.4 ^{c}

Different letters indicate statistically significant difference between groups.

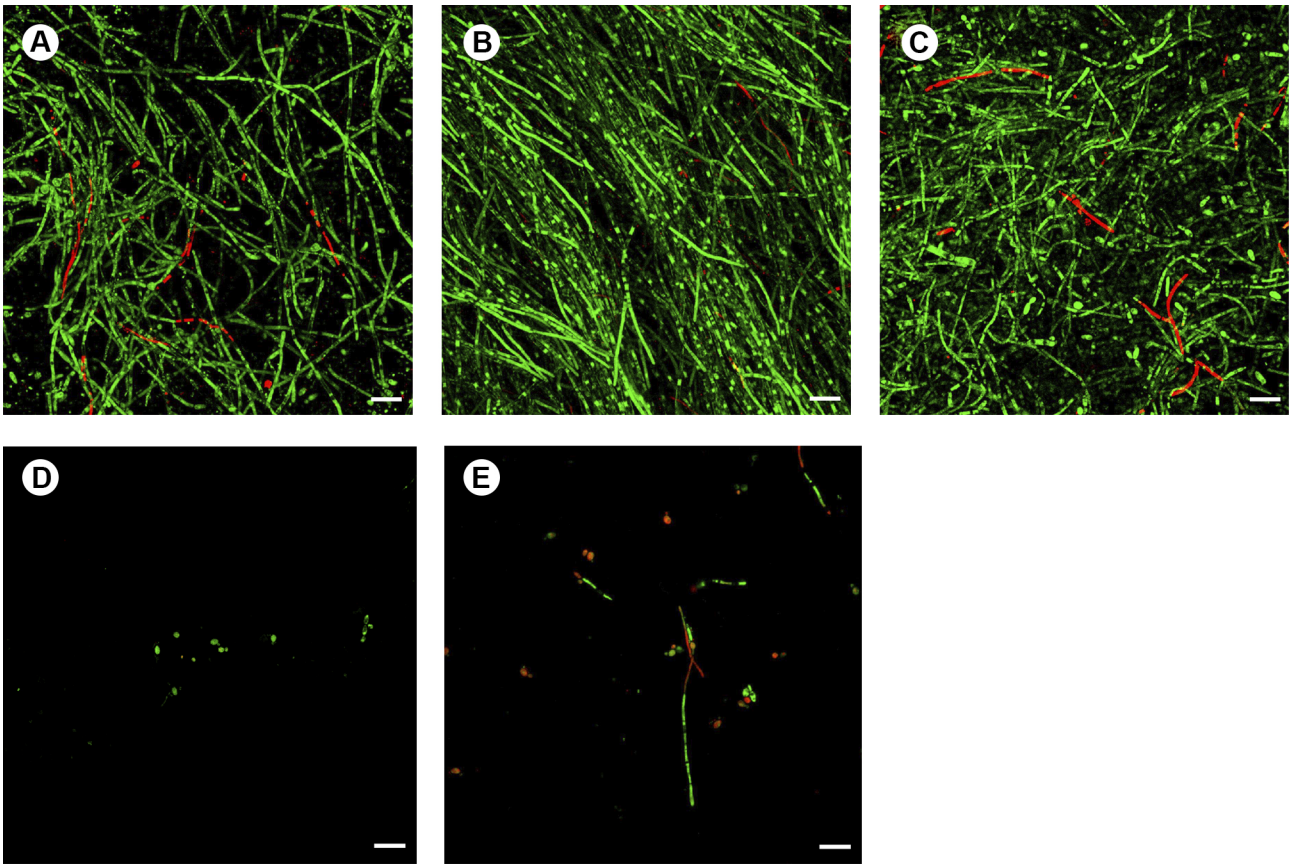

Figure 1. Confocal images using the LIVE/DEAD assay kit. The experimental groups are ordered by: A) Control group, B) Biscover before sterilization, C) Biscover after sterilization, D) ECA before sterilization and E) ECA after sterilization. At 24 h, obtained images showed that only ECAs groups showed a reduced count of viable adhered cel.

Discussion

Denture stomatitis, the most common pathology affecting denture wearers, is caused, among other factors, due to *C. albicans* biofilm adherence on denture materials (1,27). Often, denture resins have to be repaired because of ill-fitting and wear problems, and hard chairside reline resins appear as a faster and more convenient option to laboratory reline procedures. However, the absence of laboratory polishing results in greater surface roughness, increasing *C. albicans* colonization when compared to laboratory-processed resins. Thus, in the present study it was assessed whether coating of ethyl-cyanoacrylate adhesive could reduce *C. albicans* adhesion on the surface of a hard chairside self-curing reline resin, comparing with a commercial surface sealant.

In this study, hard reline resin specimen coated with ECA reduced significantly *C. albicans* biofilm formation and growth. To date, there is only one published research that investigated cyanoacrylate effect on *C. albicans* biofilm adhesion on denture materials. Similarly to our results, Ali et al. (18) observed that 2-octil cyanoacrylate (OCA) coating denture acrylic resin could completely inhibit *C. albicans* biofilms growth. ECA and OCA have similar molecular arrangements with difference in the length of their side chain, with two and eight carbon atoms, respectively. According to early studies, longer cyanoacrylates present greater biocompatibility due to its slower degradation rate to formaldehyde (28,29). However, more recent studies (22,24,30) indicate that ECA adhesives may be more compatible, mainly due to the addition of products that improve biocompatibility (31). Nitsch et al. (22) demonstrated that ECA presents´ greater in vitro biocompatibility, even when compared to OCA. Thus, ECA was selected to be the coating material in this study due to its market availability and inexpensiveness, since dentures are relatively low-price products, as well.

The mechanism via which ECA coating diminishes *C. albicans* adhesion may be due to modifications of resin´s surface characteristics, such as roughness, hydrophobicity and surface free energy. Previous researches demonstrate that *C. albicans* cells can find protection from mechanical tension in surface´s irregularities (27). Consequently, smooth surfaces are less prone to *C. albicans* adhesion and growth. Prior to ECA´ polymerization, the liquid flows

filling up grooves and cracks, leading to a smoother and more regular surface. DPE surface sealant, like ECA, is a low-viscosity resin that reduces the denture base acrylic resin roughness, similarly to laboratory polishing. Silva et al. (17) verified that DPE could reduce *C. albicans* biofilm development when coating PMMA. However, in the present study, DPE showed similar results to the CG, regarding biofilm formation. This disparity may be associated to the substrate on which the surface sealant was coated. In this study, a hard self-cured PEMA resin was used, while Silva et al. (32) used heat-cured PMMA resins, which has more wettability. Moreover, these authors coated PMMA specimens with an artificial saliva prior to biofilm development (17). While, in our study, we did not adopted any salivary coating protocol, mainly due to the controversial information presented in scientific literature, regarding the positive or negative influence of saliva on *C. albicans* adhesion. It is known that saliva coating modifies the surface free energy and affect the adhesion of *C. albicans*, due to the adsorption of salivary proteins. However, depending on its origin and type of collection, the variety of protein composition in the saliva is very wide, leading to difficulties in standardization (16,33).

Here, our data of cell viability demonstrated significant difference between ECA-coated groups and control group ($p<0.05$). However, cell viability is not less than 89% for all groups, suggesting the lack of a fungicide effect of tested materials. Our result is consistent to previous research that did not find antifungal effect of cyanoacrylate on *C. albicans* (34).

In microscopic images obtained in this study, besides the lower cell count, ECA coated surfaces showed lower hyphae/blastopore ratio. It is well-known that the presence of hyphae is a significant determinant of virulence of dimorphic fungi, mainly due to its ability to penetrate tissues (35). Furthermore, according to Mayahara et al. (36), hyphae cells are thigmotropic cell. In other words, during hyphae development, it presents a directional growth following contact with surfaces, leading to a more complex mechanical removal. In this study, we observed that after 24 h in all groups, except in ECA-coated groups, an intricate hyphae network was present.

Additionally, ECA seems to inhibit *C. albicans* biofilm formation only when covering resin surfaces, because when ECA was incorporated in resin bulk while manipulation (ECA3) all results were similar to the control group. This finding indicates that the modification of the surface features were a determining factor in the results, since in ECA3 group, the surface was kept intact.

Moreover, EO sterilization showed no influence on coating properties of ECA and DPE, possibly because EO is a low-temperature sterilization, able to maintain the coating physicochemical properties (37).

This study showed that Super Bonder®, when used as reline resin coating material, reduced *C. albicans* biofilm development, whereas Biscover® surface sealant did not have any influence on this. Therefore, these results suggest that ECA seems to be a promising product to be used in dental practice mainly in the denture bases of patients with history of DS avoiding its recurrence, which is the greatest challenge of the therapies proposed for this condition. Further in vitro studies are needed on the mechanisms of reduction of biofilm formation by ECA, its coating durability and the resistance of the product to disinfection methods employed in denture maintenance. Moreover, to the best of our knowledge, there is no information in the pertinent literature regarding the thickness of the ECA on the acrylic surface of the denture bases. Results of simultaneous research (unpublished results) demonstrated that the thickness of the ECA adhesive, when applied in acrylic resin surface, has a micrometric thickness (9.3 µm). Thus, it is strongly likely that such thickness is clinically insignificant to cause maladaptation of a base removable denture. However, clinical studies are still needed to confirm this hypothesis.

Resumo

O objetivo deste estudo foi verificar se as modificações feitas com o adesivo etil cianoacrilato, ECA (Super Bonder ®, Loctite, Itapevi, SP, Brasil) sobre as resinas acrílicas para reembasamento, poderiam inibir ou reduzir a formação de biofilmes de C.albicans sobre sua superfície quando comparado com um selante de superfície comercial (BisCover®, Bisco, Schaumburg, EUA). Amostras de resina acrílica para reembasamento foram fabricadas e divididas aleatoriamente em 6 grupos (n=8): CG (grupo controle), sem tratamento superficial; ECA1, revestimento de ECA na superfície antes da esterilização; ECA2, revestimento de ECA após esterilização; ECA3, ECA incorporado no volume da resina; DPE1, revestimento de BisCover® antes da esterilização; DPE2, revestimento de BisCover® após esterilização. Os espécimes foram inoculados com *C. albicans* SC5314 ($1x10^7$ células/mL) e incubados durante 24 h. Seguidamente, o biofilme foi corado com LIVE/DEAD® BaclightTM L7007 Kit e analisado no microscópio confocal de varredura a laser. As imagens foram avaliadas pelo software bioImageL® v.2.0, no qual foram calculados o biovolume total (μm^3), as células viáveis (%) e a área coberta (%). Os dados foram analisados estatisticamente pelos testes de Kruskal-Wallis e Dunn ($p<0,05$). Os resultados mostraram que os grupos revestidos com ECA apresentaram os melhores resultados, reduzindo a formação do biofilme de *C. albicans*. As imagens adquiridas revelaram que esses grupos (ECA1 e ECA2) apresentaram um número reduzido de células, principalmente na forma de levedura (menos patogênico), enquanto os outros grupos apresentaram um maior número de células, principalmente na forma de hifas (mais patogênicas). Com base nessas descobertas, encontra-se um efeito benéfico na aplicação do adesivo ECA sobre as superfícies das resinas acrílicas para reembasamento, sugerindo assim uma nova alternativa de prevenir a formação de biofilme fúngico em próteses dentárias.

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