Title: The Influence of Tobacco Components on *Candida albicans* Biofilm Formation on Denture Materials

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Running Title: Influence of tobacco components on fungal biofilm formation

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Abstract:
Purpose: The aim of this study was to investigate the influence of nicotine and cotinine, two major components of tobacco, on fungal biofilm formation on two acrylic resins. Additionally, the nystatin susceptibility of the planktonic and biofilm fungal cells was compared.

Materials and Method: Ivocap and Lucitone 199 polished and roughened acrylic resin discs were fabricated and randomly assigned to control, nicotine-, and cotinine-treated groups. Biofilm was prepared by incubating Candida albicans fungi (1x10^7 cells) on the resin discs in the presence or absence of tobacco components for one week at 37°C. The discs were washed to remove unbound cells. The relative number of viable fungal cells was estimated using the MTT assay and the biofilm growth was quantified by dry weight analysis. Nystatin susceptibility minimum inhibitory concentration was determined by microdilution method. Statistical significance was evaluated using ANOVA followed by Fisher's test (p<0.05).

Results: The MTT assay showed that the roughened discs had significantly greater numbers of C.albicans than the polished discs, Lucitone 199 acrylic discs promoted significantly more fungal biofilm growth than Ivocap discs, and the presence of tobacco components significantly enhanced biofilm formation on both types of acrylic resin. Dry weight analysis showed similar results. The nystatin susceptibility assay showed that tobacco treated-cells were more resistant to the antifungal treatment.

Conclusion: This study showed significant differences in fungal growth between polished and roughened surfaces and fungal growth on the different types of acrylic discs, with a higher degree of biofilm formed on Lucitone 199 surfaces; enhanced biofilm growth in the presence of tobacco components; and the biofilm fungal cells were more resistant to nystatin than the planktonic cells.

Keywords: Nicotine, cotinine, denture stomatitis, Ivocap, Lucitone 199, and nystatin.
Introduction:

Denture stomatitis, a common oral disease, can induce pathologic changes to the denture-bearing tissues. These tissue changes can vary from localized hyperemia to diffused erythema to papillary hyperplasia.\textsuperscript{1,2} Patients with severe denture stomatitis may experience pain, itching, and/or burning sensation.\textsuperscript{3-5} Studies have shown that the prevalence of denture stomatitis in the edentulous population ranges from 15% to over 70% with more cases found in the elderly, women, smokers, and immunocompromised.\textsuperscript{6-12}

\textit{Candida albicans} adherence and colonization of denture prostheses has been shown to induce denture stomatitis.\textsuperscript{13,14} In general, \textit{C. albicans} species exist as commensal organisms in about 33% of the adult dentate patients and about 75% of the complete denture patients.\textsuperscript{15} \textit{C. albicans} can exist individually in a liquid medium (planktonic) or together in a film on a surface (biofilm). They are classified as asexual diploid fungus that can exist in the form of yeasts or hyphae. Smears obtained from the intaglio surface of dentures often demonstrate the presence of the invasive filamentous hyphal form.\textsuperscript{8,16,17}

Denture base resins are susceptible to fungal colonization in the oral environment. Surface roughness is one of the factors that aids in the initial fungal attachment. Surface defects such as scratches, cracks, and porosities serve as protective surfaces for microorganisms to bind.\textsuperscript{18,19}

One of the more common denture resins used today is polymethylmethacrylate (PMMA), a polymer developed in the 1930s.\textsuperscript{20} PMMA can be classified as heat-activated or chemically-activated resin based on the method of polymerization. Heat-activated PMMA can be processed by compression-molded, injection-molded, or microwave-processed techniques.\textsuperscript{21} Physical characteristics of PMMA are dependent on the type of resin as well as the processing technique. Manufacturers of the injection-molded resin claim that the “cross-linked, high impact homopolymer” obtained after polymerization offers excellent polishability and helps reduce plaque build-up.\textsuperscript{22}

It has been reported that the use of tobacco products may cause an increase in \textit{C. albicans}-induced denture stomatitis in susceptible patients.\textsuperscript{23} According to a study by Arendorf \textit{et al.}, there are higher numbers of \textit{Candida} in tobacco smokers as compared to non-smokers.\textsuperscript{24} Salivary nicotine and cotinine are widely used in clinical and epidemiological smoking studies.\textsuperscript{25} Nicotine is a weak base with a pKa value that approaches 8. When salivary pH increases, the non-ionized form of nicotine is absorbed across the buccal and nasal membranes.\textsuperscript{26} Cotinine, the major metabolite of nicotine, has been reported to have a pKa of 5.\textsuperscript{27} The concentration of cotinine in saliva is variable depending on an individual’s nicotine metabolism and the salivary pH. Salivary concentrations of nicotine and cotinine can be affected by many factors such as cigarette brand, length of cigarette, gender, puffing behavior, and testing methods.\textsuperscript{28} Robson \textit{et al.}\textsuperscript{29} reported that salivary nicotine concentrations ranged from 0.36µg/ml to more than 4.6µg/ml and cotinine concentrations ranged from 0.0096µg/ml to more than 1.4µg/ml in individuals who
smoked 2-20 cigarettes per day. In contrast, Cote et al.\textsuperscript{28} reported each cigarette’s nicotine concentration to be between 100µg/cigarette to more than 2650µg/cigarette. While smoking has been implicated as one of the factors contributing to denture stomatitis; however, its exact pathogenic influence has yet to be demonstrated.

Nystatin, discovered and isolated in the early 1950s from \textit{Streptomyces noursei}\textsuperscript{30}, is one of the most common antifungals used to treat \textit{C. albicans}-induced denture stomatitis. It is a broad spectrum polyene agent shown to inhibit \textit{Candida} cell growth and division.\textsuperscript{31} It is available in oral rinses, creams, and slow-releasing tablets.\textsuperscript{32} Studies have shown that prophylactic oral application of nystatin may prevent systemic spread of oral candidal infection in immunocompromised individuals\textsuperscript{33} and suppress candidal adhesion to denture acrylic surfaces.\textsuperscript{34, 35} Anil et al.\textsuperscript{36} and Ellepola et al.\textsuperscript{37} have determined that the minimum inhibitory concentration (MIC) of nystatin required to inhibit fungal cell growth is within the range of 0.78-1.56 µg/ml.

Understanding the underlying factors leading to fungal adhesion to denture acrylic resin surfaces and the roles that nicotine and cotinine play in biofilm formation may help alleviate the problems associated with denture-induced stomatitis. This first aim of this study was to evaluate the effect that tobacco components had on two different denture base acrylic resins with polished and roughened surface topography. The second aim was to test nystatin susceptibility of \textit{Candida} grown in the presence of tobacco components. The null hypothesis was that the surface topography, type of acrylic resin, and tobacco treatment would not affect fungal growth.

**Materials and Methods:**

**Materials:**

Acrylic resin discs (10 mm x 1mm) were prepared from polymethyl methacrylate (PMMA) obtained from two different manufacturers (Table 1). Ten millimeter diameter vinyl polysiloxane (VPS) (Dentsply International Inc., York, PA) rods were prepared and invested in type III dental stone (Whip Mix Corp., Louisville, KY) in the respective flasks.
Table 1. Denture resins, manufacturers, compositions, and polymerization cycles used in this study.

Ivocap (Ivoclair Vivadent AG, Schaan, Liechtenstein) was processed via the injection-molded technique. Five millimeter diameter wax sprues connected the injection channel to the top of the VPS rods. The wax sprues were boiled out and the VPS rods were removed from the flasks. The two halves of the flasks were held together using the clamping frame and 3 tons/6000 psi of pressure. Twenty grams of polymer and 30ml monomer were mixed together for 5 minutes in the Cap Vibrator. Compressed air (6 bar/85 psi) was used to inject the resin into the flask. Polymerization time started when the water reached 100°C and the resin was allowed to polymerize for 35 minutes. After the polymerization cycle, with the pressure apparatus remaining unchanged, the flasks were cooled in cold water for 20 minutes. Then the pressure apparatus was removed and the flasks were cooled for an additional 10 minutes before deflasking. The resin rods were cut into sample discs (10mm x 1mm) using an electric saw (Cut-Off Saw, Harbor Freight Tools, Calabasas, CA). A roughened surface was obtained by using an acrylic bur (Komet, Rock Hill, SC) to abrade the discs. To obtain a polished surface, acrylic discs were pumiced using a combination of medium and fine pumice (Whip Mix Corp.) and polished using polishing compound (Grobet, Carlstadt, NJ).

Lucitone 199 (Dentsply) was mixed at 3.2:1 powder to liquid ratio. The VPS rods were removed after the stone was set. The acrylic resin was trial packed twice and the excess was removed before final flask closure. Lucitone 199 was polymerized using the long curing cycle. The cycle started in the water bath (Manfredi Acrydig 4-10, Torino, Italy) at room temperature and was gradually brought to 74°C/165°F and held at that temperature for 8 hours, followed by 100°C/212°F for 1 hour, and bench cooled for at least 30 minutes before deflasking. The same methods detailed above were used to obtain polished and roughened discs.

The discs were stored in purified water for seven days to remove any residual monomer. They were decontaminated by rinsing with bleach, then soaking in 70% ethanol for 2 hours, and finally were exposed to UV light over night. The discs were routinely tested for sterility by culturing in tissue culture media.

**Biofilm formation:**

*Candida albicans* 18807 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The fungus was cultured in Sabouraud’s broth (SAB, Gibco, Grand Island, NY) at 30°C for 24 hours. The cell suspensions density was measured at 540nm by a Spectrostar spectrophotometer (BMG Labtech, Ortenberg, Germany) and standardized to $1 \times 10^7$ cells/ml.

For the MTT assay, test acrylic resin discs from both manufacturers were randomly divided into two broad groups, which consisted of polished and roughened surface topography. Each polished and roughened group was further subdivided into control, nicotine, and cotinine treated
samples. The twelve groups had a total of 144 samples with 12 samples per group. The discs were placed in a 24-well culture plate (Becton Dickenson and Co., Franklin Lakes, NJ), then incubated for 72 hours with 1.0 ml of standardized C. albicans suspension along with 10µg nicotine or cotinine. Nicotine and cotinine were purchased from Sigma-Aldrich Co. (St. Louis, MO). The cultures were fed with glucose (50 mM) for another four days to facilitate biofilm formation. Discs incubated with sterile SAB served as negative control.

For the dry weight analysis, fungal biofilms were treated with 10µg of nicotine. Equal numbers of Ivocap and Lucitone 199 sample discs were divided into polished and roughened groups. The polished and roughened groups were further subdivided into control and nicotine-treated samples. The eight groups had a total of 24 samples. The discs were placed in a 24-well culture plate, and incubated for 72 hours with 1.0 ml of standardized C. albicans suspension and control or 10µg nicotine. The cultures were fed with glucose for another four days to facilitate biofilm formation.

C. albicans susceptibility to nystatin was conducted with biofilm cells prepared in the same manner as the dry-weight samples and compared to the freshly cultured planktonic fungal cells.

Quantitative measurement of biofilm growth:

A) Tetrazolium reduction assay (MTT Assay):

The relative number of the C. albicans cells grown in the twelve groups was evaluated using tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-reduction assay (Roche Diagnostics, Indianapolis, IN), which measures cell viability as well as proliferation. After biofilm formation, 50 µl of MTT solution was added to each well and incubated for 4 hours at 37°C. The unbound cells were removed by washing the wells gently with PBS. The contents of each well along with the fungal cells were collected and incubated with solubilizing agent over night. The optical density of the fluid was measured by a spectrophotometer at 540 nm. Control wells contained the medium plus MTT to determine background formazan values. All test samples were assayed three times using the same protocol.

B) Determination of dry weights:

The acrylic resin discs were pre-weighed prior to biofilm formation and the measurements were recorded. Post biofilm formation, the discs were removed from the medium, gently washed with PBS to remove non-biofilm cells, dried, and re-weighed. The difference in the weight of the discs was calculated.

C) Determination of nystatin susceptibility:

The susceptibility of Candida to ten different concentrations of the antifungal agent nystatin was determined using the National Committee for Clinical Laboratory Standards guidelines. Analytical grade nystatin powder (Sigma-Aldrich Co.) was dissolved in 100% dimethyl
sulfoxide (DMSO) and absolute ethanol (3:2 ratio). The inoculum was prepared from one week old culture of cells in SAB and adjusted to give a final inoculum of $10^5$ CFU/ml. A 1ml aliquot of the suspension was placed in 10 different sterile glass tubes with varying concentrations of nystatin (100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 7.25 µg/ml, 3.125 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml, and 0.195 µg/ml). The fungal cells were incubated for 24 hours. The minimum inhibitory concentration (MIC) of nystatin, determined visually, was defined as the lowest concentration at which there was 100% inhibition of C. albicans growth, as indicated by the absence of turbidity.

**Data Analysis:** Results of MTT assays and dry weight analyses were evaluated by ANOVA followed by Fisher’s test, with a statistical significance set at $p<0.05$. 
Results

MTT Assay

Effect of surface topography: The results (Table 2 and Figure 1) indicated that:

1. Rough irregular surfaces on Ivocap acrylic discs treated with nicotine or cotinine showed significantly greater fungal growth \((p<0.05)\) compared to the polished Ivocap discs treated with tobacco products.
2. Irregular surfaces on Lucitone 199 acrylic discs treated with cotinine showed significantly greater fungal growth \((p<0.05)\) compared to the polished Lucitone 199 discs treated with cotinine.
3. The fungal growth on the rough surfaces on Ivocap control, Lucitone 199 control, and Lucitone 199 treated with nicotine did not significantly differ from their polished counterparts.

Effect of different denture materials: The fungal cell growth on Lucitone 199 polished and roughened discs treated with nicotine and cotinine was significantly higher than Ivocap discs \((p<0.05)\) treated under the same conditions. About 85% viable fungi were viable in biofilm formed on Lucitone roughened surface but on Ivocap only 67% of fungi was measured. However, the fungal growth in the control (buffer treated) Lucitone 199 group did not significantly differ from the control Ivocap group. (Figure 1)

Effect of tobacco components treatment: The presence of nicotine and cotinine significantly affected *C. albicans* growth in the Ivocap roughened, Lucitone 199 polished and roughened groups as compared to their control counterparts \((p<0.05)\). In contrast, neither nicotine nor cotinine treatment significantly affected the fungal growth in the polished Ivocap group.

Table 2. Results from MTT assay comparing surface topography, two denture materials, and tobacco components treatment.
Figure 1. Average values from MTT assay comparing roughened vs. polished surfaces, Ivocap and Lucitone 199, and nicotine and cotinine vs. control.

**Dry weight analysis**

Table 3 and Figure 2 show average dry weight values of the eight sample groups. There were significantly greater number of cells found on the roughened surfaces than the polished surfaces of both types of resin \( p<0.05 \), more cells on Lucitone 199 than Ivocap discs \( p<0.05 \), and more cells all nicotine-treated groups than their respective control groups \( p<0.05 \).

Table 3. Results from dry weight analysis comparing surface topography, two denture materials, and tobacco components treatment.

Figure 2. Average values from dry weight analysis comparing roughened vs. polished surfaces, Ivocap and Lucitone 199, and nicotine vs. control.

**Nystatin susceptibility analysis**

Figure 3 demonstrated the minimum inhibitory concentration (MIC) of *C. albicans* to nystatin. The nystatin MIC for cells in the planktonic (non-nicotine treated and non-denture disc) group was 1.56µg/ml. The nystatin MIC for the Ivocap polished and roughened, non-nicotine treated biofilm groups was 12.5µg/ml. The MIC for the Lucitone 199 polished and roughened, non-nicotine treated biofilm groups was 25µg/ml. The MIC of all nicotine-treated biofilm groups was 50µg/ml.

Figure 3. Minimum inhibitory concentrations of fungal cells to nystatin.

**Discussion**

The null hypothesis in this study was rejected since the surface irregularity, type of acrylic resin, and treatment with tobacco components affected fungal growth significantly. However, since MTT assay results varied among the different comparison groups, this suggested that the biofilm formation could have resulted from a complex phenomenon with a multifactorial origin. Given the vast range of denture base resins available, the results of this study could not be extrapolated and applied to other compression- and injection-molded materials.
The results from the MTT assay of *C. albicans* grown on discs of different surface topography showed that some groups were affected by surface properties while others were not. For example, fungal growth on Ivocap nicotine- and cotinine-treated roughened discs was significantly greater than on their polished counterparts. In contrast, rough surfaces on Ivocap control, Lucitone 199 control, and Lucitone 199 nicotine-treated groups did not significantly enhance fungal growth compared to the polished groups. These results were in agreement with other studies on surface topography which suggest that increased fungal growth on rough surfaces may be due to greater micro surface area available for attachment. Once attached, the fungal cells were less likely to be dislodged.18, 19

In general, Lucitone 199 resin promoted more fungal growth than Ivocap. As evaluated by the MTT assay, Lucitone 199 polished and roughened denture discs treated with nicotine or cotinine promoted significantly more *C. albicans* growth than their Ivocap counterparts. However, in the absence of nicotine and cotinine, no significant differences were found between Lucitone 199 and Ivocap discs (polished or roughened). These findings may be attributed to the variations in the chemical compositions, the concentrations of different components, and the processing methods used for acrylic resins, or the interactions between tobacco components and the resins themselves.

Compared to the control groups, fungal growth in all of the nicotine- or cotinine- treated groups was significantly increased, except in the Ivocap polished groups. Due to the many factors that can prevent a study from reliably measuring nicotine and cotinine concentration intraorally as outlined by Cote *et al.*28, a pilot study was performed prior to the full study. The results suggested that a concentration of 10µg/ml of nicotine and cotinine could induce fungal growth. This concentration was higher than concentrations reported by Robson *et al.*29 but lower than concentrations reported by Cote *et al.*28

Since nicotine and cotinine elicited similar fungal responses, only nicotine was tested in the dry weight analysis. The results from this analysis showed significant differences in fungal growth between roughened and polished surfaces, the two denture materials, and nicotine-treated and control groups. In the case of the Ivocap polished discs, the results of dry weight analysis and MTT analysis were contradictory. While the MTT values of nicotine-treated polished Ivocap discs were increased, they did not significantly differ from the control, whereas the values from the nicotine-treated group did significantly increased compared to the control in the dry weight analysis. These results may be due to the different nature of the assays. Since MTT assay measured growth and viability, it may have missed the dead cells that were present. In contrast, dry weight analysis measured the weight of all cells, alive or dead.

The minimum inhibitory concentration of nystatin in the planktonic baseline group without denture disc and nicotine treatment was 1.56µg/ml, which was in agreement with previous studies by Anil *et al.*36 and Ellepola *et al.*37 When Ivocap denture discs were introduced, the nystatin MIC increased to 12.5µg/ml, three times more than baseline reading. When Lucitone
199 denture discs were introduced, the nystatin MIC increased to 25µg/ml, four times more than baseline reading. When nicotine and denture discs were added, the nystatin MIC increased to 50µg/ml, five times more than the baseline reading. While surface topography did not affect the efficacy of nystatin treatment, the denture material and tobacco treatment raised the MIC three to five folds as compared to the baseline group. This may be the first study that tested biofilm-grown cells’ susceptibility to the anti-fungal agent nystatin.

A limitation of this study was a lack of quantitative measurement of the surface topography. Further studies are needed to investigate the surface topography quantitatively, the hardness and density of the denture materials, other denture materials, and other antifungal agents.

**Conclusion**

This study assessed the influence of surface topography, denture material, and tobacco component treatment on *C. albicans* biofilm growth, and indirectly, fungal-induced denture stomatitis. This study demonstrated significant differences in fungal growth on polished and roughened surfaces, and significant differences in fungal growth on different types of acrylic discs, with a higher degree of biofilm formed on Lucitone 199 surfaces. Furthermore, tobacco components appeared to enhance biofilm formation, and biofilm fungal cells were more resistant to nystatin than the planktonic cells.

Within the limitations of this study, it suggests that when adjustments are made to dentures, they should be polished prior to placement in an attempt to reduce the degree of fungal growth. In susceptible patients, certain denture materials may increase the risk of fungal infections. Denture wearers who are also smokers may have an increased risk of fungal colonization, and the fungal organisms they harbor may also be more resistant to antifungal therapy. Therefore, the antifungal treatment in these patients may have to be more closely monitored.

**Acknowledgements:**

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References

Table 1. Denture resins, manufacturers, compositions, and polymerization cycles used in this study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
<th>Composition</th>
<th>Polymerization cycle</th>
</tr>
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<tbody>
<tr>
<td>Ivocap (injection-molded)</td>
<td>Ivoclar Vivodent AG Schaan, Liechtenstein</td>
<td>Polymer: poly(methyl methacrylate), copolymer, benzoyl peroxide, pigments</td>
<td>Heat polymerized: 35 min at 100°C</td>
</tr>
<tr>
<td>Lucitone 199 (compression-molded)</td>
<td>Dentsply International Inc. York, USA</td>
<td>Polymer, methyl methacrylate, copolymer, red azo pigment, titanium dioxide, black iron oxide, yellow iron oxide, red acetate fibers, dibenzoyl peroxide</td>
<td>Heat polymerized: 8 hours at 74°C/165°F 1 hour at 100°C/212°F</td>
</tr>
<tr>
<td>Treatment groups</td>
<td>Ivocap</td>
<td>Lucitone 199</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polished</td>
<td>Roughened</td>
<td>Polished</td>
</tr>
<tr>
<td>Control</td>
<td>1.33±0.29</td>
<td>1.73±0.42</td>
<td>1.87±0.42</td>
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<tr>
<td>Nicotine treated</td>
<td>1.77±0.53</td>
<td>2.62±0.73</td>
<td>2.88±0.55</td>
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<tr>
<td>Cotinine treated</td>
<td>1.86±0.62</td>
<td>2.93±0.55</td>
<td>2.79±0.63</td>
</tr>
</tbody>
</table>
Figure 1.

% Viable cells on denture discs

Denture material

- Ivocap Polished
- Ivocap roughened
- Lucitone Polished
- Lucitone roughened

Legend:
- Control
- Nicotine
- Cotinine
Table 3.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Ivocap</th>
<th>Lucitone 199</th>
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<tr>
<td></td>
<td>Polished</td>
<td>Roughened</td>
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<tr>
<td>Control</td>
<td>4.13±0.16</td>
<td>5.60±0.10</td>
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<tr>
<td>Nicotine treated</td>
<td>6.01±0.22</td>
<td>8.14±0.44</td>
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</table>
Figure 2.

Dry Weight Analysis

Average dry weight values (mg)

- Polished
- Roughened
- Ivocap
- Lucitone
- Nicotine treated
Figure 3.

**Minimum Inhibitory Concentration of Nystatin**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planktonic</td>
<td>1.56</td>
</tr>
<tr>
<td>Ivocap polished/roughened control biofilm</td>
<td>12.5</td>
</tr>
<tr>
<td>Lucitone polished/roughened control biofilm</td>
<td>25</td>
</tr>
<tr>
<td>Ivocap/Lucitone polished/roughened with nicotine biofilm</td>
<td>50</td>
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