Salivary Polypeptide/Hyaluronic Acid Multilayer Coatings Act as "Fungal Repellents" and Prevent Biofilm Formation on Biomaterials

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Abstract

Candida-associated denture stomatitis (CADS) is a common, recurring clinical complication in denture wearers that can lead to serious oral and systemic health problems. Current management strategies are not satisfactory due to short-acting and ineffective therapeutic effects. Here, we describe a new fungal biofilm controlling strategy using polyelectrolyte layer-by-layer (LBL) self-assembly technology on denture materials. Conventional poly (methyl methacrylate) (PMMA) denture material discs were functionalized with negatively charged poly (methacrylic acid) (PMAA) via plasma-initiated surface grafting, followed by repetitive alternating coating with the salivary antimicrobial polypeptide histatin 5 (H-5; cationic polymer) and hyaluronic acid (HA; anionic polymer). On the other hand, the H-5/HA LBL coatings (i.e., the outermost layer was H-5) inhibited fungal attachment/adhesion, significantly reduced fungal biofilm formation, and showed synergistic effects with the antifungal drug miconazole. LBL surface hydrophilicity was not the key mechanism in controlling *Candida* biofilm formation. The current approach demonstrates the utility of a new design principle for fabricating anticandidal denture materials, as well as potentially other related medical devices, for controlling fungal biofilm formation and combating insidious infections.

Graphical Abstract

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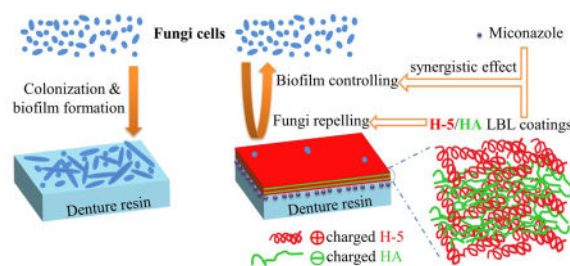
For patients missing multiple teeth or with edentulism, removable partial or full dentures are invaluable for maintenance of overall health, contributing to nutrition, speech, appearance, and quality of life.[1] Unfortunately, due to colonization of the dentures by biofilm-forming microorganisms, patients wearing dentures often develop *Candida*-associated denture stomatitis (CADS), a common, recurring disease that affects up to 67% of denture wearers.[2–4] Moreover, *Candida* and other microorganisms on the denture surface often cause difficulty to control dental diseases (e.g., caries and periodontal disease), oral and systemic infections (e.g., gastrointestinal and pleuropulmonary infections), and even death.[4–6]

Current management of CADS includes patient compliance with good denture hygiene practices (e.g., denture cleaning, disinfection), ensuring that denture fit is optimized by use of tissue conditioners/liners/rebases, topical or systemic antifungal therapy, and incorporation of antifungal drugs into denture materials.[7–9] However, none of these approaches completely eliminates *Candida* colonization and biofilm formation on the denture surface; this outcome is particularly problematic in the elderly and those who are medically or immunologically compromised and results in poor clinical outcomes.

The current study used a layer-by-layer (LBL) self-assembly approach to functionalize the denture disc surface and provide long-term anticandidal activity. The LBL technology is a simple, yet versatile technique, for alternately depositing polyelectrolytes of opposite charges on top of each other one layer at a time.[10–12] Histatin 5 (H-5), a salivary polypeptide, is the smallest cationic peptide with the highest anticandidal activity in the major histatin family.[13–16] In clinical applications, topical histatins reduce/prevent oral *Candida* infection.[17] Previous studies have adsorbed synthetic H-5 on denture materials, resulting in low amounts of peptide adsorption and weak antifungal effects.[18] Here, we used hyaluronic acid (HA) as the anionic counter ion to increase incorporation of H-5 in a LBL self-assembly multilayer coating process. HA is a water-soluble anionic linear polysaccharide consisting of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid.[19, 20] As a natural component of the extracellular matrix and synovial fluid, HA, beyond the broad applications in medical practices and cosmetics, has been widely used as a biopolymer for drug delivery, wound dressing, and tissue engineering, due to its excellent biodegradability, biocompatibility, biological activity, and high water sorption and retention ability.[19–22]

Lucitone 199 (Dentsply Intl, York, PA), a common denture base material, was used for the current studies and is representative of heat-polymerizing poly (methyl methacrylate) (PMMA)-based resins. Denture discs (1.0 cm of diameter and 0.13 cm of thickness) were

fabricated following the manufacturer's recommendations using a standard compression molding technique. Poly (methacrylic acid) (PMAA) was then grafted onto the PMMA denture discs (g-PMAA) for H-5 attachment; this was followed by alternate immersion in HA and H-5 solutions for multilayer self-assembly. Some of the g-PMAA discs were pre-charged with the anticandidal drug, miconazole, prior to H-5/HA self-assembly. The H-5/HA LBL coated discs were tested for activity against *C. albicans* (e.g., direct contact, adhesion, and biofilm formation assays) and skin fibroblasts (e.g., cytotoxicity) (See detailed procedures in Supporting Materials).

The pristine PMMA surface had a negative charge with a zeta potential of -16.8 ± 6.3 mV (Fig. 1). To further increase the anionic character of the PMMA surface for H-5 binding and subsequent H-5/HA LBL coating, PMAA was grafted onto the PMMA discs using plasma-initiated surface grafting as we described previously.[23, 24] With a grafting yield of 1.5 ± 0.2 mg/cm^2 of PMAA, the resulting denture discs (g-PMAA) had a much lower negative zeta potential (-35.1 ± 3.4 mV). After coating with one layer of H-5 (assigned as 0.5 bilayer), the zeta potential became $+17.9 \pm 5.3$ mV, indicating that positively charged H-5 successfully coated the surface. Further coating with negatively charged HA (1 bilayer) reversed the surface charge (zeta potential = -22.1 ± 6.9 mV). As predicted, surface charge polarity changed with each subsequent LBL cycle (*i.e.*, if the final layer was H-5, a positively charged surface was obtained; if the final layer was HA, the surface carried a negative charge), suggesting that multilayer coatings were successfully built onto the denture disc surface.

H-5 content in the multilayer coatings was quantified using the colorimetric micro bicinchoninic acid (BCA) protein assay.[25, 26] In screening studies, we found that after one cycle of H-5 coating, the pristine PMMA surface adsorbed 1.50 ± 0.39 $\mu g/cm^2$ of H-5. However, after PMAA grafting, H-5 adsorption on the surface (i.e., g-PMAA) increased dramatically to 19.88 ± 0.61 $\mu g/cm^2$ of H-5 (0.5 bilayer). Subsequent H-5/HA LBL cycles further increased H-5 content; with 10.5 bilayers of H-5/HA, H-5 content increased to 43.19 ± 1.14 $\mu g/cm^2$ (Figure 2). The data fitted well to an exponential growth ($R^2 = 0.958$), indicating that the H-5/HA LBL coatings grew exponentially during layer pair deposition. Numerous reports have showed nonlinear growth of multilayer coatings depending on the chemical nature of the polyelectrolytes used, e.g., exponentially growing coatings were commonly observed with polypeptides and polysaccharides; their buildup was based on the diffusion "in" and "out" of at least one of the polyelectrolytes through the coating during layer pair deposition.[27–29]

The effect of LBL coating on surface hydrophilicity/hydrophobicity was assessed using water contact angle measurements (see Fig. S1 in Supporting Materials). Pristine PMMA resin discs had a water contact angle of $85.1 \pm 4.0°$. After grafting PMAA to the surface, which introduced hydrophilic PMAA polymer chains, the water contact angle of g-PMAA significantly decreased to $66.9 \pm 1.0°$. After deposition of one layer of H-5 onto the g-PMAA (0.5 bilayer) surface, the contact angle was not significantly changed; however, after one complete cycle of H-5/HA (HA was the outermost layer), the contact angle was significantly reduced to $53.9 \pm 5.2°$, suggesting that HA, containing numerous -OH and -COOH groups, was more hydrophilic than H-5. *If we keep HA as the outermost layer,*

further increase in coating layers did not significantly affect contact angle values (around 54°) with each additional bilayer (1, 5, and 10 bilayers). However, *if the outermost layer was H-5* (0.5, 5.5, and 10.5 bilayers), the contact angle gradually decreased from 69.0 ± 2.5° to 54.9 ± 5.5°, suggesting that when H-5 was the outermost layer, with the increase of coating layers, the HA chains might protrude through adjacent layers (i.e., H-5) during LBL self-assembly and increase the hydrophilicity of the surface. If HA did not protrude, H-5 outermost layer (0.5, 5.5 and 10.5 bilayers) should have similar contact angles. This observation correlated well with the diffusion-based buildup mechanism of the exponentially growing H-5/HA LBL coatings as discussed above (Fig. 2). Also, the hydrophilicity may not account for fungal repelling of H-5 (see below).

Candida albicans (*C. albicans*, ATCC 10231) was selected as a representative fungus for evaluating the anticandidal activity of the denture materials. This strain can be isolated from patients with CADS and readily forms biofilms on various biomaterials.[2–4] A direct contact mode "sandwich assay" (i.e., incubation of *C. albicans* between two denture discs) was used to test the fungicidal effects of the multilayer coatings on g-PMAA,[30, 31] using pristine PMMA discs as controls. Interestingly, LBL coatings with H-5 as the outermost layer (e.g., 0.5 bilayer, 5.5 bilayers, and 10.5 bilayers of H-5/HA) did not show any change in colony-forming units (CFUs) with up to 8 h of contact. Since the fungicidal action of H-5 relies on its internalization into the cells to reach intracellular targets,[14–16, 32–34] these results suggest that H-5 remained bound to the coatings and did not diffuse into the surrounding environment to kill *Candida*.

Although H-5 in the LBL coatings did not provide direct fungicidal activity, it significantly reduced *Candida* initial adhesion. Initial adhesion is the first stage of biofilm formation, which is mainly affected by the surface properties of the solid materials. As shown in Fig. 3, after 1 h of immersion in a *Candida* suspension in PBS, $(1.69 \pm 0.39) \times 10^5$ CFUs of adherent *Candida* could be recovered from the pristine PMMA control. The g-PMAA disc showed a significantly lower level of *Candida* adherence, which could be caused by the hydrophilic surface.[30, 35]

In the study of the LBL coatings, *both layer numbers and the nature of the outermost polymer affected Candida initial adhesion*. For instance, when H-5 was the outermost layer, the g-PMAA discs coated with 0.5 bilayer and 10.5 bilayers further significantly reduced *Candida* adhesion, *demonstrating that the presence of H-5, not hydrophilicity, was responsible for this level of reduction in Candida adhesion*. The 10.5 bilayers had significantly lower *Candida* initial adhesion than the 0.5 bilayer, *suggesting that more H-5 would lead to higher anti-adhesion activity*. On the other hand, when the outermost layer was changed from H-5 to HA, *Candida* adhesion on the 1 bilayer coating was significantly higher than that on the 0.5 bilayer coating (although H-5 content was the same in the 0.5 bilayer and the 1 bilayer coatings), *confirming that the reduction in Candida adhesion was provided by H-5, not the hydrophilic HA*.

After 1 h of initial adhesion, the *Candida*-containing discs were individually incubated in YM broth for 2 days to allow biofilm formation. As shown in Fig. 4, $(1.23 \pm 0.42) \times 10^7$

CFUs of *Candida* were recovered from the adherent biofilm on the pristine PMMA control discs, which was nearly the same as recovered from the g-PMAA disc.

These results suggest that although the hydrophilic surface was able to temporarily reduce *Candida* initial adhesion, it was ineffective in preventing biofilm formation. A similar result was obtained with the g-PMAA disc coated with 1 bilayer (HA was the outermost layer), further confirming that the HA layer (although hydrophilic) could not control *Candida* colonization and biofilm formation. On the other hand, g-PMAA discs coated with 0.5 bilayer and 10.5 bilayers (H-5 was the outermost layer in both) had much lower levels of *C. albicans* [> 100 times less; (6.40 ± 1.24) x 10^4 CFUs for 0.5 bilayer and (2.42 ± 0.22) x 10^4 CUFs for 10.5 bilayers, respectively] than on the pristine PMMA, *suggesting that the biofilm-controlling effect was provided by H-5.* As predicted, the biofilm level on 10.5 bilayers was significantly lower than that on 0.5 bilayer. These results were consistent with the initial adhesion studies.

The biofilm-controlling performance was also preliminarily evaluated in artificial saliva and 50% human saliva, respectively, and the results were presented in Table S1 in Supporting Materials. Exposure to artificial saliva or human saliva did not significantly affect the anticandidal activity of the H-5/HA LBL coatings against biofilm formation, suggesting that the biofilm-controlling potency of H-5 in the LBL coatings was maintained in the presence of either artificial saliva or human saliva. These findings demonstrated the stability and efficacy of the H-5/HA LBL coatings in the presence of oral fluid, pointing to great potentials for real applications.

Candida that remained in the *broth* solution (i.e., not in the biofilm on disc surface) after two days of culture was also assessed and quantified. No significant differences in broth levels of *C. albicans* were observed after immersion with pristine PMMA, g-PMAA, or g-PMAA coated with H-5/HA multilayers; *Candida* levels in all cases were around (1.3 ± 0.2) x 10^7 CFUs/ml. The biofilm-controlling results suggest that while *Candida* readily colonized pristine PMMA, g-PMAA, and the g-PMAA discs coated with HA outermost layer, the yeast cells stayed in the broth and avoided the surface of g-PMAA discs coated with H-5 as the outermost layer, indicating that H-5 on the surface might have acted as a "fungal repellent" and prevented biofilm formation.

The "fungal repellent" effect of H-5 in the coatings was long-lasting. A series of g-PMAA discs coated with 10.5 bilayers of H-5/HA were immersed in PBS at 37°C with constant shaking up to 4 weeks. PBS was changed daily. The biofilm-controlling activity as demonstrated with *C. abicans* initial adhesion and YM cultures was essentially unchanged during the entire time of immersion (Fig. 5). As clinical treatments for CADS are often within 1–2 weeks,[36] this level of durability is relevant for managing CADS in real applications.

The biofilm-controlling properties of the LBL coatings were further confirmed by scanning electron microscopy (SEM). After 2 days of incubation in YM broth, pristine PMMA and the g-PMAA discs were covered with copious amounts of layered *Candida* colonies containing filamentous cells, highly indicative of fungal biofilm formation (Fig. 6a and b).

On g-PMAA discs with 0.5 bilayer of H-5/HA, only scattered adherent *Candida* could be observed (Fig. 6c). When the number of bilayers was increased to 10.5, the disc surface appeared clear and no *Candida* biofilm present (Fig. 6d). Furthermore, after immersion in PBS at 37°C for 4 weeks, the biofilm-controlling function of the 10.5 LBL coating was maintained (Fig. 6e), suggesting that the coating had potential long-term fungal repellent/biofilm-controlling activity and clinical prevention of development of CADS. Since CADS commonly occurs on the oral mucosa underneath the dentures, in real applications the coatings will present on the tissue side of the dentures. For properly fitted dentures, the tissue side has limited exposure to oral fluids with low friction/scratch with the denture for patient comfort/compliance. Thus, this level of stability of the LBL coatings on the denture materials points to good likelihood of the new technology for clinical applications. If, future clinical tests find that longevity of the coating should be further improved, one strategy would be to use covalent bonding or cross-linking to improve the durability of the coating. Another option will be to "recharge" the H-5/HA multilayer coatings, since dentures are removable and can be easily taken out for re-coating.

An attractive feature of H-5 in the LBL coatings was that its antifungal properties were synergistic with clinical anticandidal drugs and provided potent biofilm-controlling effects. To test combination of H-5/HA LBL coatings and anticandidal drugs, g-PMAA discs were pre-charged with miconazole following a procedure previously reported by us that resulted in binding of 126.8 ± 10.5 μg of miconazole per disc.[23] After drug charging was complete, 0.5 bilayer and 10.5 bilayers were built on the miconazole-containing g-PMAA discs.

The combination of H-5/HA coatings with miconazole significantly reduced *Candida* biofilm formation more than using either one alone (Fig. 7 as well as data presented in Fig. 4), suggesting that H-5/HA coatings synergized with miconazole to provide more potent anticandidal activity. Interestingly, the disc with 0.5 bilayer plus miconazole showed a greater reduction in biofilm formation than the sample with 10.5 bilayers and miconazole (Fig. 7, $p < 0.05$). We showed in our previous studies[23] that the anticandidal effects of miconazole-containing denture materials were achieved by the sustained release of miconazole, which was due to the specific interactions (e.g., hydrogen bonding) between miconazole and the grafted PMAA chains. The drug release profiles of miconazole-containing discs without coating and with 0.5 and 10.5 bilayers are presented in Table S2 in Supporting Materials. With 10.5 bilayers coating, the discs released much less miconazole compared with discs either with 0.5 bilayer coating or without coating in the biofilm testing period. Thus, the 10.5 bilayers coating might have acted as a barrier to miconazole release,[10] thereby reducing biofilm-controlling potency. These results provide important insights for the design of anticandidal dentures and other related medical devices in the future and suggest the possibility of using multiple classes of antifungal agents to control biofilm formation.

The potential cytotoxic effects of H-5/HA LBL coatings on denture materials were evaluated using rat skin fibroblasts (ATCC CRL-1213) and the XTT assay as specified by ISO 10993-5 with Triton X-100 as a positive control. Pristine PMMA and g-PMAA discs, with or without 10.5 bilayers H-5/HA coatings, were individually immersed in culture media for 1 or 3 days and the conditioned media were then added to fibroblast cultures to assess their

effect. Compared to control media, fibroblast viability was not significantly affected by any of the conditioned media, suggesting that H-5/HA LBL coatings had no cytotoxic effects on the cells (see Fig. S2 in Supporting Materials). This result was not completely unexpected since PMMA is one of the most commonly used denture base resins and has a proven safety record.[37, 38] PMAA has also been widely used in the preparation of drug delivery systems and surface modification of various biomaterials.[39, 40] H-5 is a component of saliva, and HA is a component of extracellular matrix and synovial fluid. All of these factors contribute to the outstanding biocompatibility of the LBL coatings on the denture materials. Nevertheless, the long-term safety of the coatings should be further evaluated using animal models and potentially clinical trials in future studies.

In summary, we demonstrated that fungal biofilm-controlling functionality can be introduced onto PMMA-based denture materials by grafting PMAA to the surface, followed by H-5/HA bilayer coating using LBL self-assembly technology. We found that hydrophilic surface alone was ineffective in controlling *Candida* biofilm formation. Although H-5 in the multilayer coatings did not provide fungicidal activity in the direct contact anticandidal tests, its antifungal activity significantly reduced the initial adhesion of fungal cells and effectively prevented biofilm formation. Thus, H-5 in the coatings acted as a "fungal repellent" to control *Candida* colonization and biofilm formation. Furthermore, the anti-adhesion and biofilm-controlling effects were stable for longer than 4 weeks and H-5/HA bilayer coatings synergized with clinical anticandidal drugs, such as miconazole, to provide even more potent anticandidal activity. In addition, the H-5/HA LBL coatings on denture materials demonstrated excellent biocompatibility toward mammalian cells. These results suggest that this strategy has great potential for use in developing new anticandidal denture materials and a wide range of other related medical devices to prevent fungal biofilm formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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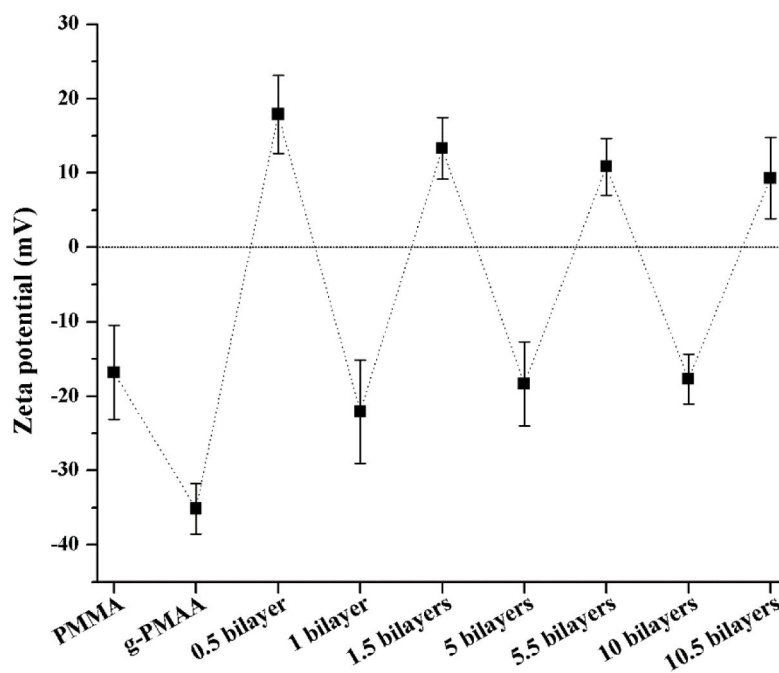40. Cheng R, Meng F, Deng C, Klok HA, Zhong Z. Biomaterials. 2013; 34: 3647–3657. [PubMed: 23415642]

Fig. 1.
Zeta potentials of the pristine PMMA, g-PMAA, and the H-5/HA multilayer coatings on g-PMAA
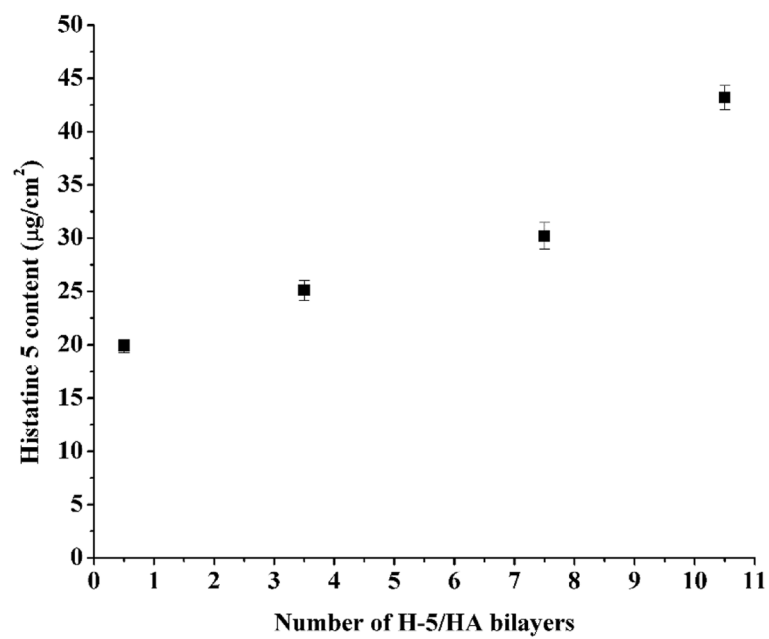
Fig. 2.
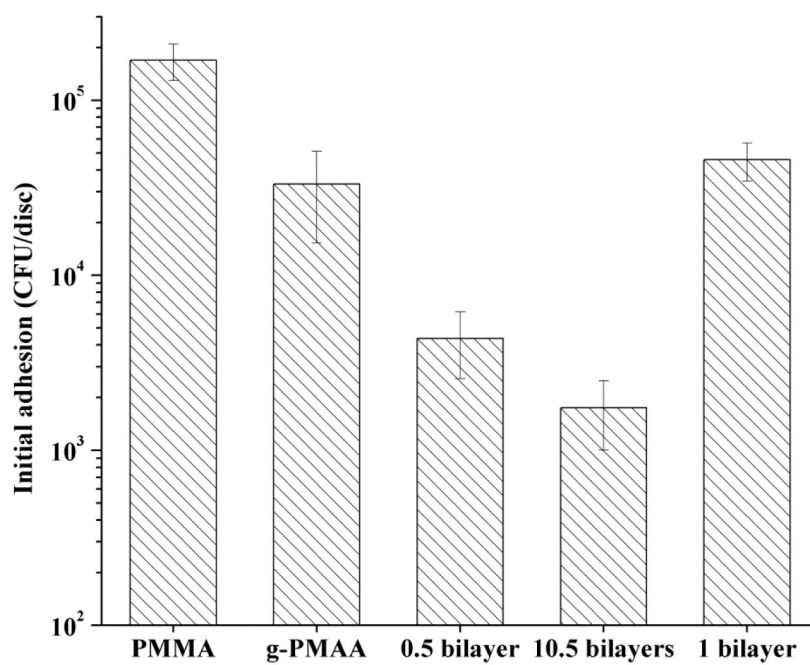H-5 content in the H-5/HA multilayer coatings on g-PMAA

Fig. 3.
Initial adhesion levels of *Candida* on pristine PMMA, g-PMAA, and H-5/HA LBL coatings on g-PMAA after 1 h of immersion in a *Candida* suspension.

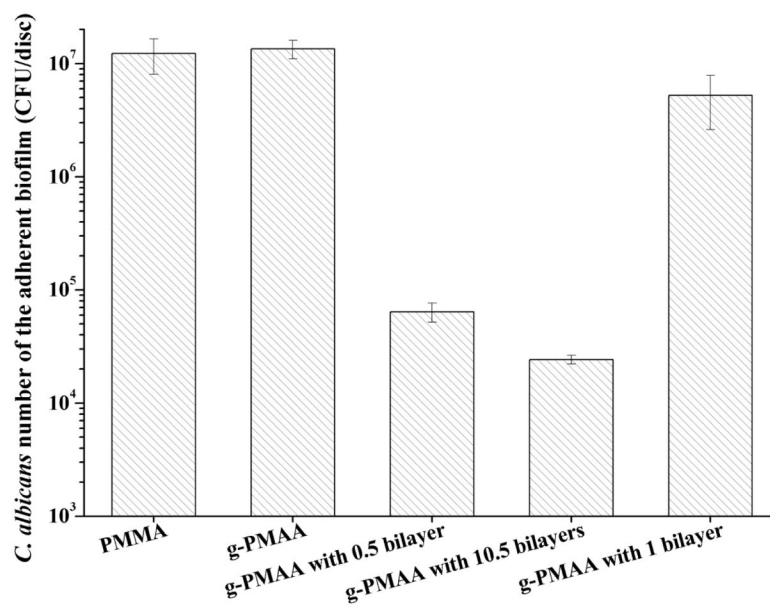**Fig. 4.**
Levels of recoverable adherent *Candida* in biofilms on different denture discs after two days of culture in YM broth
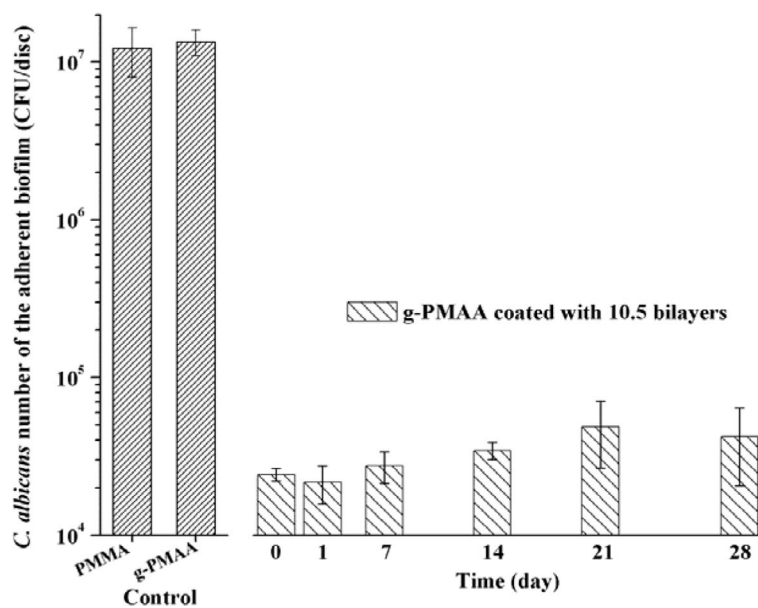
Fig. 5.
Biofilm-controlling effects of pristine PMMA, g-PMAA, and g-PMAA coated with 10.5 bilayers of H-5/HA after different periods of immersion in PBS at 37°C with constant shaking
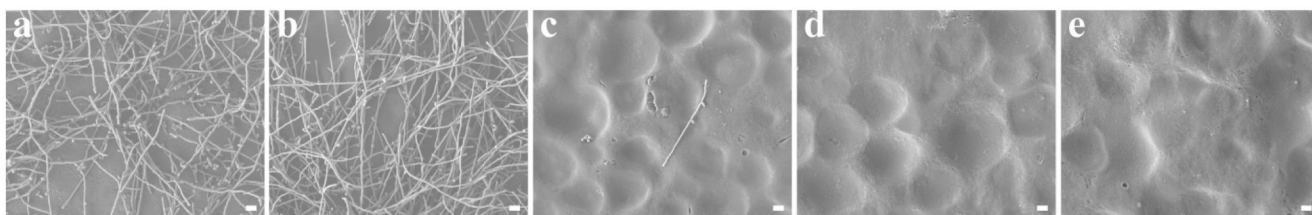
Fig. 6.
SEM images of biofilms formed on (a) pristine PMMA; (b) g-PMAA; (c) g-PMAA with 0.5 bilayer coating; (d) g-PMAA with 10.5 bilayer coating; and (e) g-PMAA with 10.5 bilayers coating after immersion in PBS at 37°C for 4 weeks (bar = 10 μm)
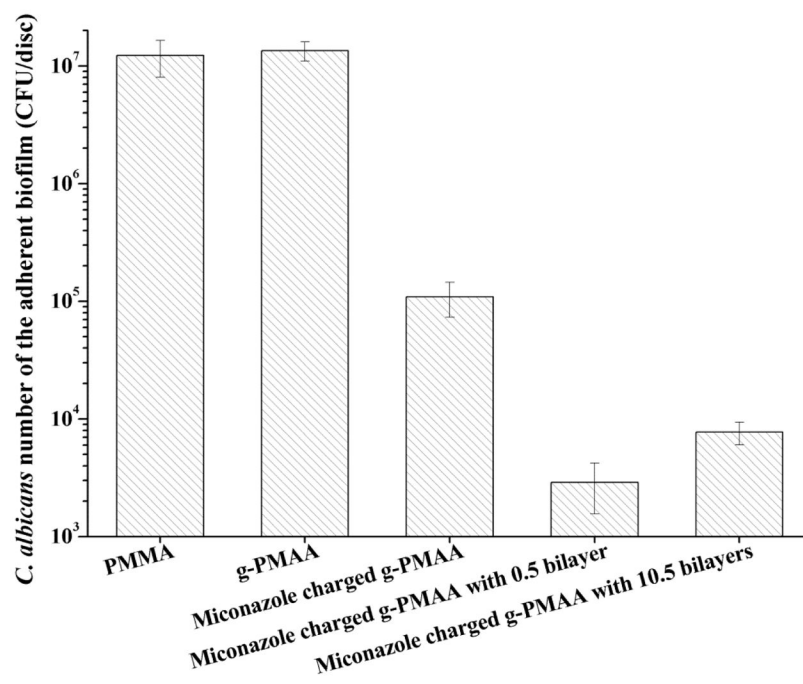
Fig. 7.
Levels of recoverable adherent *Candida* in biofilms on different denture discs after two days of culture in YM broth