

Supporting Information

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

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EXPERIMENTAL SECTION

Materials. Miconazole was purchased from Fisher Bioreagents (Fairlawn, NJ, USA). Histatin 5 (H-5; H-Asp-Ser-His-Ala-Lys-Arg-His-His-gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His- Ser-His-Arg-Gly-Tyr-OH; MW: 3036.66; > 95% purity) was purchased from Genemed Synthesis, Inc. (San Antonio, Texas). Hyaluronic acid (HA; MW of 1.01 MDa) was from Lifecore Biomedical (Chaska, MN, USA). Micro BCA™ protein assay kit was provided by Thermo Scientific (Waltham, MA, USA). XTT cell proliferation Kit II reagent was obtained from Roche Diagnostics (Indianapolis, IN, USA). All other chemicals were reagent grade or higher, purchased from Sigma-Aldrich (St. Louis, MO, USA), and used as received. *Candida albicans* (*C. albicans*, ATCC 10231) and rat skin fibroblasts (ATCC CRL-1213) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Artificial saliva was used to mimic the electrolytes and viscosity of human saliva and the formula was [CaCl₂-2H₂O (2.55 mM), MgCl₂-6H₂O (0.61 mM), KCl (16.0 mM), NaCl (14.5 mM), NaHPO₄-12H₂O (6.98 mM), sorbic acid (8.9 mM), sodium carboxymethylcellulose (5 g/L), and sorbitol (236 mM); pH was adjusted to 7.0] following previous reports.¹ Whole saliva (oral fluids) was obtained from healthy human subjects (ages 27–52 years; 4 females and 4 males) while chewing paraffin wax for 15 min with approval by the Institutional Review Board (IRB) at the University of Texas Health Science Center at San Antonio (UTHSCSA) (Protocol: HSC20120105H, renewed 02/10/2015). The collected saliva was immediately centrifuged (2000 g) for 15 min at 4 °C, the supernatant was passed through 0.20 µm filter and diluted with PBS to get 50% human saliva, which was frozen at -20 °C until use.¹

Instruments. Water contact angle was measured on a VCA optima contact angle analyzer (AST Products, MA, USA) using the sessile drop technique. Reported water contact angles were the average of measurements taken at different locations on the surface using 1 μ L pure water drops (n=6). Zeta potential of the denture disc surfaces was determined with a DelsaNano-HC particle analyzer (Beckman Coulter) with a flat surface cell, using polystyrene (PS) latex in 10 mM sodium chloride solution as the probing particles (n=4). SEM images were obtained on a field-emission scanning electron microscope (FE-SEM, JEOL JSM 7401) to check for the presence of *Candida* biofilms.

Grafting PMAA onto PMMA-based denture materials. Lucitone 199 (Dentsply Intl., York, PA), a two-component resin, composed of PMMA powder and a liquid containing methyl methacrylate (MMA), with ethylene glycol dimethacrylate (EGDMA) as the crosslinking agent, was used to fabricate poly(methyl methacrylate) (PMMA) denture discs according to the manufacturer's instruction.² Briefly, after mixing the two components, denture resin discs were fabricated through heat-polymerization (90 min at 73 °C and then 30 min at 100 °C). The resulting PMMA denture discs (1.0 cm of diameter and 0.13 cm of thickness) were functionalized with poly (methacrylic acid) (PMAA) on the surface through plasma-initiated grafting polymerization.² Briefly, PMMA denture discs were dipped individually into an acetone solution containing 20 wt% of methacrylic acid (MAA) and diurethane dimethacrylate (DUMA, 5 wt% of the monomer, as a crosslinker) at ambient temperature until the discs increased to 120% of their original weights. The discs were taken out, air-dried for 2 h, and then subjected to plasma treatment for 30 min at high RF power output in a plasma cleaner (Harrick Plasma, Ithaca, NY) on each side. Thereafter, the discs were thoroughly washed with

ethanol and distilled water, air-dried overnight, and then stored in a desiccator for 3 days to reach constant weights. Grafting yields of the discs were calculated according to the following equation (n=5):

$$\text{grafting yield} = (W_1 - W_0)/A$$

where W_0 is the weight of the original discs, W_1 is the weight of the PMAA-grafted discs (g-PMAA), and A is the surface area of the discs.

Coating H-5/HA multilayers on g-PMAA through LBL self-assembly. Stock solutions of H-5 and HA with a concentration of 0.1% (w/v) were prepared individually in phosphate buffered saline (PBS, pH 7.4). The g-PMAA discs were first immersed in the H-5 solution for 20 min, rinsed thoroughly with deionized water, and then air dried. The resulting discs coated with one layer of H-5 were designated as g-PMAA with 0.5 bilayer coating. To build multilayers on the discs using LBL self-assembly, g-PMAA/H-5 discs were immersed in HA solution for 20 min, rinsed with deionized water, and air dried. The resulting discs, g-PMAA/H-5/HA, named as g-PMAA coated with 1 bilayer (HA as the outermost layer), were coated with H-5 again using the same method to produce g-PMAA/(H-5/HA)₁/H-5, named as g-PMAA coated with 1.5 bilayers (H-5 as the outermost layer). Discs with even more layers, e.g., g-PMAA coated with 5 bilayers (HA as the final layer) or g-PMAA coated with 10.5 bilayers (H-5 as the final layer), were prepared similarly.

H-5 content in the multilayer coatings was quantified with the colorimetric micro bicinchoninic acid (BCA) protein assay following the procedure reported previously.^{3, 4} Briefly, the adsorbed H-5 in the LBL coatings was removed by shaking in 2ml of 1%

sodium dodecyl sulfate (SDS) solution for 6 h at room temperature followed by ultrasonication for 60 min. The H-5 content in the SDS solution was quantified with the Micro BCA™ protein assay kit (Thermo Scientific) by measuring the absorbance at 562 nm on a plate reader (Infinity M200 Pro, Tecan; n=3). The amount of H-5 present was determined using a standard curve prepared with H-5 at concentrations ranging from 0.5 to 200 µg/ml.

Pre-charging g-PMAA with miconazole. Miconazole, a widely used antifungal drug was selected to pre-charge the g-PMAA discs. Drug loading content was determined using a UV-vis spectrophotometer (Beckman Coulter DU 520) at 280 nm after immersing the g-PMAA discs into 0.5 mg/mL of miconazole solution in ethanol for a period of 2 days at ambient temperature (n=4).² Afterwards, H-5/HA multilayer coatings were built on the miconazole-containing g-PMAA discs using the LBL self-assembly method as described above.

Drug releasing. A series of miconazole-containing discs without coating and with 0.5 and 10.5 bilayers coatings were individually immersed in 5 mL sterile PBS at pH 7.4 and 37 °C with constant shaking (30 rpm). PBS was changed daily during the entire releasing period. Every 24 h, the contents of miconazole in the PBS solution were measured following a previously reported method (n=4).²

Direct contact mode anticandidal test. A direct contact mode “sandwich assay” was used to test the fungicidal effects of the multilayer coatings on g-PMAA, using pristine PMMA as controls. Briefly, an aliquot of 2.5 µL of a *Candida* suspension in PBS (10⁸-10⁹ CFUs/mL) was placed onto each disc surface. The disc was then “sandwiched” using

a second identical disc. Caution was taken to ensure that all of the *Candida* suspension was covered between the two discs during the experiments. After various periods of time, the entire “sandwich” was transferred to 2 mL sterile PBS. The mixture was sonicated for 5 min and then vortexed for 60 s. Our preliminary studies found that these treatments detached all *Candida* without affecting fungal growth. Antifungal activity was then determined by counting the CFUs present (after the sonication and vortexing steps) using YM agar plates incubated at 25 °C for 48 h (n=4).

Fungal initial adhesion test. A series of pristine PMMA, g-PMAA, and g-PMAA coated with H-5/HA multilayers were immersed individually in 10^{8-9} CFUs/mL of *Candida* suspension in PBS. After shaking (30 rpm) at 25 °C for 1 h, discs were aseptically removed from the *Candida* suspension, and gently washed three times with sterile PBS to remove any non-adherent fungi. The discs were individually sonicated in 2 mL of sterile PBS for 5 min and then vortexed for 60 s to transfer the adherent fungi into PBS. *Candida* initial adhesion levels were then determined by counting the CFUs after culture on YM agar plates (n=4), as described above.

Biofilm-controlling test. After 1 h of initial adhesion, the *Candida*-containing discs were individually incubated in YM broth for 2 days at 25 °C to allow for biofilm formation. Afterwards, the discs were washed with sterile PBS to remove non-adherent *Candida*. Some of the discs were used to examine the *Candida* biofilm formation level by counting the CFUs after inoculating the recovered fungi on YM agar plates as described above (n=4). The remaining discs were observed under a field-emission scanning electron microscope (FE-SEM) to check for the presence of *Candida* biofilms.

Biofilm-controlling test was also performed in artificial saliva and 50% human saliva, respectively. 10^{8-9} CFUs/mL of *Candida* suspensions in artificial saliva or 50% human saliva were prepared, and various denture discs with or without H-5/HA LBL coatings were immersed individually in these suspensions respectively for 1 h under shaking (30 rpm) at 25 °C. Thereafter, the resulting denture discs were allowed for biofilm formation for 2 days as described above.

Cytotoxicity test. The cytotoxicity of various denture materials was evaluated with rat skin fibroblasts (ATCC CRL-1213) using colorimetric assays for metabolic activity (XTT assay) according to the method specified by ISO 10993-5, using culture medium supplemented with 1% Triton X-100 as a positive control. In brief, various denture materials were individually immersed (a surface/volume ratio of 3 cm²/mL) in cell culture medium (DMEM with 10% fetal bovine serum, FBS) at 37 °C for 1 and 3 days, respectively, with shaking. Rat skin fibroblasts were cultured in DMEM (with 10% FBS) at 37 °C in a humid atmosphere with 5% CO₂. At confluence, cells were trypsinized, centrifuged, and resuspended in culture medium. An aliquot of 100 µL of the cell suspension was seeded into 96-well plates (1×10^4 cells per well) and precultured for 24 h. The culture media were then replaced with conditioned media from the denture materials. After 24 h of incubation, 50 µL of XTT reagent was added to each well and the plates incubated in the dark for another 4 h at 37 °C. Afterward, the absorbance of the solution in each well was measured at 490 nm with a reference wavelength of 690 nm using a plate reader (Infinity M200 Pro, Tecan). Cells incubated in normal culture media (without pre-conditioning by incubation with the denture materials) were tested under the same conditions to serve as negative controls (n=4).

Statistical analyses. The data were representative of the results from at least three independent experiments and were expressed as mean $\pm$ standard derivation. Statistical comparisons to identify significant differences between two groups were performed using Student's *t*-test and differences were considered significant when $P < 0.05$.

Supporting Table

Table S1. Levels of recoverable adherent *Candida* in biofilms on different denture discs after 1 h of immersion in *Candida* suspension in artificial saliva and 50% human saliva followed by two days of culture in YM broth

	PMMA (CFU/disc)	g-PMAA (CFU/disc)	0.5 bilayer (CFU/disc)	10.5 bilayers (CFU/disc)
artificial saliva	$(1.27 \pm 0.10) \times 10^7$	$(1.17 \pm 0.13) \times 10^7$	$(7.24 \pm 0.89) \times 10^4$	$(3.01 \pm 0.94) \times 10^4$
50% human saliva	$(1.36 \pm 0.18) \times 10^7$	$(3.66 \pm 2.12) \times 10^6$	$(8.61 \pm 0.69) \times 10^4$	$(4.04 \pm 1.24) \times 10^4$

Table S2. The drug release profiles of miconazole-containing discs without coating and with 0.5 and 10.5 bilayers coatings. (MIC of miconazole against *Candida*: 0.125µg/mL)

	g-PMAA (µg/mL/24h)	0.5 bilayer (µg/mL/24h)	10.5 bilayers (µg/mL/24h)
Day 1	0.317 ± 0.056	0.258 ± 0.070	0.118 ± 0.031
Day 2	0.454 ± 0.040	0.395 ± 0.053	0.179 ± 0.054
Day 3	0.487 ± 0.080	0.385 ± 0.068	0.188 ± 0.013

Supporting Figure

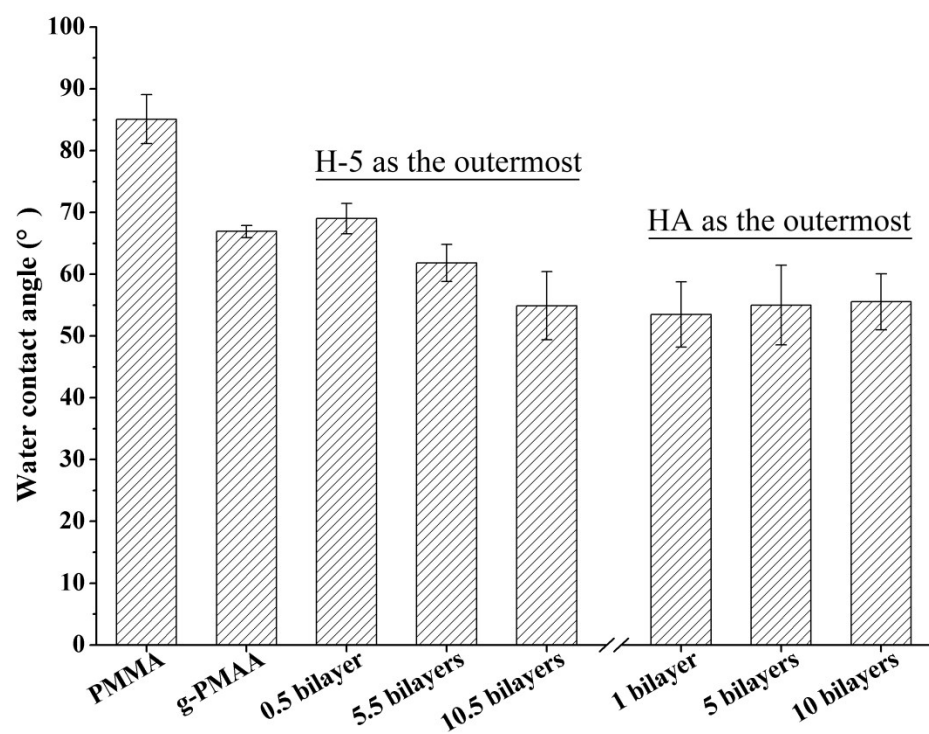


Figure S1. Water contact angles of pristine PMMA, g-PMAA, and the H-5/HA multilayer coatings on g-PMAA

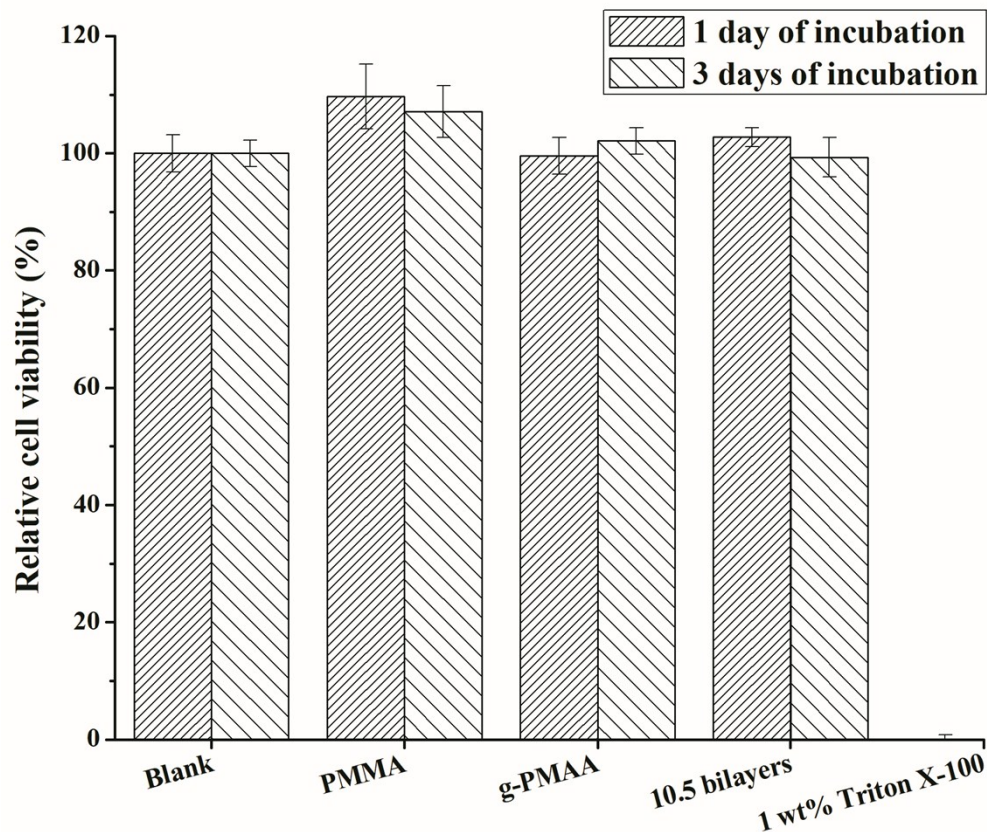


Figure S2. Effect of denture base materials and surface coatings on rat skin fibroblast viability. Discs of pristine PMMA, g-PMAA, and g-PMAA with H-5/HA multilayer coatings were conditioned in culture media for 1 or 3 days. These media were then added to rat skin fibroblast cultures to test their effect on cell viability using the XTT assay.

Data were normalized to culture media not exposed to denture resins (=100% cell viability).

References

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