



# HHS Public Access

Author manuscript

*Adv Healthc Mater.* Author manuscript; available in PMC 2016 February 18.

Published in final edited form as:

*Adv Healthc Mater.* 2015 February 18; 4(3): 469–475. doi:10.1002/adhm.201400428.

## N-trimethylchitosan/Alginate Layer-by-Layer Self Assembly Coatings Act as “Fungal Repellents” to Prevent Biofilm Formation on Healthcare Materials

**Dr. Fuguang Jiang,**

Department of Chemistry, University of Massachusetts, Lowell, MA 01854, USA

**Prof. Chih-Ko Yeh,**

Department of Comprehensive Dentistry, University of Texas Health Science Center at San Antonio, and Geriatric Research, Education and Clinical Center, Audie L. Murphy Division, South Texas Veterans Health Care System, San Antonio, TX 78229, USA

**Dr. Jianchuan Wen, and**

Department of Chemistry, University of Massachusetts, Lowell, MA 01854, USA

**Prof. Yuyu Sun**

Department of Chemistry, University of Massachusetts, Lowell, MA 01854, USA

Chih-Ko Yeh: yeh@uthscsa.edu; Yuyu Sun: yuyu\_sun@uml.edu

### Abstract

Fungal biofilm formation on healthcare materials is a significant clinical concern, often leading to medical device related infections, which are difficult to treat. A novel fungal repellent strategy is developed to control fungal biofilm formation. Methylacrylic acid (MAA) is grafted onto poly methyl methacrylate (PMMA)-based biomaterials *via* plasma initiated grafting polymerization. A cationic polymer, trimethylchitosan (TMC), is synthesized by reacting chitosan with methyl iodide. Sodium alginate (SA) is used as an anionic polymer. TMC/SA multilayers are coated onto the MAA-grafted PMMA via layer-by-layer self-assembly. The TMC/SA multilayer coatings significantly reduce fungal initial adhesion, and effectively prevent fungal biofilm formation. It is concluded that the anti-adhesive property of the surface is due to its hydrophilicity, and that the biofilm-inhibiting action is attributed to the antifungal activity of TMC as well as the chelating function of TMC and SA, which may have acted as fungal repellents. Phosphate buffered saline (PBS)-immersion tests show that the biofilm-modulating effect of the multilayer coatings is stable for more than 4 weeks. Furthermore, the presence of TMC/SA multilayer coatings improve the biocompatibility of the original PMMA, offering a simple, yet effective, strategy for controlling fungal biofilm-formation.

### Keywords

Trimethylchitosan; Alginate; Layer-by-Layer coatings; Fungal biofilm

---

Correspondence to: Yuyu Sun, yuyu\_sun@uml.edu.

## 1. Introduction

Biofilms are an accumulation of microbes, and a polymeric matrix they produce, that adheres to material surfaces bathed in fluids. Both bacteria and fungi colonize the surfaces of biomaterials used clinically and form biofilms associated with various infections. Although bacterial biofilms have been extensively studied, fungal biofilms have received much less attention.<sup>[1–8]</sup> Recently, because of the increasing use of antibiotics and indwelling devices, and the growing number of aged and/or immunocompromised patients, fungal biofilm associated infections have emerged as a significant clinical problem with devastating consequences.<sup>[2–8]</sup> A wide range of medical and dental devices such as dentures, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints, central nervous system shunts, *etc.*,<sup>[1]</sup> have been reported to be colonized by fungal biofilms, resulting in serious device-related infections with high morbidity and mortality, extended hospital stays, and high costs.<sup>[2–8]</sup> One of the most troubling characteristics of these types of infections is that the fungal cells living inside the biofilm are much more resistant to antifungal drugs than their planktonic counterparts, due to the expression of multidrug efflux pumps, alterations in membrane structure, and the effect of the extracellular matrix (*i.e.*, the biofilm) itself.<sup>[5,6,9–11]</sup>

In our continuing effort to design and fabricate antifungal materials for clinical use,<sup>[12–14]</sup> we discovered that the layer-by-layer (LBL) self-assembly of trimethylchitosan/sodium alginate (TMC/SA) multilayer surface coatings on various clinically relevant biomaterials effectively prevented fungal biofilm formation. Trimethylchitosan (TMC) is one of the most widely used water-soluble cationic derivatives of chitosan. TMC is antimicrobial, biocompatible, and bioadhesive, and has been used in tissue engineering, drug/gene delivery, and wound dressing applications.<sup>[15–17]</sup> Sodium alginate (SA) is a water-soluble anionic natural polymer isolated from seaweed and various microbial sources. It is biocompatible and mucoadhesive and has been widely used in the food industry; it has also been used in drug delivery, tissue engineering, and nerve repair applications.<sup>[18–22]</sup> LBL is a simple, yet versatile technique for assembling charged macromolecules, in which polyelectrolytes of opposite charges are placed one layer at a time alternatively on top of each other.<sup>[23–30]</sup>

This study describes the use of TMC/SA LBL coatings in inhibiting the formation of fungal biofilms. Although fungal cells readily attached to unmodified poly methyl methacrylate (PMMA) and form a biofilm, they failed to do so on the TMC/SA LBL coated PMMA surface. Thus, the TMC/SA multilayer coating acted as a fungal repellent and blocked biofilm formation. These results provide new insight into an alternative biomaterial design for reducing the risk of various device/material-related infections.

## 2. Results and Discussion

### 2.1. Fabrication of TMC/SA LBL Coatings onto PMMA-based Biomaterials

In this study, a PMMA-based polymer, Lucitone 199 (Dentsply Intl, York, PA), was used as a representative, clinically relevant material. PMMA is a versatile polymer with multiple dental and medical applications, including complete denture bases, bone cements, screws for

bone fixation, fillers for bone cavities/skull defects, and vertebral stabilization in osteoporotic patients,<sup>[32]</sup> where fungal biofilm formation has become a growing concern.<sup>[1–14]</sup> To introduce anionic groups onto the PMMA surfaces for TMC binding and subsequent TMC/SA LBL coating, methacrylic acid (MAA) was grafted onto the PMMA polymer. The grafting reaction was carried out in a plasma cleaner as described by us previously.<sup>[12]</sup> Although plasma treatment alone (without MAA) also introduces anionic functional groups, this effect is short-lived (*e.g.*, last for minutes) because the functional groups can diffuse into the bulk polymer to minimize the free energy of the surface. Thus, to maintain stable anionic groups on the surface, MAA-based anionic polymer chains were covalently bound to the PMMA surface during plasma treatment.<sup>[12]</sup>

The effect of varying the reaction conditions on MAA grafting is shown in the Supporting Information (Figure S-1). TMC/SA multilayers were then readily built on the grafted PMMA (g-PMMA) surface. Surface charge was characterized with zeta potential analysis. As shown in Figure 1a, the untreated PMMA control had a zeta potential of  $0.77 \pm 0.67$  mV, indicative of a nearly neutral surface. After MAA grafting (grafting yield:  $1.54 \pm 0.47$  wt%), the zeta potential of g-PMMA changed to  $-13.52 \pm 0.34$  mV (a negatively charged surface). Upon treatment with TMC, the zeta potential became  $12.06 \pm 3.81$  mV, indicating that the positively charged TMC had been successfully applied to the surface. The coating of SA onto the TMC surface reversed the surface charge (zeta potential =  $-11.52 \pm 3.10$  mV). Predictably, it was observed that surface polarity changed with each subsequent treatment (*i.e.*, if the final layer was TMC, a positively charged surface was obtained; if the final layer was SA, the surface carried a negative charge).

The properties of the multilayer coatings were further assessed with contact angle measurements (Figure 1b). PMMA control discs had a contact angle of  $99.23 \pm 1.78^\circ$ , indicative of a hydrophobic surface. After MAA grafting, the contact angle of g-PMMA decreased to  $87.55 \pm 5.29^\circ$ ; with one layer of TMC coating, the contact angle was  $86.54 \pm 2.59^\circ$ . As the number of coatings increased, the contact angle significantly decreased. The sample coated with 10.5 bilayers (10 TMC/SA bilayers plus a TMC final layer) had a contact angle of  $68.90 \pm 1.33^\circ$  ( $p < 0.05$ ). The resulting decrease in contact angle (and thus increase in hydrophilicity) with additional TMC/SA coating was due to the introduction of various groups, such as -OH, -COOH, -NH<sub>2</sub>, and -N(CH<sub>3</sub>)<sub>3</sub> onto the surface of the PMMA discs.

The presence of various functional groups on the surface was characterized using Fourier transform spectroscopy (FT-IR) in attenuated total reflection (ATR) mode (Figure 1c). The unmodified PMMA disc had a strong absorption at  $1732 \text{ cm}^{-1}$ , which is characteristic of carbonyl groups. After MAA-grafting and one layer of TMC coating, little difference could be detected in the ATR spectra; this could be explained by the low amount of new functional groups introduced onto the surface.

With the addition of more layers, the presence of TMC and SA functional groups became detectable. For example, the spectrum of g-PMMA with 10.5 TMC/SA bilayers showed a broad band at  $3200\text{--}3500 \text{ cm}^{-1}$ , which was related to the hydroxyl groups and amino groups of SA and TMC, in addition to the  $1732 \text{ cm}^{-1}$  band of the original PMMA, and a  $1605 \text{ cm}^{-1}$

band which was associated with the carboxylic groups of SA. All of these findings suggest that TMC/SA multilayers were successfully coated onto the PMMA-based biomaterial.

## 2.2. Effect of TMC/SA LBL Coatings on Initial Fungal Adhesion and Biofilm Formation

*Candida albicans* (*C. albicans*, ATCC 10231) was used for proof of principle studies. This is a diploid fungus that grows both as yeast and filamentous cells; it readily colonizes a wide range of materials used clinically, including PMMA, and forms biofilms which cause device-related infections.<sup>[1-11]</sup> The *Candida* adhesion study was performed for 1h in phosphate buffered saline (PBS) at 37°C and the level of adherent cells was determined by measuring general metabolic activity with the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) assay.

We used this assay instead of the CFU (colony forming unit) determination method because sessile *Candida* cells isolated from biofilms can have different modes of growth from their free-floating counterparts, making the CFU determination method inaccurate.<sup>[5]</sup> All surface-modified PMMA samples showed significantly lower amounts of *Candida* than the control ( $p < 0.05$ ; Figure 2); differences between the modified surfaces were not statistically significant.

These results suggest that polar and hydrophilic surfaces reduce initial *Candida* adhesion. In subsequent biofilm-formation studies, materials with adherent *Candida* were incubated in yeast and mold (YM) broth for 2 days to allow biofilm formation. In contrast to our expectation based on the initial adhesion studies (Figure 2), g-PMMA did not provide any noticeable effect in controlling biofilm formation; in particular, the level of biofilm was very similar to that of the original PMMA control (Figure 3). Similarly, there was no apparent anti-biofilm effect observed with surfaces containing a single layer of TMC coating. However, anti-biofilm activity gradually increased as more layers of the TMC/SA coating was added, e.g., the metabolic activity of *Candida* on g-PMMA with 5.5 bilayers and 10.5 bilayers was 54.78% and 11.96%, respectively, of the PMMA control ( $p < 0.05$ ).

These findings suggest that different surface properties affect anti-adhesion and anti-biofilm activities. It is known that *Candida* favors hydrophobic surfaces for colonization and this is one of the reasons why this fungus readily forms biofilms on PMMA-based materials.<sup>[1-12]</sup> After MAA grafting and/or TMC/SA LBL coating, the PMMA surfaces became more hydrophilic, resulting in lower initial adhesion. TMC has known biocidal activity.<sup>[15-17]</sup> To further evaluate the influence of TMC on *Candida* adhesion and growth, a “sandwich assay” was used<sup>[12]</sup> to evaluate the effect of continuous contact with PMMA (or a PMMA-modified surface) on fungal metabolic activity (Figure 4).

After one hour of direct contact with the surface coated with 10.5 bilayers (10 TMC/SA bilayers plus a TMC final layer), 77.2% of the metabolic activity of the PMMA control remained. When contact time was extended to 2h or 4h, the metabolic activity on the 10.5 bilayers decreased to 47.9% and 19.9%, respectively, of the PMMA control levels. Additional increases in contact time failed to reveal any further antifungal activity. Based on these observations, we conclude that the anti-adhesive effect of the LBL modified PMMA is primarily mediated by surface hydrophilicity (lower contact angles). Since the initial

adhesion test was only for 1h, there was likely not enough time for us to observe significant antifungal effect due to the TMC coating. This explains our observation that although all the surface-modified PMMA samples showed significantly lower level of adherent *Candida* than the control ( $p < 0.05$ ; Figure 2); differences between the modified surfaces (with different layers of TMC) were not statistically significant.

On the other hand, the hydrophilicity provided by g-PMMA appears to be ineffective at preventing *Candida* growth and/or biofilm formation since robust biofilm growth on g-PMMA was evident after the attached *Candida* had been cultured for 2 days (Figure 3). In contrast, there was a potent anti-biofilm effect displayed by the TMC/SA coated surfaces in the same assays, which was likely due to the antifungal effects of TMC. Further, both TMC and SA are polymeric chelators that can sequester divalent cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . [15–22] Since these cations are essential for stabilizing the polymeric extracellular matrix (ECM) structure of the biofilm synthesized by the fungus,<sup>[35]</sup> it is possible these chelating effects might have also contributed to the observed anti-biofilm properties.

To determine the anti-biofilm activity of the final coating (TMC or SA), PMMA control, g-PMMA coated with 10 bilayers (SA as final layer), and g-PMMA coated with 10.5 bilayers (TMC as final layer) were tested in biofilm-controlling studies following initial adhesion. After two days of incubation, the metabolic activity of *Candida* on PMMA controls was significantly higher ( $p < 0.05$ ) than on the 10-bilayer and 10.5-bilayer discs (Figure 5a), indicating a significantly higher level of adherent *Candida* on the PMMA control. The metabolic activity on the 10.5 bilayers was slightly lower (not statistically significant) than that on the 10 bilayers, suggesting that in addition to the direct antifungal activities of TMC, the chelating effects TMC and SA could also provide the biofilm-controlling activity.

The metabolic activity of *Candida*, suspended in *broth solutions* containing the corresponding PMMA-based samples, was also determined using the XTT assay (Figure 5b). Interestingly, the broth solutions containing PMMA control discs had significantly less *Candida* metabolic activity than broth solutions containing the other two types of discs ( $p < 0.05$ ). Further, the fungi in the 10.5-bilayer broth showed significantly higher metabolic activity than those in the 10-bilayer broth ( $p < 0.05$ ).

These results suggest that when *Candida* cells were given a preference between the broth and the PMMA surface (without TMC/SA coatings), the fungal cells favored the PMMA surface for growth and biofilm formation. However, if the surface of the PMMA discs is modified with the TMC/SA multilayers, biofilm formation is affected because of the antifungal effects of the TMC coating and the chelating effects of TMC/SA. In this case, the TMC/SA LBL coatings acted as fungal repellents to prevent *Candida* biofilm formation. Because TMC had both antifungal effects and chelating effects, using TMC as the final layer led to even more potent repelling effects than using SA as the final layer. To the best of our knowledge, this is the first demonstration of a process for modifying a surface that has fungal repelling activity, rather than fungicidal effect, to achieve anti-fungal biofilm properties, offering a new, drug-free strategy in controlling biofilm formation on healthcare materials.

Biofilm-controlling effects were also directly observed using SEM (scanning electron microscope). As shown in Figure 6, after two days of incubation, a large amount of *Candida* adhered to the surface of unmodified PMMA (Figure 6 A), forming layered clusters with the presence of filamentous cells (black arrows), suggesting the formation of a fungal biofilm. On g-PMMA (Figure 6 B), *Candida* adhered and aggregated on the surface, but no filamentous cells were observed. The introduction of TMC/SA coatings led to much less *Candida*; only scattered *Candida* cells were evident with no biofilm formation. Few scattered *Candida* with abnormal appearances (shapes/sizes) and its fragments were observed on the disc surfaces. These findings strongly suggest that TMC/SA multilayer coatings effectively prevented biofilm formation.

The prolonged biofilm-controlling effect of TMC/SA multilayer coatings was evaluated by immersing a series of g-PMMA discs coated with 10.5 bilayers in PBS at 37 °C for up to 4 weeks. The anti-biofilm activity of the coating was essentially unchanged during this time period (Figure S-2 in Supporting Information), suggesting that this surface modification strategy has potential for controlling fungal biofilm formation on clinically relevant biomaterials over extended periods of time.

### 2.3. Biocompatibility of the TMC/SA LBL Coatings

It is well-established that PMMA-based materials have mild to moderate cytotoxic effects due to the leaching of residual un-polymerized monomers,<sup>[33,34]</sup> which can result in cell cycle delay and death. To determine the effects of the TMC/SA LBL coatings on cytotoxicity of the resulting materials, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate the samples (Table 1). The g-PMMA coated with 10.5 bilayers had no significant effect on cell growth, as compared to the PMAA control, except on Day 7; the percent of viable cells in the multilayer conditioned media was significantly higher than that in the PMMA conditioned media ( $p < 0.05$ ). The preparation of the g-PMMA discs and the subsequent coatings may reduce the content of the residual monomers in the original PMMA. Further, the presence of TMC/SA multilayer coatings may reduce residual monomer release. In both cases, improved biocompatibility would have resulted.

## 3. Conclusions

The development of biomaterials capable of preventing fungal biofilm formation is a daunting challenge. In this report, we demonstrate that fungal biofilm-controlling functionality can be introduced into a PMMA-based biomaterial by grafting MAA onto the surface, followed by TMC/SA LBL self-assembly coatings. These modifications dramatically alter the hydrophilicity and surface charge of the resulting materials. The TMC/SA multilayers significantly reduce fungal cell initial adhesion, and effectively prevent biofilm formation. The anti-adhesion activities are likely related to surface hydrophilicity, while the biofilm-controlling functions are mediated through the antifungal activities of TMC as well as the chelating effects of TMC and SA, which act as fungal repellents to prevent *Candida* biofilm formation. To the best of our knowledge, this is the first demonstration of a fungal repellent, based on an LBL coating strategy, which prevents biofilm formation. The anti-adhesion and biofilm-controlling effects were stable for at least



4 weeks. In addition, the introduction of TMC/SA multilayer coatings improved the biocompatibility of the original PMMA-based materials. These results indicate the great potential of this strategy for developing new materials that can be used in a wide range of biomedical applications to prevent fungal biofilm formation.

## 4. Experimental Section

### Materials

A PMMA-based polymer, Lucitone 199 (Dentsply Intl, York, PA), was used as a representative, clinically relevant material. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. *Candida albicans* (*C. albicans*, ATCC 10231) and rat skin fibroblast cells (ATCC CRL-1213) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

### Instruments

FT-IR spectra of the samples were recorded on a Nicolet iS10 Mid-IR spectrometer using the ATR mode. <sup>1</sup>H-NMR spectra were collected on a 600 MHz Bruker NMR spectrometer (D<sub>2</sub>O as solvent). Contact angle was measured using the VCA optima surface analysis system (AST, MA, USA); values for each surface were obtained by averaging six different measurements, under the same experimental conditions, in quadruplicate. Zeta-potential of the solid samples was determined with a DelsaNano-C particle analyzer with a flat surface cell assembly, using polystyrene (PS) latex in 10 mM sodium chloride solution as the probe particles. SEM observations were performed on a JEOL JSM 7401 FE-SEM.

### Grafting of methylacrylic acid (MAA) onto PMMA-based materials

PMMA-based Lucitone 199 discs (6.0 mm of diameter and 0.5 mm of thickness) were fabricated according to the manufacturer's instructions. The discs were dipped into acetone containing 0–20 wt% methylacrylic acid (MAA) and diurethane dimethacrylate (DUMA, 5 wt% MMA, as a crosslinker) with 1wt% azobisisobutyronitrile (AIBN) as an initiator at ambient temperature. After a certain period of time (30–120 min), the discs were taken out, air-dried for 60 min, and put in a plasma cleaner (Harrick Plasma, Ithaca, NY) at high RF level for 5–30 min of plasma treatment on each side to graft MAA onto PMMA. The resulting discs (g-PMMA) were washed with acetone and deionized water, air-dried for 24h, and stored in a desiccator for 3 days to reach an equilibrium weight. Grafting yield of MAA onto PMMA was calculated according to the following equation:

$$\text{Grafting yield \%} = (W_1 - W_0) / W_0 \times 100$$

where  $W_0$  was the weight of the original disc, and  $W_1$  was the weight of the g-PMMA.

### Preparation of trimethylchitosan (TMC)

TMC was synthesized following a previously described procedure<sup>[31]</sup> with slight modification. Briefly, 4.0 g chitosan were hydrated in 160 mL N-methylpyrrolidone at 90 °C for 24h. Afterwards, 4.8 g sodium iodide and 22.0 mL of 15wt % sodium hydroxide solution were added to the flask, followed by the addition of 11.5 mL methyl iodide. The mixture

was stirred at 90 °C for 18h and then poured into acetone. The precipitate was collected by filtration, washed with acetone, and dried at 50°C under vacuum. The product was dissolved in 5wt% sodium chloride (aqueous) solution to transform iodide ions to chloride ions. After precipitation from ethanol, TMC was collected, dried at 50 °C under vacuum, and stored in a desiccator. The degree of quaternization (28.0%) was determined by NMR studies.<sup>[31]</sup>

### **Fabrication of TMC/alginate LBL coatings onto the surfaces of the PMMA-based materials**

Stock solutions of TMC and SA with a concentration of 0.1% (w/v) were prepared individually in Tris buffer [0.1 M, tris(hydroxymethyl) aminomethane-hydrochloric acid, pH 7.0]. The g-PMMA discs were first immersed in the TMC solution for 20 min, rinsed thoroughly with deionized water, and then air dried. The resulting discs were designated as g-PMMA coated with one TMC layer. To build multilayers on the discs, using the LBL technique, g-PMMA-TMC discs were immersed in SA solution for 20 min, rinsed with deionized water, and air dried. The resulting discs, g-PMMA-TMC-SA, or g-PMMA coated with one bilayer (SA as the final layer), were coated with TMC again using the same method to produce g-PMMA-(TMC-SA)<sub>1</sub>-TMC, or g-PMMA coated with 1.5 bilayer (TMC as the final layer). Discs with even more layers, *e.g.*, g-PMMA coated with 5 bilayers (SA as the final layer) or g-PMMA coated with 10.5 bilayers (TMC with the final layer), were prepared similarly.

### **Effect of TMC/SA multilayer coatings on fungal initial adhesion**

*C. albicans* was used as a representative example of a fungus because of its prevalence in fungal biofilms and device-related infections.<sup>[1-11]</sup> To test the effect of the TMC/SA multilayer coatings on *Candida* initial adhesion, a series of PMMA, g-PMMA and g-PMMA coated with TMC/SA multilayers were immersed individually in 10<sup>8-9</sup> CFU/mL of *C. albicans* suspended in PBS. After shaking (30 rpm) at 30 °C for 1h, each disc was aseptically removed from the *Candida* suspension, and gently washed 3 times with sterile PBS to remove any non-adherent fungi. The discs were individually sonicated in 1 mL sterile PBS for 5 min and then vortexed for 60 s to release the adherent fungi into PBS. The level of recoverable *Candida* from each disc was determined using the XTT assay (n=4), as previously described.<sup>[5,12]</sup> Briefly, 100 µL of the resulting suspension were aseptically transferred to the well of a 96-well microplate, and 50 µL of the XTT cell proliferation Kit II reagent (prepared by mixing 5 mL of the XTT labeling reagent with 0.1 mL of an electron-coupling reagent, Roche) were added to each well. The mixture was incubated at 37 °C for 20h in dark. The absorbance of each well was measured using an Infinity M200 Pro plate reader at 490 nm. Each test was performed in triplicate.

### **Effect of the TMC/SA multilayer coatings on fungal biofilm formation**

The effect of the TMC/SA multilayer coatings on *Candida* biofilm formation was evaluated in parallel with the initial adhesion study (see above). After one hour of *Candida* adhesion, each disc was aseptically removed from the *Candida* suspension, gently washed 3 times with sterile PBS to remove non-adherent fungi, and placed in YM broth solution for 2 days at 30 °C to allow biofilm formation. Afterwards, the discs were washed with sterile PBS to remove non-adherent *Candida*. Some of the discs were used in the XTT assay, after



sonication and vortexing, to determine the level of adherent *Candida* on each disc as described above. The remaining discs were immersed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and stored at 4 °C overnight. At the end of treatment, samples were washed 3 times with PBS, followed by dehydration in a graded ethanol series (25%, 50%, 70%, 95%, and 100%; 10 min each). The dehydrated samples were air dried, sputter coated with Pd-Au, and viewed in a JEOL JSM 7401 FE-SEM to check for the presence of adherent *Candida*/biofilms.

*Candida* content in the broth solution was also tested with the XTT assay. Briefly, before disc removal, 100 µL of the broth were aseptically transferred to the well of a 96-well microplate, and 50 µL of the XTT cell proliferation Kit II reagent were added to each well. The mixture was incubated and the absorbance was measured according to the procedure as described above.

### **Antifungal activities of TMC/SA multilayer coatings on PMMA-based materials**

*Candida* were grown in YM broth, washed two times with sterile PBS using a centrifuge to pellet the cells, resuspended in PBS, and diluted to  $10^8$ – $10^9$  CFU/mL. Afterwards, 2.5 µL of the *Candida*, suspended in 0.1% Triton X-100 in PBS, were placed onto the surface of a disc containing a TMC/SA multilayer coating. The disc was then “sandwiched” using another identical disc. Caution was used to ensure that all of *C. albicans* suspension was covered between the two discs during the experiments. After various periods of time, the entire “sandwich” was transferred to 10 mL sterile PBS. The mixture was sonicated for 5 min and then vortexed for 60 s. Our preliminary studies found that these treatments detached most of the *Candida* without affecting fungal growth. Antifungal activity was determined using the XTT assay.

### **Durability of the biofilm-controlling effects of the TMC/SA multilayer coatings**

A series of g-PMMA discs coated with TMC/SA multilayers were immersed in PBS at 37°C under constant shaking for 4 weeks. Weekly, some of the discs were taken out, and immersed in a suspension of *Candida* in PBS, followed by incubation in YM broth, to reevaluate the biofilm-controlling effects of the coatings, using the same procedures as described above.

### **Biocompatibility of the TMC/SA multilayer coatings**

Biocompatibility was evaluated using rat skin fibroblasts (ATCC CRL-1213), as a representative mammalian cell, with the MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. A series of g-PMMA discs coated with TMC/SA multilayers were immersed in PBS at 37 °C under constant shaking for 1, 3, 5, and 7 days to prepare “disc conditioned media”. Rat skin fibroblasts were cultured in a media (DMEM with 10% FBS) at 37 °C in a humid atmosphere with 5% CO<sub>2</sub> and 95% air. At confluence, cells were trypsinized, and 100 µL of the cell suspension ( $5 \times 10^5$  cells/mL) were added to the well of a 96-well plate. The fibroblast cultures were grown for 24h as above and then treated with media containing 50% fresh growth media and 50% (v/v) the “disc conditioned media” for an additional 24h. Afterwards, 10 µL of MTT reagent were added to each well and the mixture incubated in the dark for 4h. Subsequently, 100 µL DMSO (dimethyl

sulfoxide) were added to each well, and the absorbance of the solution at 562 nm measured using an Infinite® 200 Pro multimode reader. Cells incubated with 50% of growth medium and 50% of pure PBS (without disc immersion) were tested under the same conditions to serve as controls.

### Statistical analysis

Data shown in the figures (mean  $\pm$  standard deviation) are representative of at least three independent experiments. Data were analyzed by student's *t* test and *p* values less than 0.05 were considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

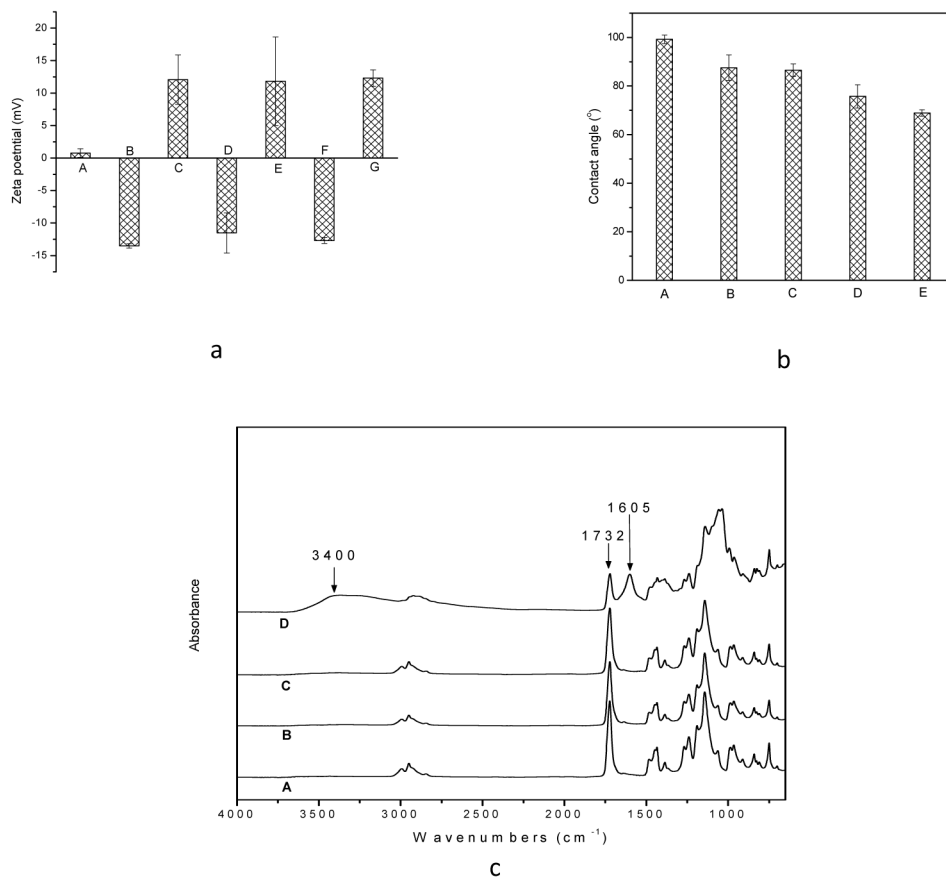
### Acknowledgments

This study was supported by NIDCR, NIH (R01 DE021084) and VA Merit Review (1101BX001103). We would like to thank Dr. David Dean (Professor, Comprehensive Dentistry, UTHSCSA) for his careful review and editing of this manuscript.

### References

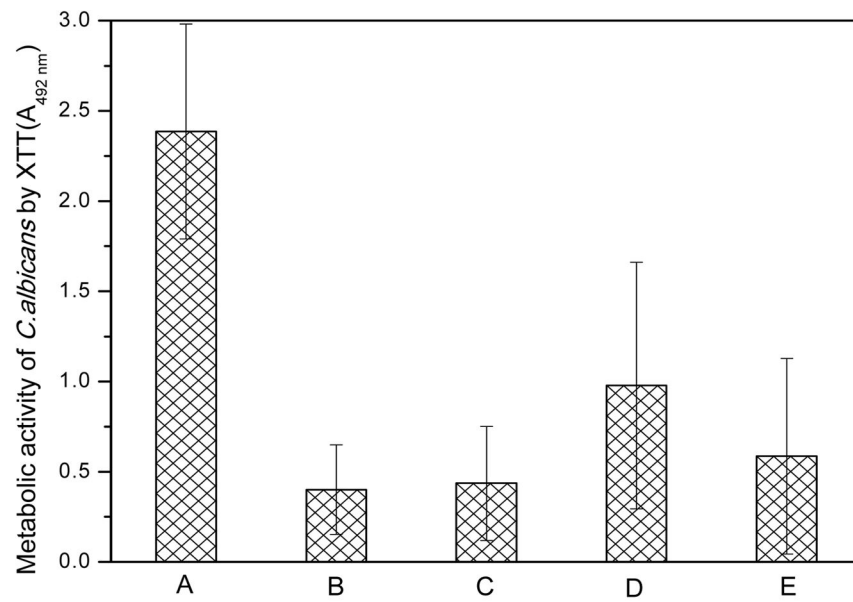
1. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. *J Bacteriol.* 2001; 183:5385. [PubMed: 11514524]
2. Blankenship JR, Mitchell AP. *Curr Opin Microbiol.* 2006; 9:588. [PubMed: 17055772]
3. Nett J, Andes D. *Curr Opin Microbiol.* 2006; 9:340. [PubMed: 16815078]
4. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. *Nat Rev Microbiol.* 2011; 10:112. [PubMed: 22158429]
5. Ramage G, Vandewalle K, Wickes B, Lopez-Ribot J. *Antimicrob Agents Chemother.* 2001; 45:2475. [PubMed: 11502517]
6. Baillie GS, Douglas LJ. *Antimicrob Agents Chemother.* 1998; 42:1900. [PubMed: 9687381]
7. Ramage G, Williams C. *Adv Appl Microbiol.* 2013; 84:27. [PubMed: 23763758]
8. Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. *Crit Rev Microbiol.* 2009; 35:340. [PubMed: 19863383]
9. Iñigo M, Pemán J, Del Pozo JL. *Int J Artif Organs.* 2012; 35:780. [PubMed: 23138707]
10. Kuhn DM, Ghannoum MA. *Curr Opin Investig Drugs.* 2004; 5:186.
11. Mukherjee PK, Chandra J. *Drug Resist Updat.* 2004; 7:301. [PubMed: 15533767]
12. Sun X, Cao Z, Yeh CK, Sun Y. *Colloids Surf B Biointerfaces.* 2013; 110:96. [PubMed: 23708753]
13. Villar CC, Lin AL, Cao Z, Zhao XR, Wu LA, Chen S, Sun Y, Yeh CK. *Oral Dis.* 2013; 19:287. [PubMed: 22957799]
14. Cao Z, Sun X, Yeh CK, Sun Y. *J Dent Res.* 2010; 89:1517. [PubMed: 20940361]
15. Mourya VK, Inamdar NN. *J Mater Sci Mater Med.* 2009; 20:1057. [PubMed: 19112609]
16. Tan H, Ma R, Lin C, Liu Z, Tang T. *Int J Mol Sci.* 2013; 14:1854. [PubMed: 23325051]
17. Belalia R, Grelier S, Benaissa M, Coma V. *J Agric Food Chem.* 2008; 56:1582. [PubMed: 18271546]
18. Augst AD, Kong HJ, Mooney DJ. *Macromol Biosci.* 2006; 6:623. [PubMed: 16881042]
19. Eiselt P, Yeh J, Latvala RK, Shea LD, Mooney DJ. *Biomaterials.* 2000; 21:1921. [PubMed: 10941913]
20. Wang L, Shelton RM, Cooper PR, Lawson M, Triffitt JT, Barralet JE. *Biomaterials.* 2003; 24:3475. [PubMed: 12809776]

21. Hariyadi DM, Lin SC, Wang Y, Bostrom T, Turner MS, Bhandari B, Coombes AG. *J Drug Target*. 2010; 18:831. [PubMed: 20958098]
22. Ahmad Z, Khuller GK. *Expert Opin Drug Deliv*. 2008; 5:1323. [PubMed: 19040395]
23. Decher G. *Science*. 1997; 277:1232.
24. Guyomard A, Dé E, Jouenne T, Malandain J-J, Muller G, Glinel K. *Adv Funct Mater*. 2008; 18:758.
25. Lawrence NJ, Wells-Kingsbury JM, Ihrig MM, Fangman TE, Namavar F, Cheung CL. *Langmuir*. 2012; 28:4301. [PubMed: 22339263]
26. Stauder M, Papetti A, Mascherpa D, Schito AM, Gazzani G, Pruzzo C, Daglia M. *J Agric Food Chem*. 2010; 58:11662. [PubMed: 21038921]
27. Follmann HD, Martins AF, Gerola AP, Burgo TA, Nakamura CV, Rubira AF, Muniz EC. *Biomacromolecule*. 2012; 13:3711.
28. Li Z, Lee D, Sheng X, Cohen RE, Rubner MF. *Langmuir*. 2006; 22:9820. [PubMed: 17106967]
29. Lichter JA, Rubner MF. *Langmuir*. 2009; 25:7686. [PubMed: 19317389]
30. Tang Z, Wang Y, Podsiadlo P, Kotov NA. *Adv Mater*. 2006; 18:3203.
31. Sieval AB, Thanou M, Kotzé AF, Verhoef JC, Brussee J, Junginger HE. *Carbohydr Polym*. 1998; 36:157.
32. Frazer RQ, Byron RT, Osborne PB, West KP. *J Long Term Eff Med Implants*. 2005; 15:629. [PubMed: 16393131]
33. Ratanasathien S, Wataha JC, Hanks CT, Dennison JB. *J Dent Res*. 1995; 74:1602. [PubMed: 7560423]
34. Vale FM, Castro M, Monteiro J, Couto FS, Pinto R, Gião Toscano Rico JM. *Biomaterials*. 1997; 18:1133. [PubMed: 9247352]
35. Sutherland IW. *Microbiol*. 2001; 147:3.



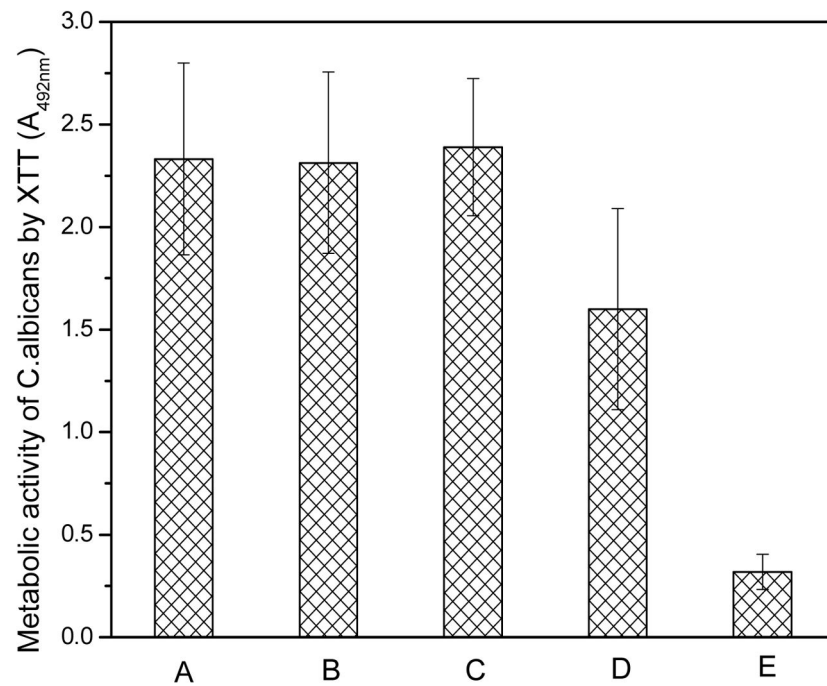
**Figure 1.**

Figure 1a, Zeta potential of the surface of (A) the original PMMA, (B) g-PMMA, (C) g-PMMA with one layer of TMC coating, (D) g-PMMA with one TMC/SA bilayer coating, (E) g-PMMA with 1.5 bilayer coating (one TMC/SA bilayer plus a TMC final layer), (F) g-PMMA with 10 bilayer coatings, and (G) g-PMMA with 10.5 bilayer coatings ( $n=4$ ); **1b**, contact angle values of (A) the original PMMA, (B) g-PMMA, (C) g-PMMA with one layer of TMC coating, (D) g-PMMA with 5.5 bilayers, and (E) g-PMMA with 10.5 bilayers ( $n=4$ ); and **1c**, ATR spectra of (A) the original PMMA, (B) g-PMMA, (C), PMMA-g-MAA with one layer of TMC, and (D) PMMA-g-MAA with 10.5 bilayers



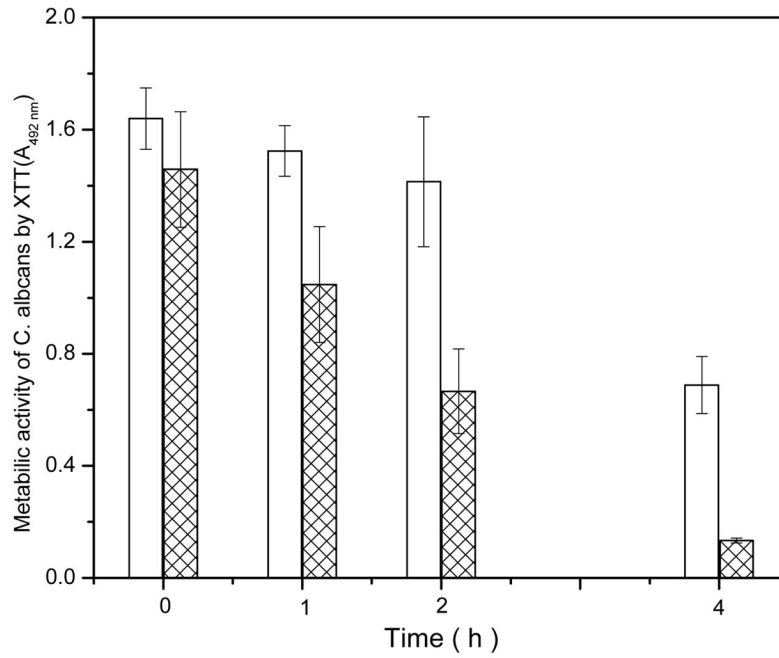
**Figure 2.**

Level of *Candida* initial adhesion on different samples evaluated by the metabolic activity of adherent *Candida* using the XTT assay on (A) the original PMMA to serve as controls, (B) g-PMMA, (C) g-PMMA with one TMC layer, (D) g-PMMA with 5.5 bilayers, and (E) g-PMMA with 10.5 bilayers, after *one hour* of initial adhesion in PBS (n=4)

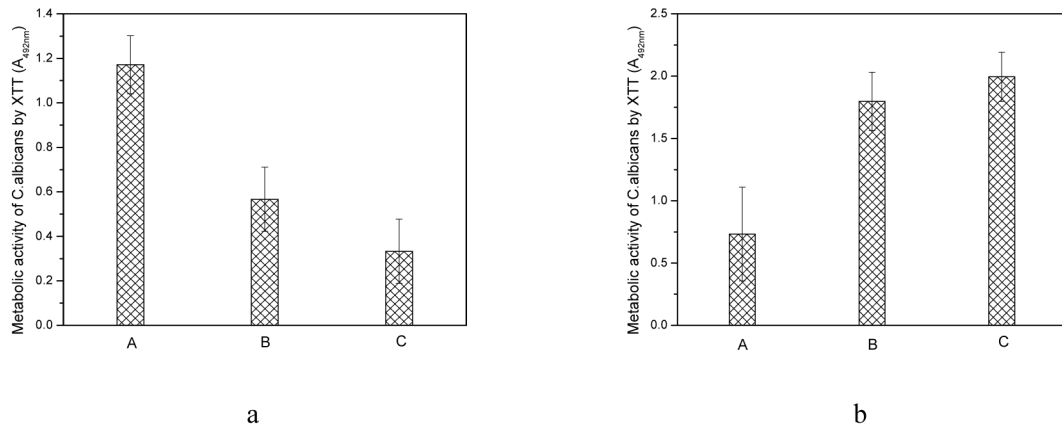


**Figure 3.** Level of *Candida* biofilm formation on different samples evaluated by the metabolic activity of adherent *Candida* using the XTT assay on (A) the original PMMA to serve as controls, (B) g-PMMA, (C) g-PMMA with one TMC layer, (D) g-PMMA with 5.5 bilayers, and (E) g-PMMA with 10.5 bilayers, after *two days* of incubation in broth solutions (n=4).



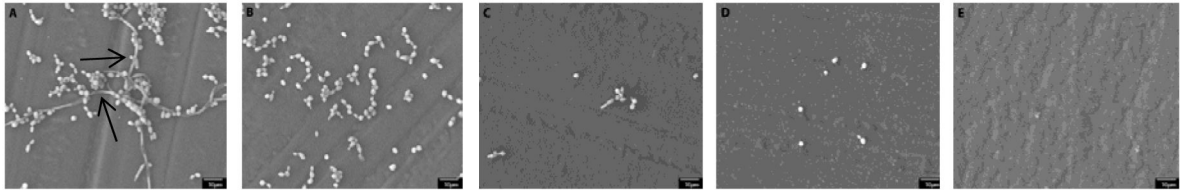


**Figure 4.** Antifungal activities of the disc samples against *Candida* after different periods of direct contact; the figure shows the metabolic activity of *Candida* on the PMMA control disc (open bar) and g-PMMA with 10.5 bilayer coatings (marked bar) after different periods of direct contact (n=4). A higher XTT absorbance indicates a higher metabolic activity of *Candida* cells.



**Figure 5.**

Figure 5a. Metabolic activity of adherent *Candida* on (A) the unmodified PMMA to serve as controls, (B) g-PMMA with 10 bilayers, and (C) g-PMMA with 10.5 bilayers, after two days of incubation in broth solutions (n=4); and **5b**, metabolic activity of free floating *Candida* in the broth solutions with (A) the original PMMA disc, (B) g-PMMA coated with 10 bilayers, and (C) g-PMMA coated with 10.5 bilayers, after two days of incubation (n=4). A higher XTT absorbance indicates a higher metabolic activity of *Candida* cells.



**Figure 6.** Representative SEM images of biofilm formed on (A) the original PMMA control, (B) g-PMMA, (C) g-PMMA coated with a one layer of TMC, (D) g-PMMA coated with 5.5 bilayers, and (E) g-PMMA coated with 10.5 bilayers, after incubation in broth for 2 days. The scale bar is 10  $\mu\text{m}$ .

**Table 1**Effect of TMC/SA multilayer coating on CRL-1213<sup>TM</sup> cell viability using the MTT assay

Days of disc conditioning	% viable cells after incubation with PMMA control conditioned media	% viable cells after incubation with the multilayer coating conditioned media
1 day	110.04±38.02	82.77±23.40
3 days	111.45±24.81	124.45±8.61
5 days	90.83±13.37	108.27±19.05
7 days	54.40±7.59	74.67±1.16*

\* Significant difference vs. the PMMA control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript