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Rechargeable Infection-responsive Antifungal Denture Materials

ABSTRACT

Candida-associated denture stomatitis (CADS) is a significant clinical concern. We developed rechargeable infection-responsive antifungal denture materials for potentially managing the disease. Polymethacrylic acid (PMAA) was covalently bound onto diurethane dimethacrylate denture resins in the curing step. The PMAA resins bound cationic antifungal drugs such as miconazole and chlorhexidine digluconate (CG) through ionic interactions. The anticandidal activities of the drug-containing PMAA-resin discs were sustained for a prolonged period of time (weeks and months). Drug release was much faster at acidic conditions (pH 5) than at pH 7. Drugs bound to the denture materials could be "washed out" by treatment with EDTA, and the drug-depleted resins could be recharged with the same or a different class of anticandidal drugs. These results suggest clinical potential of the newly developed antifungal denture materials in the management of CADS and other infectious conditions.

KEY WORDS: antifungal denture, infection-responsive, rechargeable, *Candida*-associated denture stomatitis.

INTRODUCTION

Candida colonization and biofilm formation often lead to *Candida*-associated denture stomatitis (CADS), a common recurring disease that affects approximately 11% to 67% of denture wearers (Arendorf and Walker, 1987; Radford *et al*., 1999; Douglass *et al*., 2002; Ramage *et al*., 2004; Perezous *et al*., 2005). *Candida* infection, especially in immunocompromised/medically compromised patients, has been linked to dental caries, periodontal diseases, gastrointestinal/pleuropulmonary infections, and even death (Webb *et al*., 1998; Golecka *et al*., 2006). The use of antifungal denture materials has been proposed as one strategy to control CADS. The general principle is to impregnate dentures with drugs that release from the device and inhibit microbial growth. A high antifungal concentration can be achieved under the denture and its nearby mucosa, generally exceeding the minimum inhibitory concentration (MIC) of the susceptible species. The effectiveness of these antifungal dentures for short-term use (*e.g*., days to weeks) has been examined in several studies (Schneid, 1992; Matsuura *et al*., 1997; Chow *et al*., 1999; Lefebvre *et al*., 2001; Lin *et al*., 2003; Abe *et al*., 2004; Yoshinari *et al*., 2006; Redding *et al*., 2009).

However, because the current impregnating approaches cannot incorporate enough drugs into dentures to maintain the MIC for extended periods, there are still no antifungal dentures that can offer long-term protection (*e.g.*, months to years). Further, the antifungal-releasing patterns are not controlled by whether active infection is present. These denture materials have an initially high antifungal release with a subsequent exponential decrease in drug release. After a short period of time, the drugs in the dentures are depleted, and the inhibitory effects are lost.

Because a denture will be worn for years and CADS is a recurring disease, long-term antifungal dentures that can initiate and/or cease drug release based on clinical infection would have significant health benefits. Thus, we developed infection-responsive antifungal denture materials in which covalently bound poly(methacrylic acid) (PMAA) serves as a rechargeable drug carrier. Herein we first report the formulation and fabrication of these innovative denture materials, and their anticandidal activity *in vitro*.

MATERIALS & METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Candida albicans* (*C. albicans*; ATCC 10231) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

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Fabrication of Denture Materials

Denture discs (diameter, 13 mm) were prepared by co-polymerization of methacrylic acid (MAA) with diurethane dimethacrylate (DUDMA) in aluminum molds. MAA weight percentage to DUDMA varied at 0% (control), 5%, 10%, and 20%, and the weight percentage of the initiator (azobisisobutyronitrile) to the monomers (DUDMA plus MAA) was 1%. Polymerization was carried out at 70°C for 3 hrs. We examined the specimens visually to ensure that they were free of voids.

Drug Loading

A series of PMAA-based discs was immersed in either 5% miconazole ethanol solution or 10% chlorhexidine digluconate (CG) aqueous solution for 24 hrs at room temperature under constant shaking. The discs were rinsed individually with water (3x, 10 mL, and 1 min for each rinse), air-dried, and stored in a desiccator. The drugs bound to the discs were extracted with de-ionized water in a Soxhlet extraction apparatus for 24 hrs. Drug content in the extraction solution was measured at $\lambda = 220$ nm for miconazole or $\lambda = 253$ nm for CG (Senel *et al*., 2000; Bhalekar *et al*., 2009). Each test was repeated 5 times.

Drug Releasing

Disc specimens loaded with miconazole or CG were immersed individually in 10 mL buffer containing physiological concentrations of electrolytes in saliva with slight viscosity (0.375 g/L $CaCl_2 \cdot 2H_2O$, 0.125 g/L $MgCl_2 \cdot 6H_2O$, 1.2 g/L KCl, 0.85 g/L NaCl, 2.5 g/L $NaHPO_4 \cdot 12H_2O$, 1 g/L sorbine acid, 5 g/L carboxylmethylcellulose sodium, and 43 g/L sorbitol solution) at neutral pH (Raum *et al*., 2007) or pH = 5 (adjusted with citric acid; Yap *et al*., 2004). The specimens were shaken at 37°C and 40 RPM. The buffer was changed daily. Periodically, the quantity of drugs released from each specimen was determined by UV measurements. Each test was repeated 5 times.

Antifungal/Anti-biofilm Efficacy

The antifungal efficacy of the discs was assessed by the Kirby-Bauer (KB) technique. The drug-containing discs for each drug, at various drug release times as described above, were rinsed with 10 mL phosphate-buffered saline (PBS) and placed onto the surface of a yeast and mold (YM) agar plate (Fisher Scientific, Pittsburgh, PA, USA) containing overnight culture of 1 mL *C. albicans* (10^8-10^9 CFU). After incubation at 37°C for 24 hrs, the inhibition zone was measured with a ruler.

To test anti-biofilm activity, we immersed PMAA-based discs loaded with miconazole or CG in 10 mL buffer containing 10^8-10^9 CFU/mL of *C. albicans*. The mixture was gently shaken at 37°C for 1 hr. The discs were gently washed with PBS (3x, 10 mL), immersed individually in 10 mL YM broth, and incubated at 37°C for 3 days with constant low-speed shaking. Afterward, half of the discs were rinsed with 0.1 M sodium cacodylate buffer (SCB), fixed with 3% glutaraldehyde, and prepared for scanning electron microscopy (SEM; Hitachi S-3200N) study with dehydration/critical-point-drying and sputter-coating with gold (Cao *et al*., 2009). The other half were washed with PBS (3x, 10 mL), sonicated individually for 5 min, and vortexed for 1 min in 10 mL PBS. The recoverable *Candida* were determined by CFU after incubation of the serially diluted *Candida* PBS solution on YM agar at 37°C for 24 hrs.

Drug Quenching, Drug Recharging, and Drug Switching

To test drug "quenching" (wash-out), we immersed drug-containing discs individually in 5% EDTA aqueous solution for 8 hrs at room temperature. The resin-to-quenching-solution weight ratio was 1:50. The discs were washed with distilled water and air-dried. The quantity of remaining drugs in the disc was determined by Soxhlet extraction and UV measurement.

In testing rechargeability, either the quenched resin discs were recharged with drugs again, *e.g.,* discs previously loaded with miconazole were either recharged with miconazole or switched to CG, or discs originally charged with CG were recharged with either CG or miconazole. The amount of recharged drugs on the discs was determined with Soxhlet extraction and UV measurement.

The data were expressed as means ± standard deviations, and p values for comparison of drug-loading capacity were calculated by one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test.

RESULTS

The DUDMA discs without MAA absorbed 8.2 ± 0.7 μg/cm^2 of miconazole or 5.6 ± 0.6 μg/cm^2 of CG ($n = 5$), respectively. In the DUDMA-MAA copolymers, drug-binding capacity increased with the increase of MAA content (Fig. 1A). With 10% of PMAA, the discs absorbed 59.8 ± 2.5 μg/cm^2 of miconazole or 45.7 ± 2.1 μg/cm^2 of CG, respectively ($n = 5$; $p < 0.001$ *vs.* the DUDMA control). The 10% PMAA resin discs were used for the subsequent studies.

In zone-of-inhibition tests, the DUDMA discs did not provide any measurable inhibitory zones (Fig. 1B). In contrast, the PMAA-based disc containing miconazole produced a zone of 5.0 ± 0.4 mm in diameter, and the disc containing CG generated a zone of 2.0 ± 0.2 mm (Figs. 1C, 1D). In anti-biofilm tests, the control discs were covered with *Candida* biofilm after 3 days of incubation. However, the PMAA-based drug-containing discs showed no adherent cells/biofilm (Figs. 2B, 2C *vs.* 2E, 2F). These observations have been further confirmed with recoverable *Candida* colonies on the discs. While $(26.0 \pm 4.7) \times 10^4$ CFU/cm^2 of *C. albicans* could be recovered from the DUDMA control discs, no *C. albicans* could be recovered from the drug-containing discs.

The drug release kinetic assay demonstrated a sustained release of miconazole from the PMAA-based resins. At neutral pH, the disc still contained 18.22 ± 1.2 μg/cm^2 of miconazole after 60 days of immersion in the buffer (Fig. 3A, curve A). Miconazole release was about two times faster in an acidic environment (pH = 5). In parallel, the discs were removed from the buffer at indicated times for *Candida* inhibition tests. The inhibition zone decreased as the releasing period was extended

(Fig. 3A, curve B), and reduced to 0.4 ± 0.1 mm after 60 days (pH = 7). Based on drug release kinetics and the *Candida* inhibition curve, 18.22 ± 1.2 μg/cm^2 was defined as the minimum inhibitory content (MIC) of absorbed miconazole on the discs to provide a clear *Candida* inhibition zone.

Similar pH-sensitive drug release kinetics was observed in the testing of PMAA-based resins with CG (Fig. 4). The MIC for the CG-containing PMAA disc was estimated at 17.24 ± 0.2 μg/cm^2. CG content in the disc was still above MIC after 14 days of release at neutral pH. However, at pH 5, CG on the discs reached MIC after 7 days of release.

To test whether drug-loaded PMAA-based resins can be quenched, we treated miconazole- or CG-containing discs with EDTA for 8 hrs. The remaining concentrations of miconazole on the PMAA-based disc decreased to 4.1 ± 2.9 μg/cm^2 (*i.e.*, 93.1% of the original miconazole was "washed out"). Similarly, after the EDTA treatment, the remaining CG on the disc was 2.1 ± 1.2 μg/cm^2 (95.4 % of the original CG was "washed out").

After the quenching treatment, the miconazole-depleted discs were re-immersed in miconazole solution for 24 hrs and achieved 61.8 ± 2.1 μg/cm^2 of recharged miconazole (n = 5), which generated a *Candida* inhibition zone of 5.1 ± 0.3 mm (image not shown). Similarly, the quenched CG discs were again recharged with CG, and achieved 47.5 ± 2.1 μg/cm^2 of CG (n = 5), which generated an inhibition zone of 2.2 ± 0.3 mm.

For drug-switching tests, the CG-quenched discs were recharged with miconazole, resulting in 58.9 ± 3.1 μg/cm^2 of bound miconazole. Alternatively, the miconazole-quenched discs were recharged with CG, and achieved 45.2 ± 3.0 μg/cm^2 of bound CG (n = 5).

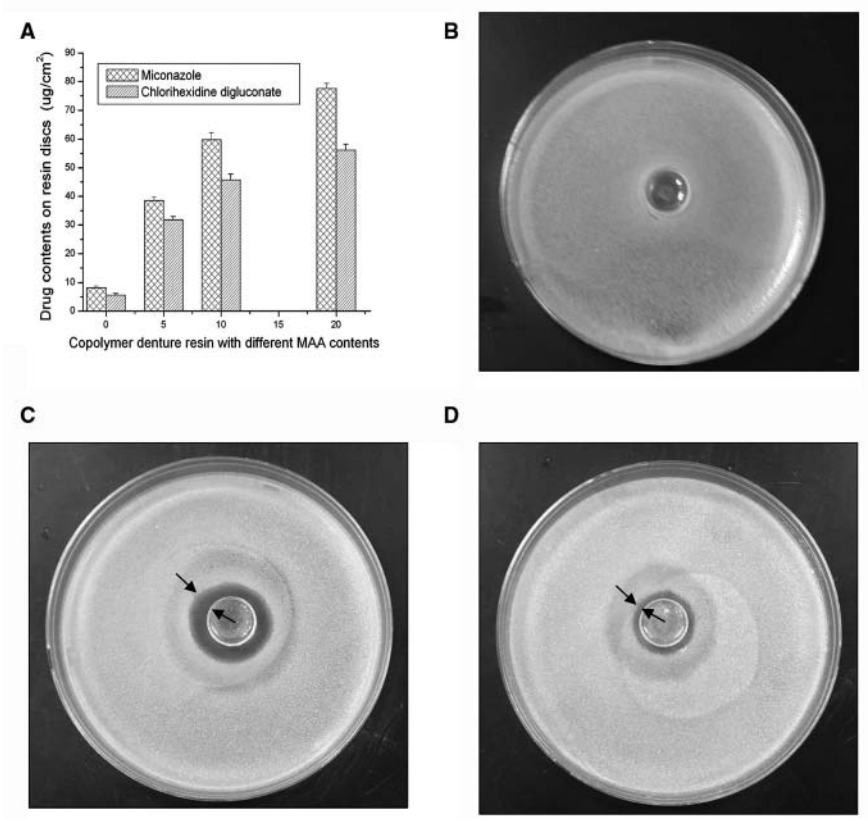

Figure 1. Drug-binding capability and anticandidal activity of the new PMAA-based resins. **(A)** Drug contents on resin discs with different weight percentages of PMAA (%), showing significant drug-binding capacity increases with the increase of PMAA contents ($p < 0.001$), and zone-of-inhibition results of **(B)** the original DUDMA disc, **(C)** PMAA-based DUDMA disc containing 10% of PMAA with 59.8 ± 2.5 μg/cm^2 of absorbed miconazole, and **(D)** PMAA-based disc containing 10% of PMAA with 45.73 ± 2.1 μg/cm^2 of absorbed CG.

DISCUSSION

Antifungal denture materials have been described in several studies (Schneid, 1992; Matsuura *et al*., 1997; Chow *et al*., 1999; Lefebvre *et al*., 2001; Lin *et al*., 2003; Abe *et al*., 2004; Yoshinari *et al*., 2006; Redding *et al*., 2009). However, most antifungal dentures have short antifungal action that cannot be controlled based on clinical needs. We developed rechargeable infection-responsive antifungal denture materials by copolymerizing DUDMA with MAA (Sun, 2010). DUDMA resins are rapidly gaining popularity as denture materials which contain 2 vinyl double bonds, allowing for cross-linking of MAA into the denture resins. We used miconazole and CG as the model drugs, since miconazole gel has already been used to treat oral *Candida* infection, and CG mouthwash is known to have anticandidal activity.

The presence of up to 10% PMAA did not negatively affect the physical properties and biocompatibility of the original DUDMA resins (data not shown). The DUDMA resin itself has very low drug-binding capacity (Fig. 1A). After copolymerization with MAA, the drugs could be bound onto the denture materials through ionic interactions with the carboxylate groups (-COO$^-$) of PMAA. Thus, drug-binding capacity increased significantly with the increase of PMAA content, resulting in potent antifungal effects (Figs. 1, 2).

The ionic interactions between the denture materials and the drugs led to a sustained drug release for weeks (CG) to months (miconazole). Since drug release was controlled by ionic interactions between the -COO$^-$ groups and the drugs, and -COO$^-$ content was affected by pH (-COOH ↔ -COO$^-$), the drug release rate from the PMAA-based denture materials was pH-sensitive (Figs. 3A, 4A). This was explored as a biological

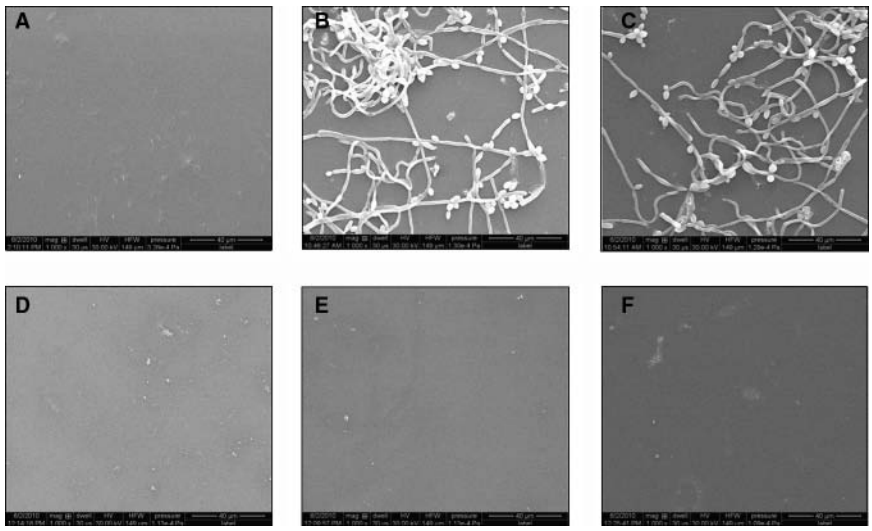

Figure 2. Representative SEM images of 5 independent experiments (magnification: 1000x) showing that although biofilm formed on DUDMA discs, PMAA-based drug-containing discs inhibited *Candida* biofilm: **(A)** DUDMA disc before immersion in *C. albicans*, **(B)** representative image 1 of DUDMA disc after immersion in *C. albicans* for 3 days, **(C)** representative image 2 of DUDMA disc after immersion in *C. albicans* for 3 days, **(D)** PMAA-based disc containing 59.8 ± 2.5 μg/cm^2 of miconazole before immersion in *C. albicans*, **(E)** representative image 1 of PMAA-based disc containing 59.8 ± 2.5 μg/cm^2 of miconazole after immersion in *C. albicans* for 3 days, and **(F)** representative image 2 of PMAA-based disc containing 59.8 ± 2.5 μg/cm^2 of miconazole after immersion in *C. albicans* for 3 days. Recoverable microbial level from the DUDMA discs was (26.0 ± 4.7) x 10^4 CFU/cm^2 (n = 5). From the PMAA-based disc containing 59.8 ± 2.5 μg/cm^2 of miconazole; however, no recoverable adherent *C. albicans* could be detected (n = 5).

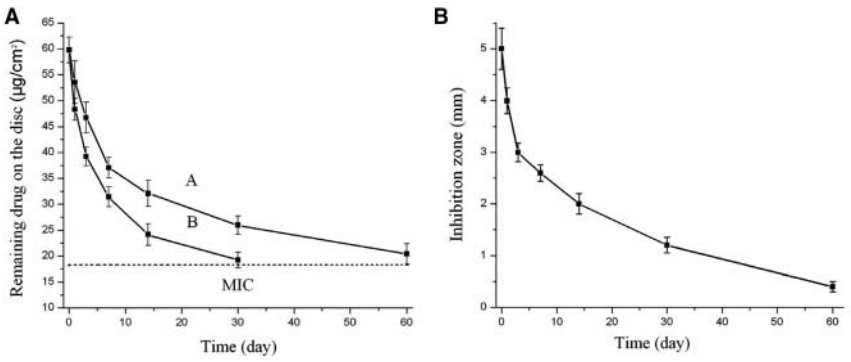

Figure 3. Sustained drug release and anticandidal effects of the new PMAA-based resins charged with miconazole. **(A)** Release curves of miconazole from the PMAA-based discs containing 10% of PMAA showing pH-sensitive drug release: curve A, neutral pH, and curve B, pH = 5.0 (original miconazole content, 59.8 ± 2.5 μg/cm^2); and **(B)** effects of drug-releasing periods from the PMAA-based denture materials on inhibition zone size against the test *Candida* cells. The resin contained 10% of PMAA and had 59.8 ± 2.5 μg/cm^2 of absorbed miconazole at the beginning of the test. The pH of the immersing solution was neutral.

"switch" in the infection-responsive drug delivery system: that is, while the normal oral environment has neutral pH, oral cavities infected with *Candia* have acidic conditions (Samaranayake *et al*., 1983; Nikawa *et al*., 1994). Therefore, the new anticandidal dentures are clinically expected to adjust drug release rates based on whether *Candida* is present and on the severity of the infections. With the same PMAA content, discs containing miconazole had much longer antifungal duration than discs containing CG (Fig. 3 *vs.* Fig. 4), which could be attributed to the higher water solubility of CG than miconazole.

Drugs on the new denture materials could be "washed out" by EDTA, a widely used chelating agent with 4 carboxylate groups on each molecule, which would compete with PMAA on the denture for the drugs. Under our quenching conditions, there were 30 times more carboxylate groups on EDTA than the carboxylate groups on the PMAA. Therefore, most of the drugs on the discs were "washed out" or "quenched" upon treatment with EDTA.

With these new denture materials, the released drugs could be repeatedly recharged to resume their antifungal activities. In subsequent recharging, drugs could be changed/switched, without affecting the drug-binding capacity. This unique "click-on/click-off" drug delivery feature is expected to revolutionize the management of CADS. For high-risk patients, the PMAA-containing denture can be worn as a conventional denture if CADS is not present. If CADS occurs, the dentures can be charged with drugs to initiate the antifungal therapy. When the infection is cleared, the dentures can be washed with EDTA to quench the therapeutic effects when no further drug release is needed. If CADS recurs, drugs can be recharged to re-initiate the treatment. In subsequent recharging, drugs can be changed/switched.

In conclusion, rechargeable infection-responsive antifungal denture materials were developed by the covalent binding of MAA onto DUDMA denture resins. The new denture materials bind and then slowly release antifungal drugs, leading to sustained, pH-sensitive drug release and prolonged antifungal effects. Drug release can be quenched by EDTA, and re-initiated by drug recharging based on disease conditions and/or the individual patient's needs. In subsequent recharging, drugs can be

changed/switched to potentially enhance inhibitory potency and/or reduce the risk of microbial resistance.

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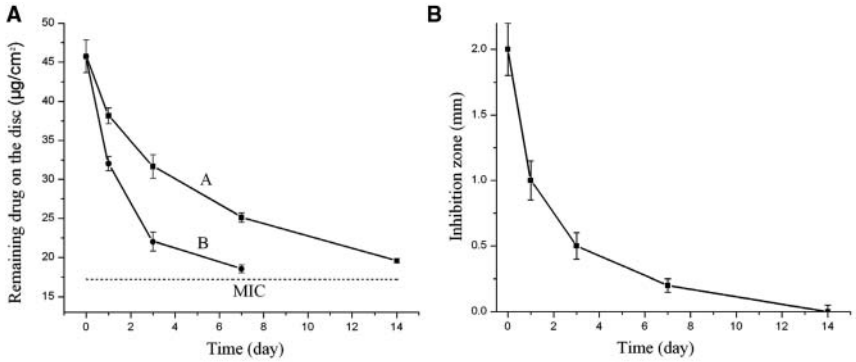

Figure 4. Sustained drug release and anticandidal effects of the new PMAA-based resins charged with chlorhexidine digluconate (CG). **(A)** Release curves of CG from the PMAA-based discs containing 10% of PMAA, showing pH-sensitive drug release: curve A, neutral pH, and curve B, pH = 5.0 (original CG content, 45.73 ± 2.1 μg/cm^2); and **(B)** effects of drug-releasing periods from the PMAA-based denture materials on inhibition zone size against the test *Candida* cells. The resin contained 10% of PMMA and had 45.73 ± 2.1 μg/cm^2 of CG at the beginning of the test. The pH of the immersion solution was neutral.

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