Antimicrobial Activity and Local Release Characteristics of Chlorhexidine Diacetate Loaded Within the Dental Copolymer Matrix, Ethylene Vinyl Acetate

Roland R. Arnold,[1,2] Hong Hong Wei,[1] Eric Simmons,[1] Padmavathy Tallury,[1] David A. Barrow,[1,3] Sid Kalachandra[1,2,3]

[1] Dental Research Center, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7450

[2] Department of Periodontology, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7450

[3] Center for Oral and Systemic Diseases, School of Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599–7450

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Abstract: ***In vitro*** **results are presented for a novel oral drug-delivery system ultimately intended for treatment of oral infections in immunocompromised patients. Test samples of ethylene vinyl acetate copolymer (EVA) containing chlorhexidine diacetate (CDA) showed desirable antimicrobial properties and steady, slow release into aqueous and other media after an initial burst of drug release in the first day of liquid exposure. By washing away this initial burst, the proposed mouthguard device should be capable of sustained delivery of locally effective CDA concentrations far below systemically toxic levels. A prolonged room temperature shelf-life of at least 1 year, and effectivity against a wide range of oral bacteria and *Candida* species was demonstrated. Drug loaded films showed a top-to-bottom asymmetry in drug release, but good lateral homogeneity, and a linear relationship between initial CDA loading concentration (from 0.63 to 10 wt %) and days 3–14 release rates in a static aqueous environment. The EVA matrix containing CDA appears to possess many suitable properties for localized oral delivery of sustained antimicrobial activity.** © 2008 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 86B: 506–513, 2008

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INTRODUCTION

The incidence of periodontal and other oral infections, particularly in immunocompromised patients, remains as a significant oral health challenge, which may in turn compromise systemic health.[1] This problem is compounded by the fact that many of the broad spectrum antibiotics such as tetracyclines may produce significant side effects. Despite the wide use of tetracyclines in numerous therapeutic applications in dental surgery and in the treatment of various clinical types of periodontitis,[2–4] because of their broad antibacterial spectrum, efficient diffusion in bone, and inhibitory effect on collagenases, they can produce a variety of side effects with long-term oral administration, such as digestive disturbances, tooth discoloration, and enamel dysplasia.[5,6] In an effort to resolve these problematic side effects, there is a great interest in the development of intraoral systems for effective delivery of novel broad spectrum antimicrobial agents. To be effective, systems should provide sustained local release of effective doses of antimicrobial agents, far below systemically toxic levels, over extended periods of time.

Several polymeric drug delivery systems have been tested with varying degrees of success for delivering effective doses of antimicrobial agents in the oral cavity. These compounds include methacrylate-based polymers,[7–11] tetrahydrofurfuryl methacrylate/poly (ethyl methacrylate) (THFMA)/PEM) polymers,[12] chitosan, poly(lactide-*co*-glycolide), and PMMA polymers as well as lactic and glycolic copolymer materials.[13–15] Another potentially interesting

Correspondence to: S. Kalachandra, Ph.D. (e-mail: sid_kalachandra@dentistry.unc.edu)
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polymeric drug delivery system is poly(ethylene-*co*-vinyl acetate) (EVA). This material is biocompatible and already has Food and Drug Administration (FDA) clearance for use in humans at different body sites-including the mouth, as the base material for mouthguards.[16,17] Recently, in a series of *in vitro* studies, this laboratory showed that after incorporation of a variety of antibacterial and antiviral agents into the EVA matrix, slow release of these agents into the surrounding medium was achieved.[18–20]

There is growing interest in the use of chlorhexidine diacetate (CDA) as an effective antimicrobial agent for use in the oral cavity. This agent has been reported to have a broad spectrum of activity against both Gram positive and Gram negative bacteria and is minimally toxic to host cells.[10,11,21,22] When released in the oral cavity, a substantial amount of CDA is likely to be retained on oral membrane surfaces because of its strong cationic charge. CDA is subsequently re-released from these reservoirs at a rate that may keep the concentration at antimicrobial levels for several hours.

This study was designed to investigate the antimicrobial activity of CDA against known oral pathogens and the functional release characteristics of this drug following incorporation into EVA.

MATERIALS AND METHODS

EVA, (Elvax; Clinical Grade containing 40 wt % vinyl acetate) was obtained from DuPont Chemicals, Wilmington, DE; clinical grade chlorhexidine diacetate (CDA) was from Metcam, Spain. Sigma CDA was purchased from Sigma-Aldrich Chemical (St. Louis, MO) and dichloromethane from Mallinckrodt Baker (Spectr AR, Paris, KY).

Preparation of Polymer Thin Films

Polymer casting solutions were prepared by dissolving first the drug and then EVA (vinyl acetate 40 wt %) copolymer beads in the appropriate ratio (for example, 40:1 for 2.5 wt % CDA) in 65 mL of dichloromethane in a stoppered conical flask. CDA loaded EVA films of 0.8 mm thickness were prepared with drug to EVA ratios of 10.0, 7.5, 5.0, 2.5, 1.25, 0.63, and 0.31 wt % for evaluation of the drug concentration dependence of release rates. Each solution was stirred with a magnetic stir rod at room temperature overnight and the entire volume was then poured into a 14 cm diameter glass PYREX® Petri dish. Each solution was allowed to dry into a film overnight in a fume hood by evaporation of residual solvent at room temperature. Three drug loaded polymer thin square films of dimension 3 cm $\times$ 3 cm $\times$ 0.08 cm were cut from the dry films and used to follow the kinetics of drug release. The square films (see Figure 1) were placed in a volume of 10 mL distilled water at 37°C to collect the drug released daily.

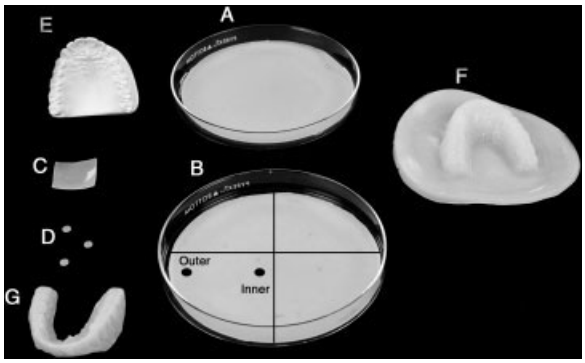


Figure 1. CDA loaded EVA films formed in Petri dishes are shown (A,B), along with a 3 cm square film (C), film disks for microbiological testing (D), a maxillary arch stone casting (E), a thermoformed mouthguard prior to trimming of excess EVA (F), and a final mouthguard device (G). Film (B) illustrates film sectioning into quadrants, and the location of inner and outer disk excision locations.

Antimicrobial Activities of CDA as Inhibition of Planktonic Growth

The antimicrobial activities for CDA prepared in deionized water were determined against two strains of *P. gingivalis* (HG405 and A7436), *F. nucleatum* 1594, *S. mutans* ATCC 10449, and a clinical isolate of *C. albicans* using two different assays. First, the CDA preparations were titrated for their ability to inhibit microbial growth in broth cultures (planktonic) measured as a change in biomass (optical density at 660 nm) over time. These assays were accomplished in a 96-well microtiter plate format.

All bacteria were grown in Wilkins-Chalgren's (W-C, Oxoid, Basingstoke, Hampshire, England) broth. *S. mutans* grew in both ambient and anaerobic atmospheres. The *P. gingivalis* strains and the *F. nucleatum* required an anaerobic atmosphere and were grown in a Coy (Coy Laboratory Products, Grass Lake, MI) flexible film dual anaerobic chamber under an atmosphere of 10% H_2, 5% CO_2, and 85% N_2. The *Candida* strains grew poorly in W-C broth and were therefore grown in Sabaroud dextrose broth (Difco Laboratories, Detroit, MI) under ambient atmosphere.

Stock solutions of CDA were serially twofold diluted in appropriate broth resulting in 200 μL volumes with a range of concentrations of CDA in the microtiter plate wells. Each well was inoculated with mid-exponential cultures diluted in broth to a microbial density of 10^6 colony forming units (CFU)/mL estimated spectrophotometrically ($A_{660\ nm}$) against strain appropriate standard curves. Plates were incubated in appropriate atmosphere and changes in biomass ($A_{660\ nm}$) were monitored over time at 37°C using a Vmax Kinetic Microtiter Plate Reader (Molecular Devices, Sunnyvale, CA). Concentrations of CDA were considered totally inhibitory if there was no increase in $A_{660\ nm}$ through 48 h. These titration assays were performed in quadruplicate with the two different sources of CDA (Sigma Chemical and clinical grade).

Antimicrobial Activities of CDA as Zone of Inhibition of Surface Growth

The second assay was designed to determine the quantitative sensitivities of the target microorganisms to inhibition of growth on an agar surface. In addition, *Candida tropicalis* and *Candida krusei* were tested in these analyses using the same growth conditions as with *C. albicans*. Standardized microbial inocula from mid-exponential cultures (100 μL adjusted to a 0.5 McFarland standard in sterile Dulbecco's phosphate buffered saline) were spread onto appropriate agar media (either W-C or Mueller-Hinton with 5% sheep blood (Anaerobe Systems, Morgan Hill, CA) for bacteria and Sabaroud dextrose or Mueller-Hinton blood agar for the yeast) using sterile disposable "hockey stick" spreaders. The plates were allowed to dry for 10 min at room temperature.

The CDA preparations were delivered evenly spaced as 5 μL spots directly on the inoculated agar surface or as 7 μL volumes to 6 mm blank paper disks (BBL^{TM}) placed on the inoculated surface. The bacterial lawns were allowed to develop by incubation at 37°C in the appropriate atmosphere. Inhibition of growth was evident as a circular zone of clearance in the lawn at the site of spot placement or around the disk. The areas of the zones of inhibition were proportional to the concentration of CDA applied and the diameters were measured using a dial caliper.

Evaluation of the Antimicrobial Activities of CDA Released From EVA as Zones of Inhibition of Surface Growth

To evaluate the proposed technique for determining the antimicrobial activity of the impregnated test material, circular disks of 6 mm diameter, 0.8 mm thickness, were cut from polymer films prepared with 2.5% chlorhexidine and from drug-free "sham" EVA films (see Figure 1, disks shown as "D"). Spread plates were prepared by distributing 100 μL of 0.5 McFarland standard suspension of exponentially growing *P. gingivalis* A7436, *P. gingivalis* HG405, *F. nucleatum* 1594, *P. intermedia* clinical isolate, *Streptococcus mutans* 10449, or *Candida albicans*. The disks were placed evenly spaced on the surface of freshly inoculated agar media as described earlier. The inoculated plates were incubated at 37°C in either 5% CO_2, 10% H_2, 85% N_2 (anaerobes) or in ambient atmosphere (aerotolerants).

The minimum inhibitory concentration (MIC) and titration kinetics for CDA as determined by surface inhibition were equivalent for all of the target bacteria. *S. mutans* was the fastest grower of the target organisms and yielded a consistent lawn that optimized interpretation of zone of inhibition. It was therefore chosen for more detailed studies of the EVA delivery of chlorhexidine. Likewise, *C. albicans* was clearly more resistant to chlorhexidine than the bacteria and was chosen for further study with the CDA-EVA.

Distribution of Antimicrobial Activities of CDA in EVA

In the pilot studies with CDA-EVA, it was noted that there were inconsistencies in the attained zones of inhibition that were apparently related to the distribution of the CDA in the EVA films. Two possible variables were addressed. The EVA castings are accomplished in a glass Petri dish with an interior diameter of 14 cm and a thickness of 0.8 mm [Figure 1(A)]. If the CDA is not uniformly incorporated into the solvent EVA mixture before casting then the resulting CDA-EVA films would result in lateral variability depending on the site of sampling. To test this possibility, the CDA-EVA sheets were divided into equal quadrants and replicate 6 mm disks were cut from the periphery and vertices of each quadrant, as shown in Figure 1(B).

In addition, in the process of casting one surface (bottom) is formed as an interface with the Petri dish and the other surface (top) is exposed to air. As the solvent evaporates, the EVA forms a matrix trapping the CDA in its resulting pores. As evaporation occurs through the air exposed surface, a bias of distribution of CDA through the thickness of the forming EVA film might be expected and was suggested by microscopic observations of CDA incorporated in methacrylate copolymer films prepared in a similar solvent evaporation process.[23]

This suspected vertical asymmetry was tested by placing pairs of disks on the *S. mutans*-inoculated lawn either bottom down or top down and determining resulting zones of inhibition. As a further test, after this initial placement, the disks were transferred to freshly inoculated plates, but with the vertical orientation reversed. Lawns were again allowed to develop and the resulting zones of inhibition were measured as diameters in mm. Student's *t*-tests were performed to determine any lateral or vertical distribution bias with $p < 0.05$ accepted as significant.

Effects of Water Elution on Release and Retention of CDA Antimicrobial Activities From EVA

EVA films loaded with 2.5% CDA were cut into equal quadrants [see Figure 1(B)] that were exposed to deionized water under different extraction conditions. In the first condition, the CDA loaded EVA quadrants were exposed to either one, two or three successive washes in 30 mL volumes of deionized water. Each wash consisted of a static, 5 min incubation in deionized water. The fourth quadrant was extracted in 30 mL of deionized water for 24 h. Replicate disks [Figure 1(D)] from the four different washed quadrants were tested for residual activity against *S. mutans* surface growth and compared to that of disks cut from untreated 2.5% CDA-EVA. Each of the resulting eluate volumes was tested for released antimicrobial activity by measuring zones of inhibition using the spot assay against surface grown *S. mutans*. The concentrations of CDA released into the elution water were determined spectrophotometrically at the wavelength of maximum absorbance (λ_{max}) 257.5 nm. In addition, estimates of the released

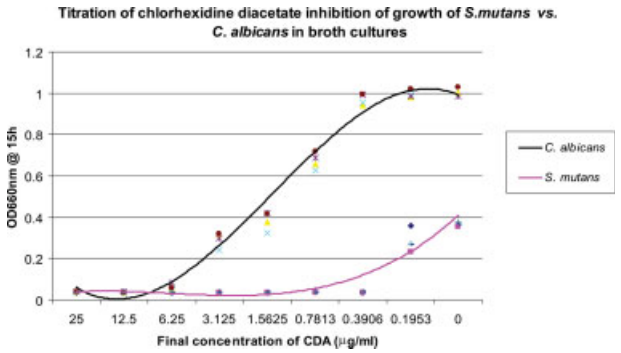


Figure 2. Chlorhexidine diacetate was titrated to the final concentrations indicated in either Wilkins-Chalgren's broth (all bacterial strains) or in Sabaroud's glucose broth (*C. albicans*). Replicate broth cultures were inoculated with 10^6 colony forming units/mL of exponential cultures of either *S. mutans*, *C. albicans*, *P. gingivalis* strains or *F. nucleatum*. The figure represents the biomass (optical density at 660 nm) achieved after 15 h incubation at 37°C of quadruplicate cultures of *C. albicans* and *S. mutans*. O.D. was monitored through 48 h with *S. mutans* failing to grow in the presence of CDA concentrations $\geq$0.39 μg/mL. *C. albicans* was totally inhibited by concentrations >6.25 μg/mL. Similar curves were generated for the other bacterial strains. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CDA concentrations were calculated using a standard curve of CDA activity vs. surface grown *S. mutans*.

Antimicrobial Activities of Different Concentrations of CDA in EVA

Disks sampled from CDA-EVA films prepared with concentrations of CDA ranging from 0.31 to 10% were tested for surface growth inhibition of *S. mutans* and *C. albicans* as described earlier.

RESULTS

Sensitivities of Oral Pathogens to CDA

Figure 2 presents the titration curves of CDA serially diluted in broth against *S. mutans* and *C. albicans*. The optical densities achieved after 15 h of growth are presented. *S. mutans* failed to grow in the presence of 390 ng/mL of CDA even after 48 h incubation. *C. albicans* recovered growth at concentrations of CDA <12.5 μg/mL, but did not grow in the presence of 12.5 μg/mL. Similar growth curves were determined for the other target bacteria and the minimum concentrations of CDA necessary for total inhibition of broth growth of each are indicated in Table I.

CDA was also tested for activities against surface growth of the target microorganisms using a spot assay with either W-C agar (bacteria) or Sabaroud's dextrose agar (*C. albicans*). The zones of inhibition for each of the microorganisms as a titration of CDA are presented in Figure 3. The bacteria showed a similar pattern of sensitivities as with the broth cultures. The *C. albicans* proved again to show greater resistance as compared to the bacteria. The

TABLE I. Relative Sensitivities of Target Oral Pathogens to Total Inhibition of Planktonic Growth by CDA as Determined by Measuring Microbial Growth as a Change in Optical Density at 660 nm as in Figure 2

Minimum Concentrations of CDA Resulting in Total Inhibition of Broth Growth of Selected Target Microorganisms	
S. mutans 10449 (aerobic)	390 ng/mL
C. albicans	12,500 ng/mL
S. mutans 10449 (anaerobic)	390 ng/mL
P. gingivalis HG405	780 ng/mL
P. gingivalis A7436	780 ng/mL
F. nucleatum 1594	390 ng/mL

antimicrobial activities of CDA were also tested against *S. mutans*, *C. albicans*, *C. tropicalis*, and *C. krusei* by delivery in blank paper disks on Mueller-Hinton blood agar plates (Figure 4). The two nonalbicans *Candida* species were not capable of growth on Sabaroud's dextrose. The inclusion of *S. mutans* on this medium assured that the differences observed earlier were not due to media effects on CDA activity. As can be seen, the three yeasts all show similar susceptibilities to CDA and the *S. mutans* proved to be more susceptible.

Distribution and Release Characteristics of the Antimicrobial Activities of CDA From EVA

Using disks cut from 2.5% CDA-EVA films, we observed that with each of the target microorganisms there was a measurable zone of inhibition of the lawn of growth with the CDA loaded EVA disks consistent with the susceptibilities of the target microorganisms to CDA (data not shown). The sham polymer had no effect on the lawn growth of any of the test microorganisms including growth under the disks. While the disks cut from 2.5% CDA-EVA films showed measurable surface activity against all of the

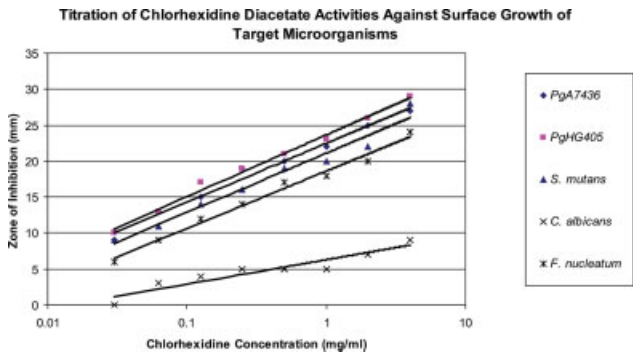


Figure 3. Aliquots of 5 μL of titrated concentrations of chlorhexidine diacetate were delivered as a spot to the spread inoculated surfaces of W-C (bacteria) or Sabaroud's Dextrose (*C. albicans*) agar plates (See Materials and Methods). Microbial "lawns" were allowed to develop in appropriate atmosphere at 37°C. Zones of inhibition of the surface growth at the sites of spot placement were measured as diameters in mm. Nearly identical results were observed with the CDA obtained from Sigma-Aldrich. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

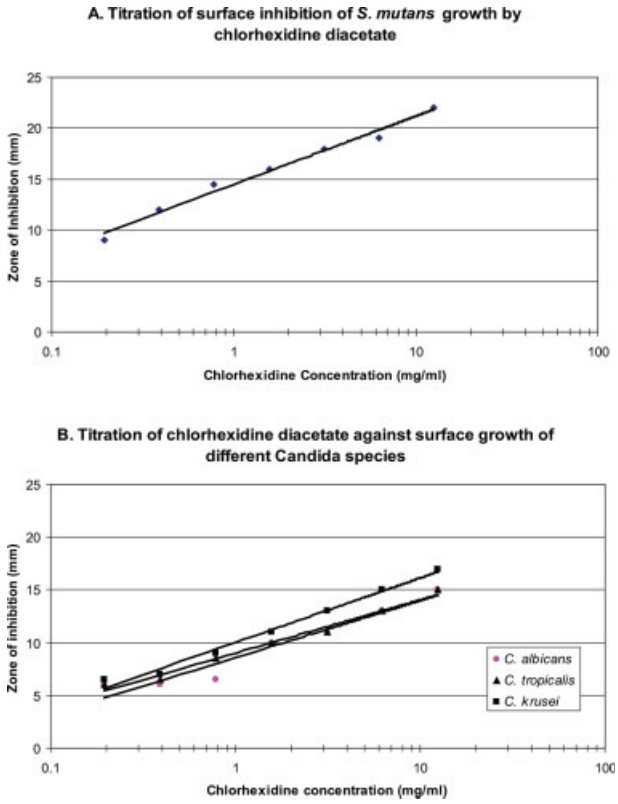


Figure 4. Antimicrobial activities of CDA against: (A) *S. mutans* and (B) *C. albicans* and non*albicans Candida*. Titrated concentrations of CDA were delivered in 7 μL volumes to 6 mm Blank Paper Discs BBL^{TM} placed on the surface of freshly inoculated Mueller-Hinton agar plates with 5% sheep red blood cells. Lawns were allowed to develop at 37°C for 24 h and zones of growth inhibition were measured as diameters in mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

target microorganisms, there was unexplained variability from the replicate disks. Disks were sampled from 2.5% CDA-EVA films from the periphery and the vertices and with attention to vertical orientation (see Materials and Methods). There were no significant differences from disks sampled from different locations in the CDA-EVA sheets as long as the vertical orientation was maintained. As can be seen in Figure 5, the CDA-EVA surface formed against the Petri dish (bottom) consistently had significantly ($p < 0.05$) higher activity (data are expressed as the zone of total inhibition minus the diameter of the CDA-EVA disk [diffusible inhibition]) against surface grown *S. mutans* than did the surface with the air interface (top). This was true whether pairs of disks were compared or if the same disk was successively tested for activity on the contralateral surface.

Titration of CDA Activities in EVA

Figure 6 presents the zones of diffusible inhibition obtained with EVA prepared with different concentrations of CDA. There was no antibacterial activity of the EVA without

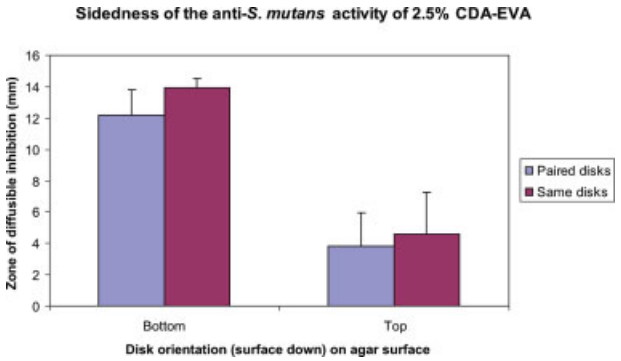


Figure 5. Three 2.5% CDA-EVA films were prepared separately. Disks were cut in pairs from either the periphery or near the center of each sheet from each quadrant. Disk orientation relative to the agar surface was alternated between pairs with half of the disks bottom down and the other half top down. In a separate set of experiments (same disks), disks were again placed either bottom or top down on the inoculated agar surface and the lawns allowed to develop for 24 h at 37°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CDA. *S. mutans* was sensitive to the lowest concentration (0.31%) of CDA tested. Consistent with the earlier data, the *C. albicans* proved to be relatively less susceptible to the CDA-EVA, with no diffusible activity with the 0.31% preparation. In fact there was yeast growth under the disk at this concentration.

Effects of Water Extraction on Retention of Antimicrobial Activity in CDA-EVA

The bar graph and the table presented in Figure 7 represent the retained antimicrobial activity, and the released CDA concentrations and activities, respectively, following the exposure conditions described in Materials and Methods. Exposure of 2.5% CDA-EVA to deionized water for either

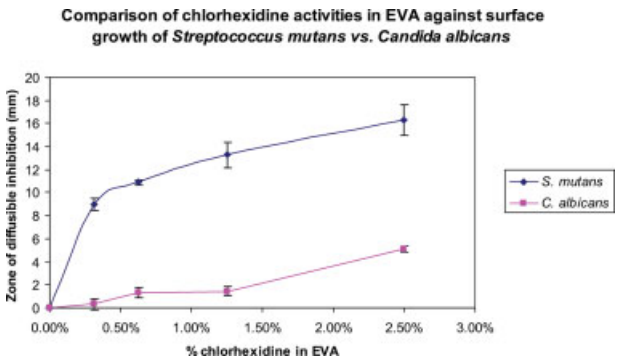
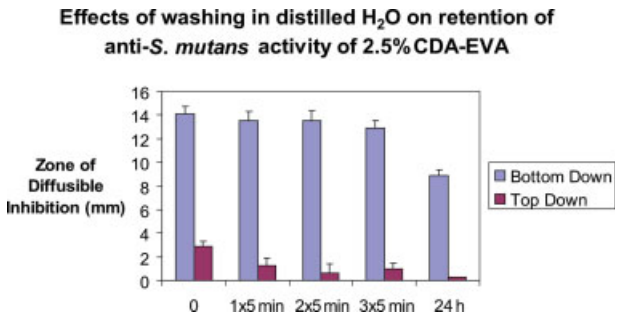


Figure 6. Influence of initial CDA concentration in the CDA-EVA film production (% chlorhexidine in EVA) on antibacterial activity against surface grown *S. mutans* in comparison to the activity with *C. albicans*. The zone of diffusible inhibition was determined by subtracting the diameter of the disk (6 mm) from the zone of total inhibition. There was growth of both microorganisms under the sham-EVA (0% CDA-EVA). In contrast, there was no growth under any of the disks made from CDA containing EVA with either of the target microorganisms. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



Effectiveness of distilled water elution of chlorhexidine from 2.5% CDA-EVA

Treatment	Mean Zone of Inhibition (mm)	S.D. of ZI	Biol. Est. of [CDA] in μg/ml	O.D. Est. of [CDA] in μg/ml
1st wash eluate	9.89	0.55	43	83
2nd wash eluate	2.47	1.49	5.4	18
3rd wash eluate	0.93	0.41	3.5	15
24 h eluate	18.5	0.71	482	849

Figure 7. Effects of sequential washing on residual antimicrobial activity of disks prepared from 2.5% CDA-EVA films (cut in quadrants) that had either not been treated (0); immersed in 30 mL of deionized water for 5 min (1 × 5 min); immersed in successive 30 mL volumes of deionized water either twice (2 × 5 min) or three times (3 × 5 min). Separate quadrants were immersed in 30 mL of deionized water for 24 h at room temperature. Data in bar graph are presented as zones of diffusible inhibition (zone of inhibition–disk diameter). The resulting elution water from each of the treatment steps was tested for activity against surface grown *S. mutans* using the 5 μL spot method (Table). These activities are given as mean zones of inhibition. These values were used to estimate the active concentrations of CDA using a standard curve derived from the titration of CDA in deionized water (Biol. Est.). This is compared to the estimate of CDA concentrations determined spectrophotometrically (O.D. Est.). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

one or two five minute periods did not result in a noticeable loss in the diffusible inhibition from the bottom CDA-EVA surface (Figure 7 graph). After the third washing (3 × 5 min) the reduction in diffusible activity of the bottom surface did achieve significance when compared with that of the unwashed CDA-EVA, but not compared to the successive washings. In contrast, the first and subsequent washing did significantly reduce the activities of the top surfaces compared to the unwashed control. Immersion of 2.5% CDA-EVA in deionized water for 24 h, resulted in significant reduction in diffusible activity from both the bottom and the top surfaces with total loss of diffusible activity from the top (Figure 7).

As predicted from the loss of retained activity, the 24 h wash resulted in a relatively high concentration of CDA and CDA activity released to the water eluate (Figure 7 table). Interestingly, the first 5 min wash resulted in significantly ($p < 0.05$) greater release of CDA activity than the subsequent washes. The anti-*S. mutans* activities detected in the extraction water and their prediction of the concentrations of CDA using a biological standard curve generally agreed with the concentrations determined spectrophotometrically.

Release of CDA Activity From 2.5% CDA-EVA on Exposure to Agar Surface

The previous data suggest that the CDA is being released in a diffusible fashion from the CDA-EVA disks into the surrounding agar matrix. Indeed, it is this diffusible release on surface contact that would allow the CDA to reach potential targets *in situ*. Figure 8 depicts the retention of CDA activity after sequential contact with a purposely septic surface. As can be seen, there were substantial reductions in the antimicrobial activities of both the top and the bottom surfaces of the 2.5% CDA-EVA disks after the first 24 h exposure compared to the initial exposure. The reductions in activities from the bottom surfaces in subsequent passages (48 and 188 h) were not significant suggesting retention of anti-*S. mutans* activity for more than 7 days. In contrast, there was total loss of diffusible activity from the top surface on the second passage (48 h).

Durability of Antimicrobial Activity of CDA-Loaded EVA Disks

The utility of the CDA loaded EVA matrices would be greatly enhanced if we could demonstrate longevity of the antimicrobial activity following prolonged storage. Three EVA films loaded with 2.5% CDA were maintained at

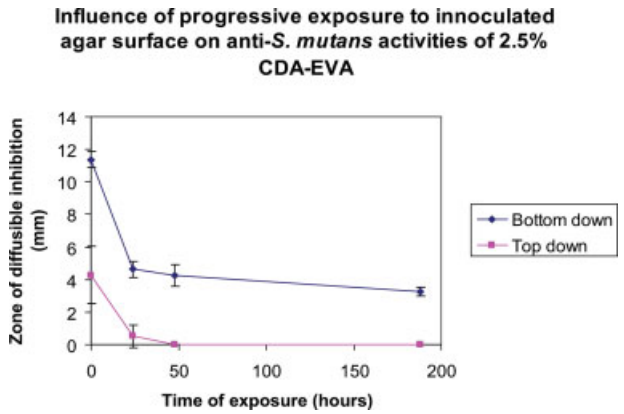


Figure 8. Comparison of the antibacterial activities of the top and bottom sides of 2.5% CDA-EVA disks after serial incubation on freshly inoculated agar plates. Disks were oriented on freshly inoculated agar surfaces as previously described. After 24 h of incubation to allow lawn development and measurement of zones of inhibition (0 h), the disks were transferred to freshly inoculated plates maintaining surface orientation and again zones of inhibition were determined (24 h). The disks were again transferred to freshly inoculated plates and zones of inhibition measured after overnight development of the lawn (48 h). The plates were returned to the incubator with disks in place until 188 h after the start of the experiment and the disks were transferred to freshly inoculated plates to determine retained activity. These experiments were performed in quadruplicate, with variation shown as ±1 s.d. error bars. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

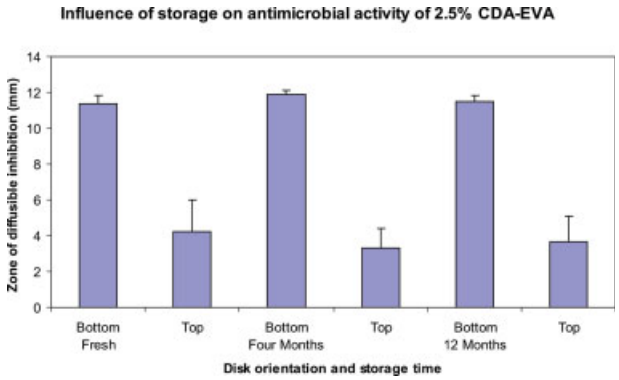


Figure 9. Prolonged room temperature storage of 2.5% CDA-EVA discs showed no discernible effect on the diffusible antimicrobial activity versus surface grown *S. mutans* over a period of 1 year. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

room temperature in the dark in a covered glass Petri dish. A minimum of eight (6 mm) disks were sampled for antimicrobial activity against *S. mutans* immediately after they were prepared, as well as 4 and 12 months after preparation. As shown in Figure 9, compared to freshly prepared disks, there was no loss in activity from either the bottom or the top surface of the disks even after 12 months of storage. Indeed, there was no loss of antimicrobial activity with EVA disks loaded with CDA either greater or less than 2.5% (data not shown). Thus, the antimicrobial activity of CDA loaded EVA samples is stable at room temperature for at least 1 year.

Effect of CDA Concentration on the Release Rate of CDA from EVA Films

We examined the rate of release of CDA when drug loaded EVA squares containing from 0.63 up to 10 wt % CDA were immersed in water at 37°C. Time release profiles generally showed a pattern of initial "burst" release of CDA during the first 1–2 days, followed by nearly constant release for each individual film for a sustained interval from days 3 to 14. Mean release rates from three individual film squares at each CDA concentration were measured by determination of the linear regression slope of the spectrophotometrically calculated CDA concentrations in samples of the daily water exchanges. As shown in Figure 10, we found a linear dose dependency in the mean rates of release of CDA from loaded EVA film squares ranging from 0.63 to 10 wt % CDA.

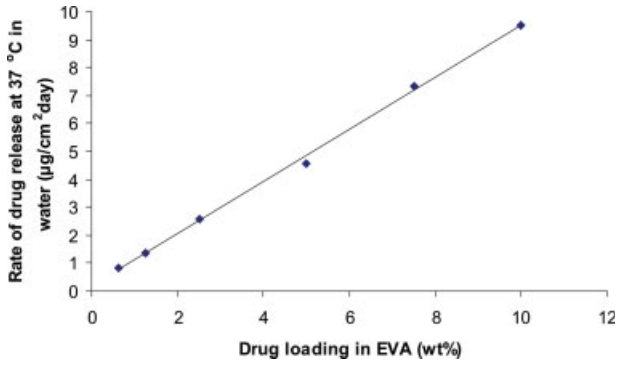


Figure 10. CDA release rates from EVA in deionized water at 37°C as a function of initial drug loading. Each point represents the mean rate determination for three film squares. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

As indicated in Table I and Figure 2, CDA released from EVA copolymer films provides demonstrable antimicrobial activity against a range of relevant target oral organisms including two strains of *P. gingivalis*, and three species of *Candida*.

Our observations of the equivalence of Clinical grade and Sigma reagent CDA assures that our previous *in vitro* studies[18,19,24] should be predictive of the behavior of the oral drug-delivery devices in clinical trials now being conducted under guidance from the U.S. Food and Drug Administration via IND 73,126.

The asymmetric release of CDA (top vs. bottom sides of disks presented in Figures 5, 7, and 8) is consistent with related observations we have made previously about this method of drug incorporation (evaporation of dichloromethane co-solvent from mixtures of CDA and other copolymers) in similar systems. Despite 24 h mixing that we feel homogeneously mixes both drug and copolymer, it is apparent that some precipitation or crystal formation of the drug occurs at the bottom surface of the film at the interface with the glass dish. The apparent zones of inhibition (with diameters roughly twice as large for the bottom sides of the film disks as compared to the tops) suggest that perhaps four times as much drug may emanate from the film's bottom surface as from the top.

We have taken advantage of this knowledge in the production of our clinical mouth guard devices by consistently placing the original film bottom surface in contact with the stone casting (see Figure 1) so that the majority of the drug release in the clinical application will occur at the intended gingival sites, and presumably less drug will escape to the lingual region of the mouth and be swallowed in the saliva prior to providing local oral effect.

As indicated in the description of the measurements underlying Figures 7 and 8, these CDA loaded EVA films typically demonstrate an initial "burst" of drug release when immersed in distilled water, lasting 1 or 2 days. Release then stabilizes at a low, nearly constant rate. The burst effect is usually attributed to a sudden release of high concentrations of drug molecules loosely bound to the surface of the films. Figure 10 shows a remarkably linear plot between CDA release rates (determined spectrophotometrically) and different initial CDA loading concentrations ranging from 0.63 to 10.0 wt %, implying a simple means to control antimicrobial release. This finding is also sup-

ported by the antimicrobial activity data shown in Figure 6. For initial clinical assessments, based partly on the MIC estimates reported here, we have chosen to use a mouthguard device containing 2.5% CDA, prepared by combining 18 g of EVA with 0.45 g of CDA. The abovementioned burst effect is circumvented by a 24 h soak of the mouthguard in 100 mL of distilled water.

CONCLUSIONS

Our studies reveal that CDA is a potent broad spectrum antimicrobial agent capable of inhibiting the growth of prominent oral pathogens as well as different species of *Candida*, several of which are resistant to killing by antifungal drugs.[25] We have also demonstrated that CDA release from EVA, and antimicrobial activity, can be maintained over an extended period of time. Finally, we found that CDA loaded EVA sheets have a long shelf-life and can retain their antimicrobial activity even after 1 year of storage at ambient temperature.

We also show that following incorporation into EVA, CDA antimicrobial activity, after an initial burst of release, is slowly and steadily released for over a week, consistent with previous reports based solely on spectrophotometric measurements of CDA dispersal in deionized water.[24] The rate of release of CDA from EVA films is proportional to the amount of CDA initially incorporated in the films. Taken together, these observations cumulatively point to EVA as an excellent matrix for slowly releasing CDA into the oral cavity to treat infections caused by a variety of oral pathogens.

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