Adhesion of *Pseudomonas aeruginosa* to collagen biomaterials: Effect of amikacin and ciprofloxacin on the colonization and survival of the adherent organisms

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Abstract: The adherence of *P. aeruginosa* to collagen membrane, sponge, and to a new anti-infective COLL dressing and the susceptibility of the organisms attached to the biomaterials to amikacin were investigated *in vitro.* After 17 h of attachment, the bacteria demonstrated an increased resistance to amikacin compared with their free-floating counterparts. Amikacin, even at a concentration exceeding 150 times the minimal bactericidal concentration (MBC) for the strain tested, did not eradicate the attached bacteria from the surface of collagen membrane. However, when the drug at a high concentration (over 16 times the minimal inhibitory concentration, MIC) was present in the incubation medium before it had been inoculated with *P. aeruginosa,* a reduction of 2 $\log_{10}$ units in the organisms adherent to the surface of collagen membrane was observed. We conclude that slow release of the antibiotic from the COLL dressing could control the bacterial colonization on the surface. In fact, the released amikacin at the final concentration of 32 times the MBC reduced the number of adherent bacteria by 6 $\log_{10}$ units. In contrast, ciprofloxacin at the same final bactericidal concentration completely eradicated the bacteria from the surface of COLL dressing. However, as ciprofloxacin is not recommended for use as a topical antimicrobial agent, a further search is needed to find an agent with a similar anti-colonization activity. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 41, 593–599, 1998.

Key words: collagen membrane; collagen sponge; adhesion; *Pseudomonas aeruginosa;* amikacin

INTRODUCTION

Collagen-based biomaterials are considered the most promising substitutes for skin regeneration. They are biocompatible materials convenient also as biological dressings and for guided tissue regeneration techniques. However, their application for temporary coverage of open wounds has some limitations because collagen-based biomaterials are particularly susceptible to bacterial colonization and enzymatic digestion.[1–3] Over twenty years ago a combination of collagen sponge and antibacterial agents as a solution in chronic wounds was proposed by Chapvil.[4] To date, several antibiotics have been investigated as agents for the reduction of wound contamination, preserving collagen from colonization by microorganisms and, consequently, improving wound healing. Amikacin has been bound covalently to implantable collagen without loss of its activity.[5] Norfloxacin and nystatin also have been selected to be used as topical antimicrobial drugs because of their low toxicity to human tissues and their efficient anti-bacterial and antimycotic activity.[6] More recently, a combination of neomycin, polymyxin B, mupirocin, quinolone, and an antifungal agent has been proposed as an experimentally optimal formulation for protection of cultured skin grafts.[7]

Despite numerous studies on the subject, only one report has demonstrated quantitatively the process of colonization of collagen membranes by bacteria. These organisms originated from strains representing the population of oral microorganisms.[8] Recently, in our laboratory a new anti-infective collagen dressing containing amikacin or gentamycin has been developed.[9] As a specific adherence of *P. aeruginosa* to bovine collagen has been documented,[10] in the present study we investigated a rate of *P. aeruginosa* colonization of collagen membrane and sponge (both materials were used to construct our dressing) and the effectiveness of amikacin in reducing the number of adherent organisms. Ciprofloxacin also was included in our experimental design because it recently has been recom-

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mended as a very potent agent in eradicating *P. aeruginosa* biofilms on biomedical devices.[11,12]

MATERIALS AND METHODS

Bacteria

Four *P. aeruginosa* clinical isolates from the collection of the Department of Clinical Bacteriology, Children's Memorial Hospital, Warsaw, Poland, and *P. aeruginosa* ATCC 27835 were tested. These strains were used in a previous study.[10] All these isolates were susceptible to amikacin (the MICs in the range of 2–8 μg/mL) and to ciprofloxacin (the MICs in the range of 0.06–0.125 μg/mL), as determined by the macrodilution method with an inoculum of 10^5 CFU of *P. aeruginosa* per mL.[13] Throughout the experiments, inoculum density standardization was performed spectrophotometrically at the wave length of 600 nm (SP6-500 UV spectrophotometer, Pye Unicam Ltd., Cambridge, England). An optical density of 0.6 was equivalent to a concentration of bacteria of about 1×10^9 CFU/mL, as verified by the dilution plating method.

Media and antibiotics

Bacto-Agar plates (Difco Laboratories, Detroit, MI) and Oxoid nutrient broth (Unipath LTD., Basingstoke, England) were used for culturing of bacteria. Antibiotics were purchased from their manufacturers as follows: amikacin sulfate (Biodacyna®) from the Institute of Biotechnology and Antibiotics, Warsaw, Poland; ciprofloxacin (Ciprobay 100) from Bayer, Leverkusen, Germany.

Collagen biomaterials

Collagen membranes produced from fibrous dermal bovine collagen by Colldres Ltd., Warsaw, Poland, and collagen sponges manufactured from bovine tendons by Tissue Bank, Morawica, Poland, were investigated in adherence studies. Also, a COLL dressing composed of two collagen sponges and an intermediate active layer with incorporated amikacin (1 mg/cm^2) or ciprofloxacin (0.04 mg/cm^2) were used in the experiments. The amount of antibiotics introduced into the active layer was standardized as described previously.[9] For comparison, a polyurethane sponge manufactured by Veenended–Schaumstoffwerk GmbH, Lichtenfeld, Holland, was included in the study.

Colonization of collagen biomaterials

Preliminary studies on the dependence of biomaterial colonization on the inoculum size showed that the bacterial density of 10^5 CFU per mL allows monitoring of the colonization rate in the presence of a constant number of the planctonic cells in the PBS medium. To observe an increase with time of the number of sessile (adherent) *P. aeruginosa* cells on the collagen membrane and sponge, the sheets of biomaterials were cut into 1 × 3 cm rectangular sections and incubated in the individual strain suspension (10^5 CFU per mL diluted with PBS) for 1, 2, 4, 17, and 24 h at 37°C. The same experimental design was used to colonize a polyurethane sponge. To evaluate the colonization rate of COLL dressing the analogous sections of the biomaterial containing antibiotics were immersed in 5 mL of nutrient broth inoculated with 10^5 CFU of *P. aeruginosa* clinical isolates per mL and incubated for 24 h at 37°C. After incubation, the number of bacterial cells adherent to the biomaterial was enumerated by a sonication and plating method (see the following paragraph). The concentration of bacteria in the incubation media (PBS or nutrient broth, respectively) was estimated by serial dilution and plating.

Enumeration of viable sessile bacterial cells

After incubation either in the bacterial suspension in PBS or in the broth culture, the sections of collagen biomaterials were washed four times for 7 min (with intensive shaking) with 8-mL volumes of PBS-T (PBS containing 0.1% Tween 80) to remove loosely bound bacteria. The biomaterial sections then were transferred to fresh vials and the adherent bacteria removed from the biomaterial into 2 mL of PBS by 32-s sonication treatment in the ultrasonic washer (64 kHz). The detached bacteria then were diluted in PBS and plated onto nutrient agar. The plates were incubated at 37°C for 24 h and the colonies then counted. The preliminary experiments showed that the sonication procedure released over 94% of tightly adherent *P. aeruginosa* organisms. Also, the viability of *P. aeruginosa* cells after such sonication treatment was examined and no lysis was observed.

Treatment of sessile *P. aeruginosa* cells with amikacin

After 1, 2, 4, 17, and 24 h of attachment of the bacteria in a *P. aeruginosa* suspension, the sections of collagen membrane were washed extensively in PBS-T to preserve only tightly bound bacteria, and then they were placed into tubes with a fresh, sterile broth containing amikacin at various concentrations. Then the tubes were incubated for 20 h at 37°C.

Preventing collagen membrane colonization by amikacin

The sections of collagen membrane were placed inside the tubes containing 5 mL of the *P. aeruginosa* clinical strain suspension at a concentration of 10^5 CFU per mL of nutrient

broth. Amikacin immediately was introduced inside the tubes at a final concentration corresponding to 0 (control), 1, 2, 8, 16, and 100 times the MIC for the strain tested. The tubes were incubated for 20 h at 37°C. The number of viable sessile cells was enumerated by sonication and plating. The concentration of planctonic cells was estimated by serial dilution and plating.

Evaluation of a concentration of antibiotic released from COLL dressing

A section (1 × 3 cm) of COLL dressing with amikacin or ciprofloxacin was placed inside the tube containing 2 mL of PBS or nutrient broth and incubated for 24 h at 37°C. Then the COLL dressing was removed and the concentration of amikacin or ciprofloxacin in the incubation medium was determined by the serial dilution method, with *P. aeruginosa* ATCC 27853 inoculated at the concentration of 10^5 CFU per mL. The concentration of released antibiotic was calculated as follows: the concentration of the drug released = the MIC value of the drug tested/a dilution coefficient in the tube in which the growth of bacteria completely was inhibited. Measurements were performed in triplicate and repeated two times.

RESULTS

In this study of collagen biomaterials, an adhesion of *P. aeruginosa,* a common organism infecting wounds, to collagen membrane and sponge has been investigated. In Table I, a comparison of the number of bacteria attached from five *P. aeruginosa* strains to these biomaterials is presented. Polyurethane sponge was included in the study because in order to compare microbial colonization of different biomaterials, the surfaces of materials available for cell attachment should be similar.[14] However, collagen membrane and sponge have different surface microtopographies: the sponge is a porous material in which bacteria can be entrapped without employment of any specific mechanisms of adhesion. The polyurethane sponge has approximately the same surface area as the collagen sponge. However, in contrast to collagen fibers,[15] polyurethane is unable to bind bacterial cells via ligand–receptor interactions. Thus by comparing these two materials one could observe whether or not the structure of the substratum influenced the adherence results. In our experiments, the adherence to collagen membrane was significantly greater than to either collagen sponge or polyurethane sponge ($P < 0.0001$) despite their greater colonization areas. The collagen sponge attracted over one $\log_{10}$ unit more organisms than did polyurethane sponge after the first 4 h of incubation. After 24 h, we observed a partial degradation of collagen sponge by enzymes produced by *P. aeruginosa* strains; therefore further comparison of these two sponges was not possible. However, the attachment of bacteria to collagen membrane still was significantly higher than it was to polyurethane sponge.

TABLE I
Comparison of Adhesion of *P. aeruginosa* to Biomaterials

Biomaterial	Number of Adherent Bacteria (Log CFU/Dressing)* after Incubation for 4 h	24 h
Collagen membrane	4.11 ± 0.20	6.46 ± 0.14
Collagen sponge	3.17 ± 0.34	partly dissolved
Polyurethane sponge	2.55 ± 0.40	5.01 ± 0.56

*Data represents the mean ± SD of the results obtained for five tested strains. Experiments were performed twice in duplicate for each strain.

In Figure 1 a kinetics of the collagen membrane colonization by organisms of five *P. aeruginosa* strains is displayed. Only slight variations in the colonization rates among the strains tested were observed. The number of attached (sessile) bacterial cells increased and peaked at 24 h for all except one strain. Similar colonization curves for collagen and polyurethane sponge were obtained (data not shown).

In the subsequent set of experiments, the suscepti-

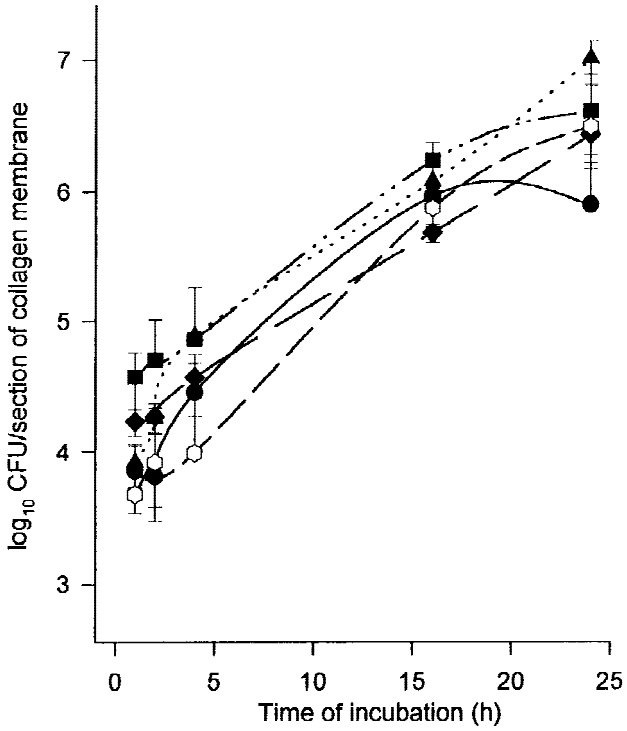

Figure 1. Adherence of *P. aeruginosa* cells to collagen membrane as a function of contact time. The collagen membranes were immersed in *P. aeruginosa* cell suspensions of five clinical strains (10^5 CFU/mL). After a 24 h incubation, the sections of membrane were washed, sonicated, and detached bacteria were counted by plating.

bility to amikacin of the bacteria attached to the surface of collagen membrane was investigated. The first question considered was the time required for bacteria to attach and become resistant to the bactericidal action of the antibacterial agents. To elucidate this, we inserted the sections of collagen membrane into the bacterial suspension for 1, 2, 4, 17, and 24 h to allow colonization and microcolony formation, and then, after extensive washings, the sessile cells were challenged with amikacin at a bactericidal concentration. As shown in Figure 2, after the first 4 h of colonization, *P. aeruginosa* cells still were susceptible to amikacin; almost a total killing of the attached cells was observed. In contrast, for the longer time periods of microcolony growth on the membrane surface, the $\log_{10}$ units were reduced by 2.3 after 17 h to 0.9 after 24 h compared to the control, drug-free samples. Therefore, we examined whether higher concentrations of amikacin—up to 150 times MBC for the strain tested—could eradicate the sessile cells from the surface of collagen membrane. As displayed in Figure 3, even the highest concentration of amikacin was not effective against *P. aeruginosa* growing on the membrane.

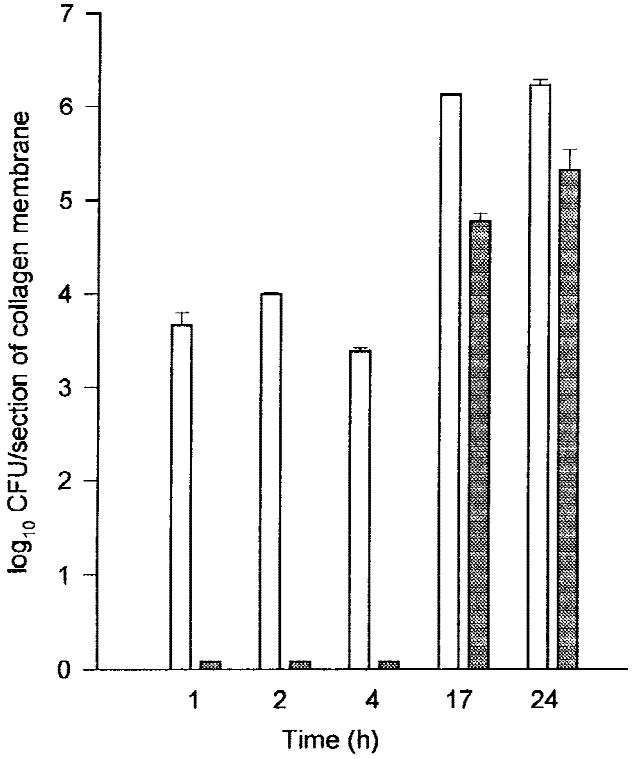

Figure 2. Susceptibility of sessile cells of *P. aeruginosa* attached to collagen membrane to amikacin at eight times the MBC. The biomaterial was exposed to a cell suspension of a clinical strain for 1, 2, 4, 17, and 24 h and, following incubation at 37°C, challenged with drug for 20 h at the same temperature (shaded bars). The other biomaterial sections colonized at the same time intervals were not challenged with an antibiotic but were incubated in fresh nutrient broth for 20 h (open bars) and served as controls. The counts of viable cells adherent to the biomaterial were estimated by sonication and plating.

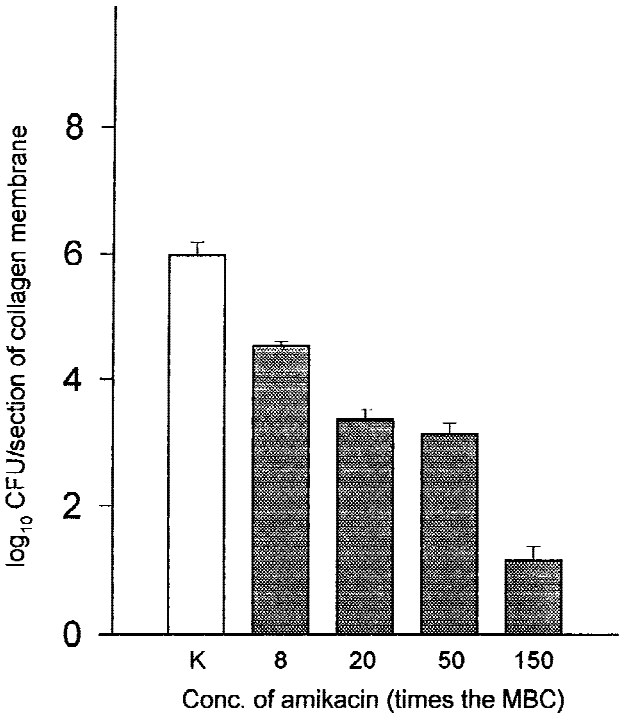

Figure 3. Killing of sessile *P. aeruginosa* cells on collagen membrane by amikacin at 8, 20, 50, and 150 times the MBC. The collagen membranes were incubated in a bacterial suspension of 10^5 CFU/mL for 24 h, then extensively washed and the sessile cells challenged with amikacin at various concentrations (shaded bars). Three sections of collagen membrane were not treated with amikacin but incubated in nutrient broth (control—open bar). After 20 h of incubation, the number of viable bacteria was estimated by sonication and plating.

Aminoglycosides influence the cell wall structures of gram-negative bacteria and consequently inhibit the attachment of *P. aeruginosa* to collagenous substrata.[7] Thus we examined whether amikacin introduced at various concentrations to the bacterial broth before inoculation with *P. aeruginosa* could prevent bacterial colonization. As shown in Figure 4, the mean number of viable bacteria adhering to biomaterial decreased gradually with increasing concentrations of amikacin, reaching a reduction of over 2 $\log_{10}$ units at a drug concentration above 16 times the MIC. At the same time an effective killing of planctonic (free-floating) cells of the colonizing strain with an increasing concentration of amikacin was seen.

Our new COLL dressing prepared with collagen sponges was designed to preserve a sustained concentration of released antibiotic in the infected tissue. To analyze whether a continuous release of amikacin from COLL dressing prevents collagen sponge from degradation and/or colonization by *P. aeruginosa*, we immersed the sections of COLL dressing in a bacterial broth culture of clinical *P. aeruginosa* strain. As sum-

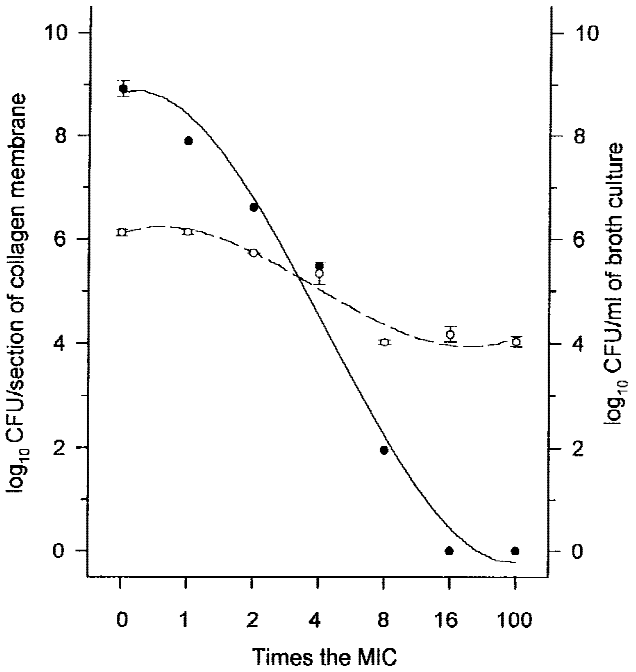

Figure 4. Effect of amikacin on inhibition of the attachment of *P. aeruginosa* to collagen membrane. The sections of biomaterial were placed in the tubes with nutrient broth containing various concentrations of drug and inoculated with 10^5 CFU of a *P. aeruginosa* clinical strain per mL. Viable counts of bacteria were estimated by sonication and plating. (○) Bacteria on the surface of collagen membrane; (●) bacteria in the broth culture.

marized in Table II, a significant reduction in bacterial counts to 10^2 CFU/cm^2 on the surface of COLL dressing and 10^3 CFU/mL of the broth was observed compared to control samples in which COLL dressing without the drug had been partly dissolved and the planctonic cells grown up to an average of 10^9 CFU/mL. However, viable *P. aeruginosa* organisms still were present on the surface of COLL dressing. Thus we introduced another antibacterial agent, ciprofloxacin, into the active layer of COLL dressing. The concentration of ciprofloxacin released from dressing after 24 h was comparable to that of amikacin when expressed as the MBC for the strain tested. Ciprofloxacin proved to be a more effective drug against the sessile *P. aeruginosa* cells; we observed almost a total killing of bacteria both on the surface of membrane and in the broth when treated with the final concentration of ciprofloxacin equal to 32 times the MBC.

DISCUSSION

Collagen attracts bacterial cells of many species, such as *Staphylococcus aureus, Staphylococcus epidermidis,* and *P. aeruginosa.* These bacteria attach to the collagen fibers via specific interactions.[10,16,17] As collagen is a very attractive biomaterial for application to healing wounds, special attention should be paid to preventing bacterial colonization and enzymatic degradation of the collagen matrix. Aminoglycosides have been considered to be good candidates as agents for controlling bacterial growth on collagen-based biomaterials. They offer a broad spectrum of activity against gram negative and gram positive bacteria and are relatively nontoxic to tissues.[7,9]

In the present study we demonstrate some data on the effectiveness of amikacin in controlling *P. aeruginosa* growth on collagen membrane or sponge. The results of the introductory experiments prove that *P. aeruginosa* colonize the surfaces of the materials tested, with the highest bacterial counts detected on the surface of collagen membrane. Collagen sponge presented a higher colonization score than did the polyurethane sponge during the first 4 h of the experiment. Assuming that the colonization area of these two sponges is comparable, the difference observed is explicable by a higher avidity of interaction between bacteria and collagen than between bacteria and polyurethane. Wang and colleagues[8] demonstrated that collagen membrane has significantly higher values of *Streptococcus* mutant adherence than other non-tissue-derived membranes used in a guided tissue regenera-

TABLE II
Effect of Amikacin and Ciprofloxacin on Viable Counts of *P. aeruginosa* on the Surface of COLL Dressing and in the Broth culture

			Bacteria (CFU) per:			
		Conc. of the Antibacterial Released	Section of COLL Dressing Without Antibacterial		Section of COLL Dressing with Antibacterial	
Antibacterial Agent	Concentration (mg/cm^2)	(mg/mL/Times the MBC)	1 cm^2 of COLL surface	1 ml of broth	1 cm^2 of COLL surface	1 ml of broth
Amikacin	1000	64/32	partly dissolved	8.2×10^8	1×10^2	3×10^3
Ciprofloxacin	40	8/32	partly dissolved	2.4×10^9	<10	<10

Biomaterial was incubated in *P. aeruginosa* culture for 24 h, then extensively washed and sonicated. The number of bacterial cells was estimated by plating. The number expressed is the mean of two experiments in triplicate.

tion technique. These authors also observed the process of collagen membrane degradation by the following organisms: *Porphyromonas gingivalis, Prevotella melaninogenica,* and *Treponema denticola,* which are known as collagenase producers. In our experiments, a very fast degradation of collagen sponge in the suspensions of all the strains tested was observed. The collagen membrane remained more resistant to the attack of bacterial enzymes. The tensile strength and chemical resistance of collagen sponge may be enhanced by chemical or physical means,[3] but we preferred to avoid such a treatment to preserve the sponge elasticity and its good biocompatibility. Hence the collagen sponge has to be combined with antibacterial agents for its protection and also to improve its effectiveness in reducing infection rate.

P. aeruginosa organisms grown as microcolonies on collagen membrane increased in number with time and became resistant to the bactericidal action of amikacin, as observed after 17 h of bacterial growth on the surface of collagen membrane. We conclude that after that period of time a *P. aeruginosa* biofilm is formed that becomes resistant even to the concentration of amikacin exceeding many times the MBC value, which was estimated for free-floating organisms of the strain tested by conventional susceptibility testing. The phenomenon of biofilm formation and the subsequent increase in antibiotic resistance of sessile bacteria has been described for many medical devices and has become a serious medical problem.[18,19] It has been shown that the first few hours before the formation of the complete biofilm and its coating with the slime layer are critical for the further antibiotic treatment of sessile bacteria.[20] We observed a similar phenomenon when amikacin in a high dose (over 16 times the MIC) was present in the incubation medium before the bacterial adhesion had started; then a reduction of over 2 $\log_{10}$ units of bacteria attached was observed. Thus we believe that an early and sustained release of a drug(s) at a high concentration may be an optimal approach for controlling the microbial colonization of collagen biomaterials. Amikacin introduced into an active layer of COLL dressing was released continuously during 24 h, reaching a concentration equal to 32 times the MBC for the strain tested. It protected the collagen sponge from degradation and also from massive *P. aeruginosa* colonization, but it did not eradicate completely either sessile or planctonic cells from the *in vitro* environment of COLL dressing. Similar results have been shown for corneas treated with gentamycin-impregnated collagen shields.[21]

Recently, many investigators have suggested that fluoroquinolones are potent drugs in the treatment and prevention of infection associated with a *P. aeruginosa*-biofilm formation.[11,12] Our results showed that ciprofloxacin completely kills both the sessile and planctonic *P. aeruginosa* cells in the COLL dressing environment. However, we are aware that application of ciprofloxacin as a topical agent, despite its high bactericidal activity against *P. aeruginosa* biofilm, may promote the generation of resistant organisms.[22] A serious risk is created for patients because ciprofloxacin often is applied as a parenteral antimicrobial agent. We believe that further investigations are needed either to establish whether very high doses of ciprofloxacin that can be applied topically safely may eradicate sessile organisms more resistant (as measured by conventional susceptibility testing with free-floating organisms) to ciprofloxacin than we have examined in this study or to identify a different antibacterial agent that is comparable with ciprofloxacin in its anticolonization and bactericidal activity.

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