

## Phosphatidylcholine Coatings Deliver Local Antimicrobials and Reduce Infection in a Murine Model: A Preliminary Study

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### Abstract

**Background** Phosphatidylcholine coatings have been shown to elute antibiotics for several days. A recently developed biofilm inhibitor, cis-2-decenoic acid (C2DA), has been shown to exhibit synergistic activity with several common antibiotics. This study aims to evaluate the effectiveness of C2DA and amikacin dual drug delivery from a phosphatidylcholine coating.

**Questions/purposes** (1) What are the in vitro elution profiles of amikacin and C2DA from phosphatidylcholine-coated coupons in incubated phosphate-buffered saline? (2)

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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

The in vivo animal model was performed at the University of Arkansas of Medical Sciences, Little Rock, AR, USA. All other work was performed at the University of Memphis, Memphis, TN, USA.

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Does the presence of C2DA in eluate samples lower the amount of amikacin needed for bacterial inhibition in overnight bacterial turbidity assays? (3) Does addition of amikacin and C2DA result in decreased colony-forming units (CFUs) on wire implants and bone when compared with phosphatidylcholine coatings alone in a mouse model of periprosthetic joint infection?

**Methods** Effects of loading concentrations were assessed during 7-day in vitro elution studies for coatings containing all mixtures of 0%, 5%, 15%, and 25% wt of amikacin and C2DA (n = 4) through quantitative high-performance liquid chromatography concentration determination and plotting concentration eluted over time. Antimicrobial activity was assessed by overnight turbidity testing of elution study samples against *Staphylococcus aureus* or *Pseudomonas aeruginosa*. In vivo efficacy was assessed using phosphatidylcholine-coated wire implants in a murine (mouse) model of infection (n = 3). Wire implants were coated with phosphatidylcholine containing no antimicrobials, amikacin alone, C2DA alone, or amikacin and C2DA and then inserted into the intramedullary femur of each mouse and inoculated with *S aureus*. The number of viable bacterial colonies on the implant surface and in the surrounding bone was determined after 1 week with the goal of achieving complete bacterial clearance. Total viable CFU count and proportion of samples achieving complete clearance were compared between groups.

**Results** Elution samples showed a burst response of amikacin and C2DA for 1 to 2 days with C2DA release continuing at low levels through Day 4. All tested eluate samples inhibited *P aeruginosa*. Samples from coatings containing 25% amikacin or 15% amikacin and any amount of C2DA were able to inhibit *S aureus* formation, but all coatings with 5% amikacin or 15% amikacin but no C2DA were not inhibitory. All in vivo treatment groups

achieved complete bacterial clearance on the wire implant, and the C2DA alone and amikacin alone coatings cleared all CFUs in bone (pin: phosphatidylcholine only one of three; amikacin three of three, C2DA three of three, amikacin + C2DA three of three,  $p = 0.04$  [Fisher's exact test]; bone: coating only: zero of three; amikacin: three of three; C2DA; three of three; C2DA + amikacin: one of three;  $p = 0.03$  [Fisher's exact test]).

**Conclusions** Phosphatidylcholine coatings elute antimicrobials in vitro under infinite sink conditions for up to 4 days in phosphate-buffered saline and were able to reduce bacterial colonies in a preliminary in vivo model. Turbidity testing with eluate samples containing varying amounts of C2DA and amikacin agrees with previous studies showing synergy between them.

**Clinical Relevance** Used as an adjunctive to systemic therapy, C2DA-loaded phosphatidylcholine coatings have potential value as a prophylactic infection prevention measure. Future studies may include different antibiotics, animal studies with larger sample sizes and more controls, and advanced coating delivery methods.

## Introduction

Bacteria in biofilm-based implant infections are up to  $\times 1000$  more resistant to antimicrobials than their planktonic counterparts, making systemic antibiotic therapy ineffective in the treatment of periprosthetic joint infection (PJI) [3]. Local antibiotic delivery methods have been investigated for several decades as a means of increasing antimicrobial concentration at the site of the wound while keeping systemic levels low, avoiding potential side effects. Antibiotic-loaded polymethylmethacrylate (PMMA) cement has been used for both prevention and treatment of PJI and has been shown to reduce the risk of infection in orthopaedic procedures [9, 15, 29]. The primary disadvantage of PMMA is that it is not biodegradable, so a second procedure is often needed to remove the device. Biodegradable delivery systems have been developed that offer similar drug elution profiles but completely degrade over a specified time interval, including collagen fleece, calcium sulfate, polymers such as lactic acid, and chitosan [5, 29]. The phospholipid phosphatidylcholine (PC) has been used in both purified form and as a PC-sesame oil gel to deliver antibiotics with favorable reductions in bacterial colony-forming units (CFUs) during in vivo PJI models [13, 19]. PC has already been approved by the FDA as an additive to demineralized bone matrix to enhance bone ingrowth, suggesting that it would not induce adverse effects when used for drug delivery applications.

Naturally occurring biofilm dispersal agents have gained attention as a result of their ability to revert biofilm

communities back into the planktonic state, eliminating the antimicrobial tolerance conferred by the biofilm [11, 16, 17, 24]. Among the recently investigated dispersal agents, cis-2-decenoic acid (C2DA) has shown potential as a result of its cross-species and cross-kingdom activity, effectiveness at low concentrations, good cytocompatibility, synergistic activity with many antibiotics, and its ability to inhibit biofilm formation when added to planktonic bacteria [4, 14, 16–18, 23, 25]. Despite promising results, there is currently a research gap between in vitro studies and development of effective local delivery devices for in vivo use. There is also a lack of in vivo studies examining the antibiofilm efficacy of C2DA at surgical wounds or confirming in vivo synergistic responses to antibiotics. The amphipathic structure of PC may make it an ideal carrier of both a hydrophobic fatty acid like C2DA and water-soluble antibiotics. PC has been previously used to deliver amikacin [13], which exhibits synergistic activity with C2DA in vitro [18] and has been shown to be cytocompatible with osteoblasts at concentrations up to 2000  $\mu\text{g/mL}$  [22].

Therefore, we asked: (1) What are the in vitro elution profiles of amikacin and C2DA from phosphatidylcholine-coated coupons in incubated phosphate-buffered saline? (2) Does the presence of C2DA lower the amount of amikacin needed for bacterial inhibition in overnight bacterial turbidity assays? (3) Does addition of amikacin and C2DA result in decreased CFUs on wire and bone when compared with phosphatidylcholine coatings alone in a mouse model of PJI?

## Materials and Methods

Elution of antibiotics was first evaluated using in vitro elution studies. Antimicrobial activity was confirmed by turbidity testing using the eluate samples. Coating efficiency then was tested in vivo using a murine model of PJI.

### Fabrication

C2DA and amikacin (MP Biomedical, Santa Ana, CA, USA) were incorporated into 90 g Phospholipon® (Lipoid, Ludwigshafen, Germany) by mixing 0 g, 50 mg, 150 mg, or 250 mg C2DA and 0 g, 50 mg, 150 mg, or 250 mg amikacin with enough PC to achieve a total mass of 1 g [12]. Individual coatings were mixed at room temperature through a chopping and kneading process until the drug payload was homogeneously distributed throughout the PC. Drug-loaded PC mixtures were made 24 hours before study use and stored at  $-8\text{ }^{\circ}\text{C}$ .

## Elution and Activity

In vitro drug elution studies were performed to determine the effects of drug loading levels on release profile before in vivo experiments. Six-well plate CellCrown™ inserts (Scaffdex, Tampere, Finland) were fitted with 41- $\mu$ M nylon filters and inserted into six-well plates. Three-quarter inch titanium coupons were cut in house; polished by successive wet grinding with 400, 800, and 1000 grit sandpaper; sonicated in acetone for 30 minutes; and autoclaved for 30 minutes at 121° C. Each coupon was coated by dragging the phosphatidylcholine mixture across the implant surface under gentle pressure to leave a thin coating (n = 4 per group). Each coupon was inserted into a separate CellCrown™ fitted well and each well received 3 mL phosphate-buffered saline (PBS). An adhesive cover was applied to each well plate and each plate was wrapped heavily with parafilm. The plates were placed in a 37 °C incubator on a Belly Dancer™ shaker (IBI Scientific, Peosta, IA, USA). Samples were taken every 24 hours with complete media refreshment for 7 days.

Amikacin and C2DA concentration in the eluate samples were measured using high-performance liquid chromatography (Thermo Scientific™ Dionex™ Ultimate® 3000, Waltham, MA, USA) with a C18 column (Thermo Scientific BDS Hypersil™ C18 150 × 4.6 mm, particle size 5  $\mu$ m). C2DA was measured at 209 nm using an isocratic buffer of 50.4% acetonitrile, 21.6% tetrahydrofuran, and 28.1% of 1% phosphoric acid [6]. Amikacin concentration was determined using a charged aerosol detector (Dionex Corona™ Veo™ Charged Aerosol Detector RS; Thermo Scientific) with an isocratic buffer of 98% 55 mM trifluoroacetic acid, 1% acetonitrile, and 1% methanol [28].

PC eluate activity was determined using a turbidity growth inhibition assay. *Staphylococcus aureus* (UAMS-1) and *Pseudomonas aeruginosa* (PA-ATCC 27317) were grown overnight in tryptic soy broth (TSB). UAMS-1 was diluted 1:50 and PA-ATCC 27317 was diluted 1:200. Representative samples from each elution group were selected for turbidity testing. In a 96-well plate, each well received 130  $\mu$ L TSB, 20  $\mu$ L of the appropriate eluate sample, and 50  $\mu$ L of bacterial inoculum (a 1:10 final dilution for each eluate sample). Each sample was tested against UAMS-1 and PA-ATCC 27317 in triplicate. The top row of each plate contained controls with six inoculated (negative controls) and six uninoculated (positive controls) wells receiving PBS instead of the eluate sample. The plates were incubated overnight at 37 °C and then turbidity was measured as absorbance at 540 nm (ELx800; Biotek, Winooski, VT, USA). The cutoff value for growth inhibition was set at 10% of the difference between positive and

negative controls, plain TSB and inoculated TSB without antimicrobials, respectively.

## In Vivo Efficacy

In vivo efficacy was tested using a pilot murine study at the University of Arkansas for Medical Sciences. PC was sterilized by gamma radiation, C2DA was filter-sterilized with a 0.20- $\mu$ m syringe tip filter, and powdered amikacin was sterilized by brief exposure to UV light. Implant wires were autoclave-sterilized at 121 °C before use. PC mixtures with no antimicrobials, 5% wt amikacin, 10% wt C2DA, or 5% wt amikacin, and 10% wt C2DA were made using an aseptic technique 24 hours before the surgery and stored at -8 °C.

Eight- to 12-week-old C57BL/6 mice weighing 16 to 18 g were anesthetized with isoflurane and Avertin (0.4-0.6 mg/g). A small incision was made over the left knee and a hole was bored into the left distal femur using 26-gauge and then 23-gauge syringe needles. A sterile 1-cm x 0.6-mm diameter stainless steel Kirschner wire was coated with the appropriate PC mixture (n = 3/group) and inserted into the femur. The wound was inoculated with approximately 10<sup>4</sup> CFUs of UAMS-1 and closed. The animals were humanely euthanized after 1 week, at which time the wire implant and appropriate femur were removed to determine the number of viable bacterial CFUs on/in each. The quantity of attached bacterial colonies on wire implants was determined by sonicating and vortexing in sterile PBS, plating the PBS on agarose, and counting the number of colonies after overnight incubation at 37 °C. The surrounding bone was homogenized and suspended in sterile PBS. The number of viable bacterial colonies in the bone was then counted by plating the homogenized solution on agarose, incubating overnight, and counting the number of bacterial colonies, similar to the process used for the wire implants. Bacterial clearance for an implant or bone was defined as a viable bacterial CFU count of zero.

SigmaPlot (Systat Software, Inc, San Jose, CA, USA) was used for all statistical analysis testing. Kruskal-Wallis analysis of variance (ANOVA) on ranks was used to test for differences in bacterial counts for each group, including control, the implants, and the surrounding tissue. Dunn's multiple comparison test versus controls was used for post hoc analysis. Fisher's exact test was used to test for differences in the proportion of complete bacterial clearance between nonantimicrobial control coatings and combined results of antimicrobial coatings. A significance level of 5% was used for determining statistical significance between groups.

## Results

The PC-coated coupons eluted C2DA and amikacin in a burst pattern over the first 1 to 2 days that tapered off by Day 3 (Fig. 1A). Amikacin elution stopped after Day 3 in most groups, but there was an increase in elution at Days 6 and 7 in some groups (Fig. 1B). C2DA eluted at a faster rate in coatings containing 15% or 25% wt amikacin (Fig. 1B). It is worth noting that only 48% of the incorporated C2DA and 57% of incorporated amikacin were recovered from the eluate samples.

The presence of C2DA in the eluate lowered the amount of amikacin needed to inhibit UAMS-1 growth; an eluate with no C2DA and 156 µg/mL amikacin was unable to inhibit UAMS-1 growth, but a sample with 19 µg/mL amikacin and 88 µg/mL C2DA was inhibitory (Table 1).

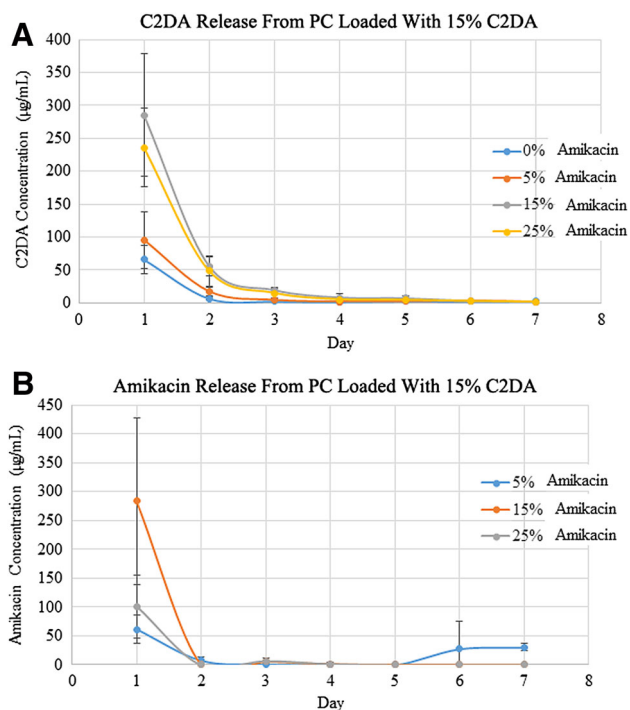
The eluates from coatings containing 5% wt amikacin did not inhibit UAMS-1 regardless of C2DA, and eluates from coatings with 15% amikacin and no C2DA were also uninhibitory. However, all eluates from coatings containing either 25% amikacin or 15% amikacin and any concentration of C2DA were able to inhibit UAMS-1. All tested eluates were effective against PA-ATCC 27317.

No attached bacterial colonies were detected on the wire implants from antimicrobial-loaded PC groups, which is lower than colonies found on coatings only (Fig. 2). The proportion of implant clearance was different between

antimicrobial-loaded groups and unloaded PC (coating only: one of three; amikacin: three of three; C2DA: three of three; C2DA + amikacin: three of three;  $p = 0.045$ ). The proportion of bone clearance was also higher between all antimicrobial-loaded groups and coating alone (coating only: zero of three; amikacin: three of three; C2DA: three of three; C2DA + amikacin: one of three;  $p = 0.03$ ). There were differences in CFU counts on implants between the treatment groups and controls, although not statistically different at the  $p < 0.05$  significance level using Kruskal-Wallis ANOVA ( $p = 0.088$ ). All nonloaded coatings had viable CFUs of bacteria in the surrounding bone (Fig. 2B). Kruskal-Wallis ANOVA detected a statistically significant difference in bacterial counts from the surrounding bone ( $p = 0.033$ ), and the amikacin alone and C2DA alone groups contained significantly less bacteria than the control group ( $p = 0.035$ ). The C2DA and amikacin combination coating did not produce a significant reduction in bacterial count based off of ranked testing ( $p = 0.620$ ; CFU count: coating only:  $1.6 \times 10^6 \pm 2 \times 10^6$ ; amikacin:  $0 \pm 0$ ; C2DA:  $0 \pm 0$ ; C2DA + amikacin:  $8200 \pm 1 \times 10^4$  CFUs).

## Discussion

Local antibiotic delivery devices offer an effective method to achieve high local antibiotic concentrations at surgical sites for prophylactic treatment of biofilm-based PJI. Bacterial adhesion is often cited as the critical event in the establishment of biofilm on implant surfaces [2], making this process a potential target for future infection prevention strategies. The biofilm inhibitor C2DA has been previously shown to induce dispersion of mature biofilms, inhibit the formation of new biofilms from cultures of planktonic bacteria, inhibit bacterial growth at higher concentrations, and exert synergistic activity with common antibiotics. These properties suggest that C2DA may be able to inhibit biofilm formation on implants if delivered alongside antibiotics at the surgical site. There is a current gap in research between promising in vitro studies and the development of effective local drug delivery devices to administer them at a surgical wound bed. PC, a biocompatible and biodegradable phospholipid, may be used to incorporate various antimicrobials and applied to implant surfaces for local drug delivery. PC is advantageous for prophylactic implant protection because it rapidly degrades, can deliver a wide variety of drugs without the harsh loading or preparation steps seen in PMMA polymerization, and, as a coating, provides better implant coverage compared with collagen sponges that must rely on diffusion to deliver antimicrobials to the implant surface [19, 27]. This work suggests that phosphatidylcholine coatings can effectively release amikacin and C2DA for 2



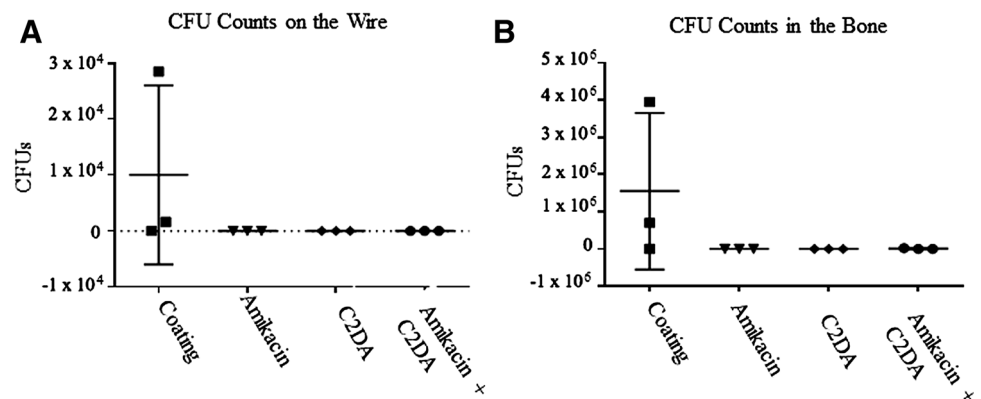
**Fig. 1A–B** Graphs show elution profiles for (A) C2DA and (B) amikacin from phosphatidylcholine coatings with 15% wt C2DA (mean  $\pm$  SD).

**Table 1.** Eluate turbidity testing against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Amikacin loading	C2DA loading	Measured amikacin ( $\mu\text{g/mL}$ )	Measured C2DA ( $\mu\text{g/mL}$ )	Inhibited UAMS-1	Inhibited PA-ATCC 27317
Low	None	156	0	N	Y
Low	Low	61	47	N	Y
Low	Medium	94	129	N	Y
Low	High	62	99	N	Y
Medium	None	65	0	N	Y
Medium	Low	19	88	Y	Y
Medium	Medium	232	283	Y	Y
Medium	High	254	150	Y	Y
High	None	237	0	Y	Y
High	Low	248	94	Y	Y
High	Medium	158	291	Y	Y
High	High	154	685	Y	Y

Eluates were diluted 1:10 during the assay; C2DA = cis-2-decenoic acid; N = no; Y = yes.

**Fig. 2A–B** Scatterplots show the number of viable bacterial CFUs recovered from (A) wire implants and (B) surrounding bone tissue (mean  $\pm$  SD).



to 3 days from orthopaedic implants to prevent bacterial colonization. From a clinical perspective, these coatings have potential for prophylactic treatment of PJI.

Although the amikacin and C2DA-loaded PC coatings show promise in the animal model, there are some limitations with this study. The elution study method shows the drug elution pattern for our system but is not a fair representation of the various processes occurring in vivo, in which the degradation rate may be different. The animal model was only a pilot study with three animals per group and limited controls, making it impossible to draw firm conclusions about the effectiveness of the coating or local delivery of C2DA. The results of the in vitro turbidity assay and the in vivo bacterial counts are conflicting with combinations of C2DA and amikacin performing better in vitro, whereas the combination group was the only treatment group to test positive for bacteria in the surrounding bone in vivo. As a result of the preliminary nature of this study and limited sample size, it is inappropriate to make comparisons among the antibiotic, C2DA, and combination

coatings; however, statistical analysis does suggest that drug-loaded coatings were significantly better than the PC-only control. It is currently unknown how well these coatings will withstand aggressive implantation procedures or how coating will influence host-implant interactions, so these results may not translate into human studies. Although limited, the results do provide pilot data to support and plan further extended preclinical studies regarding the effectiveness of PC coatings and C2DA. It should also be noted that this coating is meant to rapidly degrade and will offer no protection from hematogenous seeding of bacteria from distant infections after it has degraded.

Elution study results suggest that PC coatings can elute amikacin and C2DA over several days with a rapid burst pattern over the first 2 days that quickly tapers off. The rapid burst of antibiotic will ensure high concentrations of antibiotic in the wound bed, and incorporation of C2DA may delay bacterial attachment long enough for antibiotics or the innate immune system to kill any invading bacteria. This burst release does, however, remove most of the drug



payload quickly and shortens the overall effective elution period. The burst release also increases the chances of achieving cytotoxic levels of antimicrobials during the first day and therefore limits the amount of drug that may be incorporated. Interestingly, high-performance liquid chromatographic analysis shows only half of the incorporated antimicrobials were recovered in the eluate samples. This is likely related to bulk erosion of PC from the implant coating [10]. We believe that, during the initial elution period, large chunks of PC will erode from the coating and become dispersed in the PBS media as liposomes with encased C2DA and amikacin. These liposomes were removed when the media was changed daily, and the antimicrobials were not detected by the high-performance liquid chromatography because they were still encased in PC. This is advantageous because the liposomes will remain in the wound bed and continue to elute antimicrobials as the PC hydrolyzes and degrades, further extending the elution pattern beyond what was determined by high-performance liquid chromatography. During elution of amikacin, a second burst release was observed for the 5% amikacin group at Days 6 and 7, which has been seen in previous antibiotic elution studies using PC [7]. It was previously suggested that this is related to PC hydrolysis; however, additional research is needed to understand why this phenomenon was not observed in groups with higher antibiotic loading concentrations.

C2DA has also shown synergistic effects with many common antibiotics and will lower the amount of drug needed to kill bacteria [18, 21], which is reflected in the results of our turbidity testing. Coatings loaded with only 15% amikacin were unable to inhibit UAMS-1 growth; however, eluates from coatings with 15% amikacin and C2DA were inhibitory. This supports previous findings showing combinatorial effects between amikacin and C2DA. Coatings with 5% amikacin were unable to inhibit UAMS-1 growth regardless of the presence of C2DA, suggesting that this level of loading is too low for clinical use. All tested samples were able to inhibit PA-ATCC 27317, regardless of the presence of C2DA. *S aureus* and *P aeruginosa* are the most common Gram-positive and Gram-negative pathogens present in orthopaedic infections, respectively [1, 8, 26], suggesting that local C2DA and amikacin release can provide broad-spectrum protection against a variety of potential pathogens.

The *in vitro* findings are in accordance with the findings of our pilot animal study, in which all treatment groups were able to prevent bacterial attachment to the wire implant during the 1-week study. Although previous research shows that 500 µg/mL C2DA is needed to inhibit bacterial growth [14, 23], the C2DA-only coating was able to completely inhibit bacteria on the wire implant and in the surrounding bone. *In vitro* elution data

suggest that the peak C2DA concentration should be approximately 100 µg/mL or 20% of the minimum inhibitory concentration. One possible explanation for the complete clearance of bacteria on the wire implant and in the bone tissue is that the presence of C2DA delayed bacterial attachment long enough for the murine innate immune system to respond. This finding does call into question the use of antibiotics, because the fatty acid was able to achieve complete bacterial clearance on the pin and in the surrounding bone. Turbidity testing with the eluate samples still suggests that there is a combinatorial or synergistic effect between amikacin and C2DA, and the limited sample size of the *in vivo* groups limits the validity of any observations between treatment groups. However, this finding was not expected and does warrant an additional *in vivo* study with increased sample sizes to provide more statistically relevant data. The presence of bacteria in the surrounding bone of a limited number of animals suggests that the distance of antibiotic elution may be limited to the tissue adjacent to the implant. Although the number of CFUs recovered from bone tissue in the combination group was lower than that of the controls, bacteria could rebound and cause infection. Nevertheless, the antimicrobial-loaded coatings were able to increase the proportion of bacterial clearance when compared with the control. Because this coating would likely be an adjunct preventive measure to systemic prophylactic antibiotics [20], this risk of infection in surrounding tissue will be minimized.

In conclusion, phosphatidylcholine coatings can be used as local drug delivery devices for prevention of bacterial colonization on implant surfaces. The high early antimicrobial elution, as seen with *in vitro* elution studies, kills any contaminating bacteria during the initial wound healing time period. Local delivery of C2DA may prevent bacterial attachment and increase antibiotic effectiveness, preventing colonization and formation of biofilm. Our pilot *in vivo* murine study supports these findings and warrants testing with more antibiotics, animal studies with larger sample sizes and additional controls, and improved methods of clinical application of coatings to implants.

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