Published in final edited form as:

J Biomed Mater Res A. 2012 October ; 100(10): 2732–2738. doi:10.1002/jbm.a.34209.

Development of a Broad Spectrum Polymer Released Antimicrobial Coating for the Prevention of Resistant Strain Bacterial Infections

KD Sinclair^{1,2,*}, TX Pham², RW Farnsworth², DL Williams^{1,2}, C Loc-Carrillo^{1,2}, LA Horne², SH Ingebretsen², and RD Bloebaum^{1,2}

¹Department of Veterans Affairs Salt Lake City Health Care System, Salt Lake City, UT

²University of Utah, Salt Lake City, UT

Abstract

More than 400,000 primary hip and knee replacement surgeries are performed each year in the United States. From these procedures, approximately 0.5–3% will become infected and when considering revision surgeries, this rate has been found to increase significantly. Antibiotic resistant bacterial infections are a growing problem in patient care. This *in vitro* research investigated the antimicrobial potential of the polymer released, broad spectrum, Cationic Steroidal Antimicrobial-13 (CSA-13) for challenges against 5×10^8 colony forming units (CFU) of methicillin-resistant *Staphylococcus aureus* (MRSA). It was hypothesized that a weight-to-weight (w/w) concentration of 18% CSA-13 in silicone would exhibit potent bactericidal potential when used as a controlled release device coating. When incorporated into a polymeric device coating, the 18% (w/w) broad-spectrum polymer released CSA-13 antimicrobial eliminated 5×10^8 CFU of MRSA within 8 hours. In the future, these results will be utilized to develop a sheep model to assess CSA-13 for the prevention of perioperative device related infections *in vivo*.

Keywords

Ceragenin; CSA-13; Antimicrobial agents; Drug delivery; Controlled release device

INTRODUCTION

Over the past two decades, the United States has seen a significant increase in the number of total joint replacement procedures performed annually. This growth can be attributed to advancements in medical technology, improved patient care, but most significantly an active, aging population.¹ Projections have suggested that, by 2030, over 3.5 million total hip and knee replacement surgeries will be performed annually in the United States.² Total joint replacement (TJR) technology has greatly improved the quality of life for many patients, but these procedures are not without risk. While the risk for primary TJR infection is low (0.5–3%),³ periprosthetic joint infections pose a serious procedural complication that can ultimately result in patient trauma and device removal. Based upon the current rate of primary periprosthetic joint infection, it is estimated that TJR related infections would exceed 35,000 per year in the United States by 2030.²

Methicillin-resistant organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are responsible for an increasing number of surgical site infections;^{2,4}

*kristofer.sinclair@hsc.utah.edu.

specifically, these organisms are responsible for more than 46%^{2,4,5} of the positive cultures in revision hip and knee surgeries. Management of methicillin-resistant bacterial infections often results in inadequate treatment and prolonged hospitalizations.² The added difficulty of eradicating these antibiotic resistant pathogens results in perioperative infections accruing significantly higher in-hospital costs.² Annually incurred expenses to manage TJR and fracture fixation device related infections exceed \$1.8 billion in the U.S.⁴

Increased use of antibiotic therapy has resulted in isolates of *S. aureus* demonstrating reduced susceptibility. In recent years methicillin-resistant *Staphylococcus aureus* (MRSA) infections have commonly been treated with the antibiotic vancomycin, and more recently with linezolid, to eradicate the pathogens. However, the ability of these bacterial organisms to change their resistance patterns has rendered many modern antibiotics useless for the elimination of these bacterial infections.^{4,6} It is imperative that an alternative antimicrobial strategy, one that circumvents bacterial resistance, be developed.

Membrane-active antimicrobials may provide one solution to antibiotic resistant bacteria.⁷ The bacterial membrane makes for a captivating target as a result of numerous membrane structures conserved between Gram-positive and Gram-negative bacteria. Acquired resistance to these antimicrobials is less likely due to the mode of action, rapid kill rate, and the fact that dramatic remodeling of the bacterial membrane would be required. Ceragenins, a class of facially amphiphilic compounds designed to mimic naturally occurring antimicrobial peptides (AMPs).⁸ These compounds have demonstrated broad-spectrum bactericidal capabilities equipotent to, and in some cases exceeding, those of naturally occurring AMPs.⁸ This makes them especially useful against MRSA and other multidrug resistant pathogens.⁹ In addition to direct bactericidal activity, the ceragenins permeabilize the membranes of bacterial cells and sensitize them to hydrophobic antibiotics.^{8,10–14} This action is purportedly due to the affinity of ceragenins for the bacterial membrane.⁹

The ceragenin, Cationic Steroidal Antimicrobial-13 (CSA-13), is a synthetic analogue of a novel broad-spectrum AMP. This molecule was devised to address the increasing resistance of bacteria to current antibiotic therapies. When compared for antimicrobial potential, CSA-13 and representative AMPs demonstrated no difference in their bactericidal mechanism;⁹ however, ceragenins are less likely to be deactivated through associations with mucins, DNA, and F-actin as compared to the natural AMPs.^{9,16} Furthermore, because CSA-13 is not peptide based and cannot be a substrate for proteases, it is stable under physiological conditions.

CSA-13 mitigates antibiotic resistance by introducing a novel approach to eliminate bacteria that is not dependent upon molecular binding with receptor sites on the bacterial membrane. When critical concentrations of the synthetic antimicrobial compound accumulate, the bacterial membrane is rapidly depolarized and cell death soon follows. It is believed that the cationic portions of the molecule insert themselves into the negatively charged bacterial cell wall causing a severe disruption that subsequently kills the microorganism.⁸

The goal of this work was to develop an antimicrobial polymer released, combination device coating for the prevention of perioperative related TJR infections. CSA-13 was used in this experiment to determine whether it was effective in eliminating planktonic bacterial infections *in vitro*. Specifically, it was hypothesized that the broad-spectrum CSA-13 antimicrobial would exhibit potent bactericidal activity against MRSA *in vitro*. Furthermore, it was hypothesized that the broad spectrum CSA-13 antimicrobial incorporated within a silicone polymer coating with a weight-to-weight ratio (w/w) of 18% would exhibit desirable bactericidal properties when used as a polymer released device coating. To test

these hypotheses, a CSA-13 doped silicone polymer device coating was developed to challenge 5×10^8 colony forming units (CFU) of MRSA in an *in vitro* model.

MATERIALS AND METHODS

Implant Production

Combination coatings of 0, 16, 18, and 20% (w/w) were fabricated through the incorporation of CSA-13 with a one part room temperature vulcanizing (RTV-1) medical grade silicone (NuSil Silicone Technology; Lafayette, CA USA). This range of weight ratios was selected based upon the results generated from previous pilot studies, which utilized combination coatings ranging from 1 – 15%. The results of these studies suggested that the range of 16 – 20% CSA-13 in silicone polymer would likely demonstrate bactericidal potential. The CSA-13 antimicrobial was manufactured by the research team's collaborator, Paul Savage (Brigham Young University, Provo, UT USA). Following manufacturing, CSA-13 was micronized using a 00 Jet-O-Mizer jet mill (Fluid Energy, Telford, PA USA) and the resultant particle size distribution was measured using the NS500 (NanoSight, Wiltshire, United Kingdom) for nanoparticle characterization.

The coating was prepared by dispersing CSA-13 with 1 mL of naphtha and 2 mL of the RTV-1 silicone polymer for 30 minutes. After mixing, titanium alloy plugs (8 mm wide $\times$ 25 mm long) with a commercially pure titanium porous coating (1 mm thick and 7 mm wide) (Thortex; Portland, OR USA) were dip coated in the RTV-1 silicone polymer/CSA-13 solution to uniformly coat regions A and C (Figure 1a). To further facilitate a uniform coating thickness the dip coated devices were cured on a rotating electric wheel at 13 rpm for seven days under ambient conditions (per manufacturer recommendation) in a clean environment.

Bacterial Preparation

A clinical strain of MRSA was isolated from a patient who suffered from an MRSA infection and stored in 20% glycerol at -80°C . Before each experiment, bacteria were grown onto Luria Broth (LB) plates at 37°C overnight. Fresh bacteria was subcultured in 3 mL of Brain Heart Infusion (BHI) broth for 2 hours at 39°C in a 200 rpm shaker/incubator to stimulate bacterial growth. The rationale for performing this work at 39°C was to model the body temperature of sheep. A 1 mL sample was transferred to a flask containing 100 mL of BHI and allowed to grow under agitation in the incubator for another 4 hours. The exponentially grown cells were washed three times by centrifuging the cells into a pellet, discarding the supernatant, and re-suspending in phosphate buffered saline (PBS). The final pellet was suspended in 10 mL of PBS and the viable count (i.e. bacterial concentration) was determined through serial dilution. The bacterial suspension was diluted to 5×10^8 CFU/mL prior to inoculation.

The inoculum concentration of 5×10^8 CFU was selected based upon previously published methods^{17,18} where the ability to infect a sheep model was demonstrated. As it is the goal of our team to use the knowledge acquired from this *in vitro* research for an *in vivo* challenge utilizing a sheep model, a translatable inoculum was desired.

Time-Kill Analysis

Experiments were performed with CSA-13 and the clinical isolate of MRSA through six iterations with each group. For this work, three controls were utilized (Groups 1–3) in addition to the experimental test groups (Groups 4–6). Groups 1–3 consisted of: 1) MRSA in BHI to assess the standard bacterial growth rate, 2) MRSA in BHI with the bare titanium plug to characterize bacterial growth in the presence of titanium, and 3) MRSA in BHI with

the silicone polymer coating on the titanium plug to assess the effects of silicone on bacterial growth. Groups 4–6 consisted of CSA-13 doped silicone polymer coatings on titanium plugs with weight ratios of 16, 18, and 20%.

The coated titanium plugs were placed into capped-glass tubes containing 28.8 mL of BHI and inoculated with 1.2 mL of a BHI suspension containing 5×10^8 CFU of MRSA. The tubes were then incubated overnight at 39°C on a shaker plate (150 rpm) overnight. Tubes containing only MRSA in BHI were also prepared at this time for the experimental control. Aliquots of 100 μ l were removed from the cultures at 0, 1, 4, 8, and 24 h and were serially diluted in Dey-Engley (D/E) broth. At the 24 hour time point an additional 1 mL sample was also extracted and plated without dilution. The presence of antimicrobial residues has the potential to influence experimental outcomes. Therefore, it was necessary to use a neutralizing broth to deactivate these compounds to prevent antibiotic or antimicrobial carryover.^{19,20} Neutralization of these compounds allowed the ability to distinguish between an antimicrobial that may be bactericidal versus bacteriostatic. The lower limit of detection for the colony counts was 10 CFU/mL for the 0, 1, 4, and 8 hour samples and 1 CFU/mL for the 24 h samples. Time-kill curves were constructed by plotting the mean colony counts ($\log_{10}$ CFU/mL) versus time.

Bacterial Enumeration

One hundred microliter aliquots were collected from each group for each time point (0, 1, 4, 8, and 24 hours) and diluted in D/E neutralizing broth. At the 24 hour time point an additional 1 mL sample was extracted and plated without dilution. The samples were serially diluted from 10^{-1} to 10^{-7} using ten-fold serial dilution in 900 μ L of D/E broth per dilution. A 100 μ L aliquot from each dilution was then spread in duplicate on Trypticase Soy Agar (TSA - Difco Laboratories, Detroit, MI USA) plates and incubated at 37°C overnight. Following incubation, colonies on each TSA plate were enumerated and recorded.

Cation adjusted Mueller-Hinton broth (Difco Laboratories, Detroit, MI) supplemented with magnesium (12.5 mg/liter) and calcium (25 mg/liter) was used for all micro-dilution sensitivity testing and time-kill analyses. TSA plates were used for growth and quantification of organisms.

Antimicrobial Elution Kinetics

High pressure liquid chromatography-time of flight mass spectrometry (LC-MS) was used to analyze the broth samples collected at the time points of 0, 1, 4, 8, and 24 hours. Two hundred fifty microliters of the extracted sample was diluted with an equal volume of methanol to dissolve any residual or precipitated CSA-13. Deuterated CSA-13 was added as an internal standard and samples were vortexed for one minute. Fifty microliters of a 10% sodium hydroxide solution were added to the sample and vortexed for an additional minute. Five hundred microliters of dichloromethane was added to the mixture, the sample was vortexed for two minutes, and an aliquot was extracted. The previous steps were repeated and the two extracted aliquots were combined. The LC-MS was run on a C₁₈ column with acetonitrile and water as the mobile phase.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) techniques were utilized to evaluate the surface topography of the polymer released antimicrobial device coating and to characterize the dispersion and particle aggregate size of CSA-13 throughout the polymer matrix. Ten titanium plugs with the CSA-13/silicone coating were randomly selected and viewed with an SEM (JEOL 6100, Peabody, MA USA) in secondary mode.

Ten regions of interest were selected on each plug and recorded. The ten regions on each of the ten CSA-13/silicone coated plugs were also evaluated using energy-dispersive X-ray spectroscopy (EDX) techniques. This SEM coupled function enabled visualization of CSA-13 within the polymer matrix. For this approach the chlorine (Cl) channel was utilized, owing to the fact that chloride anions conjugate with the cationic ammonium ions of the CSA-13 antimicrobial to form ammonium salts.

Statistics

Data were expressed as the means $\pm$ standard deviation (SD). Statistical differences were analyzed using the Mann–Whitney *U* test and were balanced with the Bonferroni–Holm test if more than two groups were compared. $p < 0.05$ was considered statistically significant. All tests were performed five times. SPSS software 10.0 was used for statistical analysis.

RESULTS

Polymeric Device Coating

The jet milled CSA-13 antimicrobial was measured to have a particle size range of 100 – 200 nm with an average particle size of 118 nm. The broad spectrum CSA-13/silicone combination device coating was evaluated using scanning electron microscopy techniques. Evaluation of the surface of the polymeric device coating revealed apparent homogeneous distribution of CSA-13 throughout the polymer matrix in all concentrations of the CSA-13 doped silicone polymer coatings (Figure 2a). These findings were confirmed through the use of EDX. In Figure 2b, EDX was used to indicate the presence of CSA-13 via its conjugation with chloride anions; which form ammonium salts with the compound's three primary amines.

Following surface characterization, polymer thickness was measured. The average coating thickness was measured to be $150 \mu\text{m} \pm 75 \mu\text{m}$ in the CSA-13 doped silicone samples and $75 \mu\text{m} \pm 25 \mu\text{m}$ in the pure silicone polymer samples.

Time-Kill Analysis

The bactericidal potential of the broad spectrum CSA-13 doped silicone polymer device coatings (16, 18, and 20%) on titanium plugs was challenged against an MRSA bacterial inoculum of 5×10^8 CFU (Table 1). The results of these experiments indicated that Group 1, the experimental control, (MRSA in BHI) exhibited normal bacterial growth, consistent with preliminary growth curves, over the 24 hour experimental incubation period (Figure 3). Experiments conducted with Group 2, the bare titanium plugs, (no combination coating) demonstrated bacterial growth consistent with the experimental control ($p=0.34$). Group 3, the titanium plugs with a coating consisting of only silicone, “0% CSA-13”, did not demonstrate any bactericidal potential ($p=0.42$ when compared to the control). Instead, it was seen that bacterial growth was accelerated within the first few hours then tapered off to a growth rate consistent with that of the experimental control.

The test groups consisting of combination coatings of 16, 18, and 20% (w/w) all demonstrated the ability to reduce the bacterial population and possessed statistically greater bactericidal activity than the three controls ($p < 0.001$). Group 4, the titanium plugs containing the 16% (w/w) CSA-13 coating, were observed to have greater than a 2-log reduction within the first hour. However, after 24 hours viable bacteria were still observed with approximately 16 CFU/mL remaining. Group 5, the CSA-13 18% (w/w) combination coating, presented the greatest bactericidal effects, eliminating the most bacteria within 8 hours. After 24 hours there was no detectable bacterial growth. Group 6, titanium plugs that received the 20% (w/w) CSA-13/silicone polymer coating, also demonstrated the ability to

reduce the bacterial population. Although, after 24 hours it was observed that approximately 39 viable CFU of MRSA remained.

Comparisons of the three CSA-13 containing combination coatings indicated that the 18% (w/w) coating possessed statistically greater bactericidal potential when compared to the activity of the 16% (w/w) ($p=0.02$) and 20% (w/w) ($p=0.03$) combination coatings. Evaluation of the bactericidal potential of the 16 and 20% (w/w) combination coatings revealed no statistical difference ($p=0.13$) between the two weight percentages.

CSA-13 Rate of Elution

The elution rate of CSA-13 from the silicone polymer device coating was evaluated by collecting 100 μL aliquots at time points of 4, 8, and 24 hours from each of the three polymer/antimicrobial weight ratios (Table 2). Analysis with LC-MS indicated the elution rate for the 16% (w/w) CSA-13 combination coating was 6.11 ± 1.80 , 7.16 ± 1.88 , and 12.22 ± 3.31 $\mu\text{g/mL}$ for measurements at 4, 8, and 24 hours, respectively (Figure 4). For the 18% (w/w) combination coating elution rates recorded at 4, 8, and 24 hours were 9.04 ± 2.11 , 10.48 ± 1.56 , and 14.84 ± 1.21 $\mu\text{g/mL}$, respectively. The 20% (w/w) CSA-13 demonstrated release kinetics of 10.10 ± 2.97 , 14.35 ± 5.26 , and 24.90 ± 3.59 $\mu\text{g/mL}$ for the time points of 4, 8, and 24 hours, respectively.

Statistical analysis revealed significant difference ($p=0.04$) between the elution rates of the 16 and 18% (w/w) combination coatings at 4 hours. However, no statistical difference ($p=0.29$) was observed between the rates of elution of the 18 and 20% (w/w) coatings at 4 hours. It was not until the 24 hour elution reading that significantly different rates were observed between the 18 and 20% (w/w) combination coatings. At this time point, the 20% (w/w) coating was observed to elute significantly greater ($p=0.001$) amounts of the CSA-13 antimicrobial.

DISCUSSION

The goal of this study was to establish a combination device coating capable of eliminating MRSA bacterial challenges. It was hypothesized that the broad spectrum polymer released CSA-13 antimicrobial would demonstrate potent bactericidal activity against MRSA *in vitro*. The results of this study supported the stated hypothesis, as it was observed that the concentrations of 16, 18, and 20% (w/w) all possessed significant ($p<0.001$) antimicrobial activity when challenged against MRSA inocula of 5×10^8 CFU/mL. The 18% (w/w) concentration possessed the most consistent bactericidal activity at the lowest concentration, exhibiting greater than a 7-log reduction (greater than 99.99999% bacterial elimination) within 8 hours.

Preliminary *in vitro* testing with combination coating concentrations ranging between 1% and 15% (w/w) demonstrated increasing antimicrobial potential; however, these concentrations did not exhibit complete kill. Although, these experiments did suggest that a bactericidal concentration might exist within the range of 16 – 20% (w/w). Therefore, these concentrations were utilized to fabricate polymeric device coatings with the CSA-13 antimicrobial and the medical grade RTV-1 silicone.

Evaluation of the prepared samples demonstrated distribution of CSA-13 throughout the polymeric matrix. However, CSA-13 was observed to be dispersed as aggregates varying between 0.5 μm and 20 μm (Figure 2b) in diameter. Once placed in solution these samples exhibited a continuous release of the CSA-13 antimicrobial from the silicone polymer coating over the 24 hour experimental time frame. These results were suggestive of a uniform rate of delivery, given the linear relationship of the rate of elution over time (Figure

4). Furthermore, a peak release of 14.35 $\mu\text{g/mL}$ was observed with the 18% (w/w) CSA-13 combination coating, as compared to rates of 10.10 and 24.90 $\mu\text{g/mL}$ for 16 and 20% (w/w) respectively.

Comparisons of the elution values for the 16, 18, and 20% (w/w) CSA-13 combination coatings revealed that all concentrations were well above the antimicrobial's reported MIC and MBC.^{10,21} However, the 18% concentration was the only coating to demonstrate rapid and complete bacterial elimination after 24 hours. The significance of these results was highlighted by the work of Fletcher et al.²² where it was demonstrated that the first 24 hours were the most essential for the prevention of perioperative infections. Additionally, the results of this elution study suggested that the antimicrobial potential of CSA-13 was consistent with drugs exhibiting a concentration-dependent pattern of antimicrobial activity. These observations were also made by Chin et al. (2007) who noted CSA-13's behavior was consistent with concentration-dependent antimicrobial activity. While susceptibility of the pathogen to the antimicrobial is often MIC dependent, to understand the relevance of a particular dosage, the pharmacokinetic and pharmacodynamic characteristics must be integrated.²³ It has been previously demonstrated, that for certain agents, concentration dependent bacterial killing occurs over a narrow range of drug concentrations.²⁴

The results of this *in vitro* investigation of the polymer released CSA-13 prove to be promising and suggest that this system may have the potential to prevent *in vivo* perioperative orthopaedic device related infections when used as a combination coating. Before clinical trials can commence certain limitations will need to be addressed. First, the mechanical properties of the CSA-13 containing polymeric device coating will have to be characterized to ensure that the coating is robust enough to be driven into bone. Second, the bond strength of the combination coating to the titanium implant will also need to be evaluated to make certain that the coating will remain in place and not shear off of the device during implantation. Third, one or more animal models will be required to determine if the CSA-13 combination coating possesses the same bactericidal potential *in vivo* as was demonstrated in *in vitro* experiments and whether the combination coating demonstrates good biocompatibility with no adverse effects on the rate of bone remodeling. Finally, the antimicrobial potential of CSA-13 must be challenged against conventional antibiotics to determine whether its properties are consistent with or superior to currently available drugs.

CONCLUSIONS

In conclusion, the CSA-13 antimicrobial is a powerful bactericidal agent when used against planktonic MRSA bacteria *in vitro*. This antimicrobial has demonstrated the ability to eliminate high inocula of antibiotic-resistant bacteria within hours when used as a combination device coating. Incorporation of this novel broad spectrum compound^{10,15,25} into combination device coatings may have the potential to significantly reduce the number of perioperative device related infections. In the future, the 18% (w/w) CSA-13 antimicrobial combination coating will be challenged against high inocula of bacteria in a large animal model utilizing a total joint replacement-like approach. The 16 and 20% coatings will also be utilized to account for differences in living tissue versus the simulated *in vitro* environment. Future studies will also be conducted to evaluate the mechanical properties and strength of the polymeric, antimicrobial combination coating for *in vivo* applications.

Acknowledgments

This material was based on work supported (or supported in part) by the Department of Veterans Affairs (VA) Office of Research and Development, Rehabilitation Research and Development Service, VA Salt Lake City Health Care System, Salt Lake City, UT. The authors would like to thank the National Institutes of Health (Award number:

1R01AR057185-01) and the Albert and Margaret Hofmann Chair and the Department of Orthopaedics, University of Utah School of Medicine, Salt Lake City, UT for their financial support. The authors would also like to thank Vinod Chaudhary for his assistance with Liquid Chromatography-Mass Spectrometry analysis, Bryan Haymond for his technical assistance, and Gregory Stoddard for assistance with and interpretation of statistical analysis.

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Figure 1.
a) Porous coated titanium plug evaluated in *in vitro* experiments. Regions A and C received the CSA-13/silicone polymer coating. **b)** Hollow lumen of plug to accommodate bacteria inoculum.

Figure 2.

a) A representative SEM image of the surface of a CSA-13/silicone polymer device coating (18% w/w). **b)** SEM-EDX image with red pseudo-coloring highlighting the CSA-13 – chloride anion conjugated regions.

Figure 3. The bactericidal activity of various concentrations of the CSA-13/polymer combination coating over 24 hours. The 18% (w/w) CSA-13/silicone polymer coating was the only combination coating to completely eliminate the bacterial burden within 24 hours.

Figure 4. Recorded elution rates for the polymer released CSA-13 from device coatings consisting of 16, 18, and 20 percent CSA-13 in a silicone polymer matrix.

Table 1

MRSA colony forming units observed at 0, 4, 8 and 24 hours for Groups 1–6. This table indicates the bactericidal potential of the 18% (w/w) combination coating evidence by the paucity of bacteria found at the 8 and 24 hour time points.

Time-Kill Analysis				
	0 hr. (CFU/mL)	4 hrs. (CFU/mL)	8hrs. (CFU/mL)	24 hrs. (CFU/mL)
<i>Control</i>	$2.2 \times 10^7 \pm 2 \times 10^6$	$4.6 \times 10^8 \pm 6 \times 10^7$	$3.9 \times 10^8 \pm 9 \times 10^7$	$7.3 \times 10^8 \pm 7 \times 10^7$
<i>Titanium</i>	$2.8 \times 10^7 \pm 2 \times 10^6$	$6.6 \times 10^8 \pm 1 \times 10^8$	$8.6 \times 10^8 \pm 10 \times 10^7$	$9.9 \times 10^8 \pm 3 \times 10^7$
0%	$2.6 \times 10^7 \pm 5 \times 10^5$	$7.2 \times 10^8 \pm 6 \times 10^7$	$6.4 \times 10^8 \pm 5 \times 10^7$	$7.5 \times 10^8 \pm 10 \times 10^7$
16%	$2.1 \times 10^7 \pm 2 \times 10^6$	$4.3 \times 10^3 \pm 2 \times 10^3$	$1.1 \times 10^3 \pm 5 \times 10^2$	16.0 ± 7
18%	$2.5 \times 10^7 \pm 3 \times 10^6$	48.0 ± 17.2	1.0 ± 1	0.0 ± 0
20%	$2.6 \times 10^7 \pm 1 \times 10^6$	$6.0 \times 10^2 \pm 3 \times 10^2$	$1.7 \times 10^3 \pm 7 \times 10^2$	39.0 ± 20

Table 2

The amount of CSA-13 eluted from the various combination coatings over the 24 hour experimental time frame. The rate of elution was observed to increase for each combination coating over the 24 hour experimental time frame.

24 Hour Elution Data				
	0 hrs. ($\mu\text{g/mL}$)	4 hrs. ($\mu\text{g/mL}$)	8 hrs. ($\mu\text{g/mL}$)	24 hrs. ($\mu\text{g/mL}$)
16%	0.00 ± 0.0	6.11 ± 1.8	7.16 ± 1.9	12.22 ± 3.3
18%	0.00 ± 0.0	9.04 ± 2.0	10.48 ± 1.6	14.84 ± 1.2
20%	0.00 ± 0.0	10.10 ± 3.0	14.35 ± 5.3	24.90 ± 3.6