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14. ABSTRACT
The purpose of this project was to investigate the feasibility of antibacterial plasma treatment of biological tissue. A low-temperature plasma was produced in air that killed 1E5-1E6 bacterial populations on synthetic skin in under 20 seconds. The bacteria used was Staphylococcus epidermis and the skin surface consisted of human keratinized cells. While there was no visible damage to the skin, caution is advised in applying this to live organisms until more research is done. Further work on electroporation of skin in the presence of electric fields needs to be considered. The electrical resistance of the skin relies on intact layers, so particular care should be used in considering antibacterial electrical treatments for wound management. For intact skin, plasma treatment shows potential for controlling bacterial growth in situations where an imbalance in the normal microbiota has occurred. For this application, cycled treatment may be optimum.

15. SUBJECT TERMS
plasma deactivation of bacteria, plasma sterilization, surface decontamination, plasma disinfection of biological tissue

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Plasma Sterilization Experiments
Final Report
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Plasma Sterilization Experiments- Final Report
Contract #FA9550-07-C-0147 December 14, 2008

Introduction

The purpose of the project was to demonstrate the feasibility of using plasma treatment to drastically reduce the population of bacteria on biological surfaces. To show antibacterial properties, a three-order of magnitude reduction in bacterial population is required. The surface suggested as the one of most interest was human skin. The intention of this project was to consider the affect of low power plasma on the surface of human skin while efficiently decontaminating it.

The bacteria chosen for the experiments was *Staphylococcus epidermis*. This species normally resides on healthy human skin, but can become a problem if overgrowth occurs (e.g. biofilm formation) or if it gets into the blood stream, where it can cause severe damage and become life-threatening (Wilson, Michael, Microbial Inhabitants of Humans, Cambridge University Press, 2005). For this reason, it is very important to sterilize the skin before puncturing it, otherwise this operation could carry skin bacteria into the bloodstream. Unless the skin is penetrated, it may not be necessary or even desirable to kill all the bacteria present but only to control excess growth. Skin contains a balance of microbiota, some of which serve to keep more infectious agents under control. According to some medical researchers, the goal of skin hygiene practices should be to "provide adequate protection from transmission of infecting agents while minimizing the risk for changing the ecology and health of the skin" (see, e.g., "Hygeine of the Skin: When is Clean Too Clean?" by Dr. Elaine Larson, *Emerging Infectious Diseases*, Vol.7, No.2, March-April 2001).

To investigate plasma treatment of bacteria on skin, we obtained artificially cultured skin that mimicked human skin morphologically. Of particular interest for these experiments was the similarity of structure of the top layer (stratum corneum) of the artificial skin to that of human skin, because the bacteria were deposited on the surface prior to treatment in the plasma reactor. The stratum corneum is composed of keratin-rich dead cells surrounded by lipids. This outer layer is tens of microns in thickness and forms a tough penetration barrier under normal circumstances. There have been reports of small pores opening temporarily in skin exposed to pulsed electric fields (Gallo, S. et al., Biophysical Journal, Vol. 76, 2824-2832, 1999). This is a dynamic phenomenon, difficult to capture for analysis, but bears further investigation before electrical treatment methods would be considered for clinical application. The electrical resistance of the stratum corneum relies on the layer being intact. Research using intact skin (Fridman, G. et al. Plasma Chemistry and Plasma Processing, Vol 26, No.4, pp. 425-442, 2006) does not automatically apply to situations where the skin has been breached, e.g. in wound management.

The direct treatment of surfaces with ionized gas plasmas to clean and decontaminate has been extensively studied. The proposed mechanisms of action of plasma on bacterial cells include UV light (destruction of nucleic acids) and reactive oxygen species (degradation of cells walls, subsequent attack of other cell components). Much work has been done to demonstrate the activity of ozone and other oxygen species generated in an oxygen-containing gas plasma for sterilizing and decontaminating surfaces (Thanomsub, Benjamas et al., "Effect of ozone treatment on cell growth and ultrastructural changes in bacteria," *J. Gen. Appl. Microbiol.*, **48**, 193-199, 2002; Sato, T. et al. "Sterilization mechanism for E. Coli by plasma flow at atmospheric pressure," Appl Phys. Lett. **89**, 073902, 2006; Ma, Yue et al, "Chemical Mechanisms of Bacterial Inactivation Using Dielectric Barrier Discharge Plasma in Atmospheric Air,: IEEE Plasma Science, **36**, issue 4, 1615-1620, 2008). The most recent work concludes that reactive oxygen species play a prominent role.

The plasma reactor used was made by MicroStructure Technologies, Inc. The AC electrical discharge plasma used for these experiments is an atmospheric pressure, low temperature plasma in air. The operating parameters were adjusted to achieve a uniform glow rather than micro-streaming or arcing that could damage the skin. High-strength dielectric materials between the electrodes were used to prevent dielectric breakdown (arcing) and still achieve treatment times of a few tens of seconds. This short treatment time prevented the skin from drying out excessively. The plasma test stand used in these experiments has been developed from the beginning to consist of portable components, useful for a wide variety of applications. The device produces a non-equilibrium, capacitively-coupled electrical discharge plasma. The energetic work of the plasma is accomplished with only a few percent ionization, so the operational temperature is relatively low, and no gas containment is required. These plasma characteristics enable treatment in air of delicate organic tissue.

Experimental

Bacterial source

Staphylococcus epidermidis was purchased from ATCC (stock number 33501), and was used to inoculate trypticase soy broth (TSB). The culture was grown at 37°C for a period of 48 hours, at which time the culture was at maximum concentration and used for the experiment. The culture was also used to inoculate a new tube of TSB.

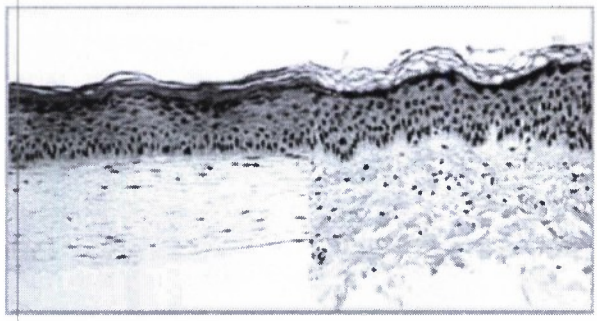
For use in an experiment, an aliquot of the S. epidermidis culture was withdrawn and used as the common source. The concentration of the common source was measured with each experiment by performing serial dilutions, followed by plating. 2.00±0.05µL of the common source was deposited into 2.00±0.04mL of isotonic saline (0.85% NaCl in H2O) (1 in 1000 dilution), then with 1.000±0.004mL of the diluted sample into 9.0±0.1mL of isotonic saline (1 in 10 dilution), then finally with 0.250±0.004mL of the last dilution into 24.75±0.10mL of isotonic saline (1 in 100 dilution), for a total of 1E6 dilution. Trypticase soy agar (TSA) plates were then inoculated in triplicate using

0.200±0.004mL of the final dilution for each plate. Typical concentrations were found to be in the 10^9 cfu/mL range.

Sterilization

Sterilization of all non-sterile materials was performed using a commercially available microwave steam sterilizer. Sterilization was checked by exposing vials containing solutions of 1E6 cfu/mL S. epidermidis to various sterilization procedures. For our microwave 7 minutes at 70% power was found to kill all the bacteria.

Substrate

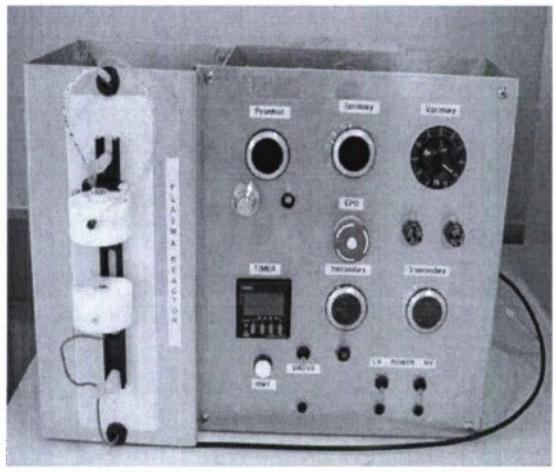


The substrate used in the experiments was "Apligraf" Test Skin from Organogenesis. The stained cross-sections of human skin (on the right) and the artificial skin (on the left) are shown for comparison; the thickness of the overall structure of the artificial skin is ~0.5 mm. The underlying dermis in artificial skin is condensed collagen containing human fibroblasts, and the epidermis covering contains human keratinized cells. The top (outer) layer of the epidermis, the stratum corneum, is composed of keratin-rich dead cells surrounded by lipids. The stratum corneum is tens of microns in thickness.

Samples were prepared by using a sterile, disposable 6-mm round tissue punch to cut uniform samples from the Test Skin II. These samples were then transferred to Transwells (Organogenesis) and were suspended over an isotonic saline solution to keep the skin samples from drying out.

2.00±0.05µL of the S. epidermidis source solution was deposited onto each of the substrates in the Transwells. The skin with the deposited solution was then allowed to equilibrate to the point where the drop deposited on the surface was no longer visibly wet. At the same time, the isotonic saline beneath the Transwells kept the skin from dehydrating. The inoculated substrates were then transferred onto quartz slides, ready for plasma treatment.

Plasma treatment



Treatment was performed using the plasma test stand shown to the right. The inoculated skin samples on quartz slides are placed between the electrodes and a plasma is initiated between the two electrodes. Key design criteria for the plasma reactor included slide-mounted sample positioning, precise electrode spacing, and easily cleaned surfaces. To ensure sterile surfaces, a vacuum holder was integrated into the HV electrode to enable rapid replacement of the quartz cover slip. The instrument panel included primary voltage control, secondary voltage and current control, secondary voltage measurement, power measurement, and electronic timer activation to provide ease of operation.
Before each session, plasma operating parameters were verified using a clean quartz slide without a test skin sample deposited on it. The primary voltage was set to 90% for these experiments. The typical voltage secondary voltage reading was 1.5±0.1 kV, and typical treatment times varied from 10 seconds to 20 seconds. With longer treatment times, the skin samples would dry out enough that the edges curled, bridging the air gap (which was less than 1 mm). Electrode spacing was fixed at 3.62mm, with the quartz slide acting as a dielectric at the lower electrode (1.0mm thick), and a quartz cover slip (0.5mm thick) as well as a mica disc (0.25mm thick) acting as the dielectric at the upper electrode. Plasma reactor surfaces were sterilized daily using isopropyl alcohol.

After the inoculated substrates were transferred to the quartz slides, they were immediately placed in the plasma reactor. The treatment time was then set, and the plasma was activated. After treatment, the substrate was transferred into a 5mL conical vial containing 2.00mL isotonic saline. Bacterial cells were then extracted from the skin by placing the vial in an orbital shaker set at 1000 RPM for a period of 10 minutes. TSA plates were then inoculated in triplicate using 0.2mL of the extraction solution for each plate. The extraction and plating process was repeated for each of the treated substrates.

Additionally, controls were run in triplicate each day to monitor the extraction process. The substrates were inoculated as described and transferred to quartz slides, but received no plasma treatment. The substrates were then transferred into a 5mL conical vial containing 2.00mL isotonic saline, and were extracted from the skin at 1000 RPM for a period of 10 minutes. Unlike the plasma-treated samples, the control samples were then diluted by three orders of magnitude (a 10:1 followed by a 100:1 dilution). TSA plates were then inoculated in triplicate using 0.200mL of the extraction solution for each plate. The extraction and plating process was repeated for each of the triplicate controls. The controls not only were a measure of extraction, they were a measure of how many viable cells were left on the substrates at the time the experiment was performed, in case some of the culture had died during the sample preparation process. An extensive discussion of experimental error can be found in Appendix A.

Results and Discussion

First experiments used an active plasma with micro-streaming (small arcs). These conditions were deemed too non-reproducible and risked damaging the sensitive organic tissue. The plasma conditions were changed to achieve a uniform glow that was active enough to kill all the bacteria on the skin samples in under 30 seconds. To ensure that something else (besides plasma treatment) in the experimental procedure was not killing all the bacteria, a program goal was set to demonstrate that a few bacteria were left alive.

Experimentally, this translates to having a few hundred colonies on the culture plates from the plasma-treated sample solution (for reasons enumerated earlier, minimizing error). The controls were diluted an extra three orders of magnitude compared to the plasma-treated samples, so having the same number of bacterial colonies on the control plates and the plasma-treated plates corresponds to a three-order of magnitude reduction. This was demonstrated by data runs such as those shown in Appendix B.

Over many runs, 20 samples showed the condition of three orders of magnitude reduction. Additionally, 38 samples showed four orders of magnitude reduction, and 40 samples had no viable colonies left. The samples that had no viable bacteria correspond to five orders of magnitude reduction, considering the number of bacteria per sample was in the 1E6 range and the cumulative error was no more than an order of magnitude.

For typical data runs, the amount of bacteria deposited on a skin sample was ~2 million cells. This was the practical maximum because of several factors. First, the maximum *Staph. epidermis* concentration of actively growing cells was ~ 1E9 cfu/ml. Deposition of this culture onto the skin sample was performed using a precision microliter syringe, set to deposit 2 microliters onto each sample. In earlier experiments, we were inoculating the skin with 20 microliter drops of water. These larger drops tended to bead up into hemispherical domes on the surface of the skin, leading to uncertainty in the uniformity of plasma treatment. With only a few microliters deposited on the skin, the bacterial solution spread out and more uniformly wet the surface. Care was taken not to allow excess liquid to spread out beyond the skin sample edges. The size of the skin samples (6-mm round, produced from sterile biopsy punches) was chosen to ensure the entire sample was within the plasma treatment area (as defined by the electrodes).

For the control samples, the inoculated skin with its ~2 million bacteria was immersed in 2 ml of isotonic salt solution and shaken to extract the bacteria. This yields a maximum concentration of 1E6 cfu/ml (if the extraction efficiency were 100%). Extraction efficiency was actually closer to 50%, so concentration of the controls prior to dilution was ~5e5 cfu/ml. Three orders of magnitude dilutions leaves ~5e2 cfu/ml. Using 0.2 ml of this solution to plate gives ~100 colonies/plate, which is the right order of magnitude (high enough to minimize error but small enough to count).

For the plasma-treated samples, the bacteria left alive were extracted into 2 ml of solution and then plated (using 0.2 ml of solution). To show three orders of magnitude reduction in bacterial population, maximum concentration of the extraction solution for treated samples would be 1E3 cfu/ml. For 50% extraction, it would be 5e2/ml, or ~100 cfu per plate. To give the desired range of countable colonies on the plates requires reducing the bacterial population from millions to ~ a thousand, with a resolution of hundreds of organisms. This resolution was achieved on some runs, as shown in Appendix B. In Appendix C are data runs with low and high exposures indicating that the plasma treatment is capable of greater than three orders of magnitude reduction of bacterial population. Generally, 18 seconds or more of plasma treatment resulted in no viable bacterial colonies.

The low power density plasma operates at a fixed frequency of 20KHz with a maximum applied potential of 6kV(AC). With nominal power coupling, about 50 watts can be deposited into the secondary or HV side of the system. An average of 49 watts is observed. With a typical E field of 3000 V/cm, the estimated E/n value for the system during this study is $1.2X10^{-16}$ V·cm².

Initiating the plasma requires about 5.25 KV applied AC voltage. Maintaining the plasma requires about 1500 volts. To assess the treatment to which the various components of the plasma cell are subjected, voltage drops across the plasma cell can be estimated as well as approximating the power dissipated by each component. By analogy, the E field requirements for plasma display panels (PDP) can be applied to the characterization of the plasma filling the gap above the skin sample. Boeuf found that a sustaining voltage of 142 VDC was required for a 10% Xenon-Neon mixture at 560 Torr in a 100 μm gap of a PDP (Boeuf, J.P., "Plasma display panels: physics, recent developments and key issues," J. Phys. D: Appl. Phys. 36, 2003, R53-R79). Thus, the E field of 14,200 V/cm for a PDP device is about 4.7 times greater than the plasma cell used in this study. Consequently, a local voltage difference for the air gap of the plasma cell can be estimated to be less than 300 volts. Note that the added activity of the AC current reduces the voltage requirement for dielectric breakdown. Considering the dielectric properties and dimensions of the other components, the mica-quartz barrier above the gap and the quartz barrier below the gap provide essentially equal resistance to current flow. Estimating a 300 volt drop across the gap, the drops across the upper and lower barriers are approximately 610 volts and 590 volts, respectively. Therefore, the artificial skin with a thickness of about 300 μm is subjected to nearly 120 volt potential. However approximate, this is important information for two reasons: 1) serious electroporation of skin has been observed with a potential of 80 VDC applied to skin with a 300 μsec pulse (Gallo, S. et al., Biophysical Journal, Vol. 76, 2824-2832, 1999), and 2) significant heating affects can disrupt and degrade the skin. Considering the epidemiology, the affects observed with electroporation resembled a localized vesicular or tubercle inflammation. While the affects appeared temporary, the results of multiple pulses or extended application of voltage need to be carefully investigated. The heating affect is observed in this study with a significant desiccation of the skin sample. With plasma treatment for 20 seconds, the sample loses about 50% of its volume (as thickness). Assuming that water loss is the primary mechanism of volume change, about

0.55 watt is required to evaporate the equivalent volume. As mentioned previously, approximately 49 watts of power is coupled into the plasma cell. With 1500 VAC secondary, nearly 33 mA current is driven through the plasma cell. Therefore, a voltage drop of about 17 volts (and 1.1 percent of the total power) is attributed to the evaporation of water from the skin sample. Where is the rest of the power being dissipated? The primary culprits are the dielectrics (i.e. heating) and the chemical reactions promoted within the gas plasma. In the reference above, the Xenon-Neon mixture has similar ionization potentials as the oxygen-nitrogen mixture of air. Given the bond energy of oxygen, it is anticipated that significant dissociation occurs in the energetic environment created by the aforementioned conditions. During plasma operation, the lavender-blue glow associated with oxygen bond dissociation (and minimal nitrogen bond dissociation) is vividly apparent. Therefore, the skin is also treated by the oxygen species generated in the plasma.

Conclusions

The goal of the project was to investigate the feasibility of plasma-based antibacterial treatment of organic tissue. The research is at the beginning stages of what could be a large project. MicroStructure Technologies, Inc. has demonstrated that its test stand could produce a plasma that did not visibly damage the biological surface but did kill bacteria on the surface within a few tens of seconds. Using the experimental parameters discussed in this report, the destruction of bacteria occurred rapidly, with a cutoff of a second or two. The exact exposure required for three orders of magnitude reduction in bacterial population varied within a larger window than the kill threshold, indicating the need for multiple treatments to ensure the desired sterilization. Of course, longer treatment times could be employed, but this can lead to desiccation of the organic surface. Pulsed treatment may have the advantage of allowing recovery of the underlying tissue while keeping the bacterial populations under control.

The findings of this study not only demonstrate a high level of biocidal activity by a low power plasma on a skin substrate but reveal areas of consideration for a practical plasma device which does not compromise the integrity of this delicate and complex substrate. Practical design would suggest a coplanar electrode configuration with a ground plane between the plasma and the surface of the skin. MicroStructure Technologies has begun development of a practical flexible, coplanar-electrode device.

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APPENDIX A

Error Calculations

Error in our results from bacterial processing came from three sources: the extraction process, the dilution process, and the plating process. Efforts were made to reduce variation seen in each of these areas, but due to the biological nature of the samples, variation was still present.

The largest variations were seen with the extraction process. Variation was measured by comparing concentration of *S. epidermis* in the extraction wash from the control samples on each day. There is an average variation of 14% within the control groups for each day. This could be partly due to the test skin surface, which is not perfectly smooth, allowing spaces where the *S. epidermis* cells could become trapped.

Error due to dilution and dispensing of solutions was minimized by using pipettes of the appropriate size for each measurement. For the delivery of the 2.00±0.05µL volume of source, we verified that it could precisely deliver the volume desired.

The repeatability of the micropipette used to deliver the 2-microliter drop of the common source was verified by performing parallel dilutions of the common source. Each serial dilution was made by pipetting 2.00±0.05µL into 2.00±0.04mL of isotonic salt solution (1:1000), followed by a mixing step and subsequent dilutions (1:10 and 1:100).

TSA plates were inoculated in triplicate with the final dilution (1:1E6 total dilution) from each of the parallel dilutions. We then used the plate counts to calculate the source concentration. When ANOVA was run on 18 plates from six parallel dilutions, the result was that none of the six dilutions were statistically different, as shown to the right. The standard error between the 6 groups was calculated to be 2%.

µP A	µP B	µP C	µP D	µP E	µP F
277	313	274	243	317	212
243	270	239	227	275	264
225	225	236	263	231	278

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
µP A	3	745	248.3333	697.3333
µP B	3	808	269.3333	1936.333
µP C	3	749	249.6667	446.3333
µP D	3	733	244.3333	325.3333
µP E	3	823	274.3333	1849.333
µP F	3	754	251.3333	1209.333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2311.111	5	462.2222	**0.429043**	0.819931	**3.105875**
Within Groups	12928	12	1077.333			
Total	15239.11	17				

The Analysis Toolpack in Excel was used to perform the data analysis ANOVA (analysis of variance). This analysis tool is used to test the null hypothesis Group A = Group B = Group C etc. The result of this operation is expressed in the value of F, which gets compared to a critical value of F (F crit) at the 0.05 level of significance. Because our computed test statistic F = 0.43 is less than F crit = 3.11, the null hypothesis is accepted. We conclude that there is no evidence of significant difference in the number of colony forming units between the six dilutions.

Error from the pipetting in the dilution steps was calculated using the uncertainty in the readings from the pipettes. The calculation was performed using standard error

propagation formulas. Included in the calculation is the1.000±0.004mL into 9.0±0.1mL dilution (1:10), the 0.250±0.004mL into 24.75±0.1mL dilution (1:100), and the final delivery of 0.200±0.004mL onto a TSA plate. All together, these steps account for 2.2% uncertainty.

Finally, there was the variation seen within the plating process. Within the triplicate plating process of the control groups, there was a median difference between plates of 7 colonies, with an average of 254 colonies per plate. This represents a variation of 3% within the plating process when colonies per plate are approximately 250. However, if the number of colonies per plate was significantly lower, even a small variation could have greater effect on the percent variation. For example, if the triplicate group had an average of 10 colonies, a difference between plates of only 1 colony would yield 10% variation within the group. Because of this, efforts were made to keep the number of colonies per plate greater than 200, but low enough that the colonies could easily be distinguished (<500).

It is important to note that the 14% variation observed in the extraction measurement actually includes the source delivery error, the dilution error, and the plating error. To find the error from the extraction step, we would need to use the formula for error propagation of a function. For function f(a,b,…,z),

$$\delta_f = \sqrt{\delta_{fa}^2 + \delta_{fb}^2 + \cdots \delta_{fz}^2} .$$

Using this formula, we can approximate that the error in the extraction step is actually closer to ±13%. This value is only slightly lower than the ±14% due to the fact that the error contribution from the other steps is quite small.

Experimental Error

Another source of variations in the results stems from the experimental setup. Care was taken to eliminate as many variables as possible, such as using a fixed power setting, constant electrode spacing, and the use of an integrated digital timer. Some variations in secondary voltage were observed. There were also slight variations in the thickness of the samples. Research-grade, artificially grown skin was used, but it does have variations in thickness and texture. We have kept the skin sterile, but we do not know what affect the sample-to-sample differences have on plasma treatment. Variations in the amount of water retained in the skin samples could also affect treatment. To minimize this variation, we kept the inoculated substrates in Transwells until just before processing. However, if the skin itself holds different amounts of water due to change in porosity etc., we would not be able to remove this sample variation.

Fortunately, the measurement error is low (±14%), so when we observe several orders of magnitude reduction in bacterial colonization of the plasma treated sample, this significant change can be attributed to the plasma treatment, and not to measurement variability.

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Error Calculation for parallel source dilutions

Error Propagation of parallel dilutions

	10^(-6) Plate Counts: S3C-Dilution B			
	Plate 1	536		
	Plate 2	577		
	Plate 3	609		
	Ave	574		
	Std Dev	30		
	Std Err	17		
	Plating:	counts/0.2mL		
		Value	Error	Rel. Err.
	Counts	5.74E+02	17	0.030
/	0.2mL	0.200	0.002	0.010
=	1E-6 dilution (cfu/ml)	2.87E+03	9.E+01	0.032
	3rd dilution:			
		Value	Error	Rel. Err.
	1E-6 dilution (cfu/ml)	2.87E+03	9.E+01	0.032
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	7.18E+04	2.E+03	0.032
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	1E-4 dilution (cfu/ml)	2.87E+05	1.E+04	0.036
	2nd dilution:			
		Value	Error	Rel. Err.
	1E-4 dilution (cfu/ml)	2.87E+05	1.E+04	0.036
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	7.18E+06	3.E+05	0.036
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	1E-2 dilution (cfu/ml)	2.87E+07	1.E+06	0.039
	1st dilution:			
		Value	Error	Rel. Err.
	1E-2 dilution (cfu/ml)	2.87E+07	1.E+06	0.039
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	7.18E+08	3.E+07	0.040
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	**S. e. Source (cfu/ml)**	2.9E+09	1.E+08	0.043
	Source Concentration: (cfu/ml)	2.9E+09	1.E+08	0.043

	10^(-6) Plate Counts: S3C-Dilution C			
	Plate 1	529		
	Plate 2	570		
	Plate 3	632		
	Plate 4	602		
	Ave	558		
	Std Dev	35		
	Std Err	17		
	Plating:	counts/0.2mL		
		Value	Error	Rel. Err.
	Counts	5.58E+02	17	0.031
/	0.2mL	0.200	0.002	0.010
=	1E-6 dilution (cfu/ml)	2.79E+03	9.E+01	0.033
	3rd dilution:			
		Value	Error	Rel. Err.
	1E-6 dilution (cfu/ml)	2.79E+03	9.E+01	0.033
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	6.98E+04	2.E+03	0.033
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	1E-4 dilution (cfu/ml)	2.79E+05	1.E+04	0.037
	2nd dilution:			
		Value	Error	Rel. Err.
	1E-4 dilution (cfu/ml)	2.79E+05	1.E+04	0.037
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	6.98E+06	3.E+05	0.037
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	1E-2 dilution (cfu/ml)	2.79E+07	1.E+06	0.040
	1st dilution:			
		Value	Error	Rel. Err.
	1E-2 dilution (cfu/ml)	2.79E+07	1.E+06	0.040
*	Final Volume (ml)	25.0	0.1	0.004
=	cfu in aliquot	6.98E+08	3.E+07	0.040
/	vol. of aliquot (ml)	0.250	0.004	0.016
=	**S. e. Source (cfu/ml)**	2.8E+09	1.E+08	0.043
	Source Concentration: (cfu/ml)	2.8E+09	1.E+08	0.043

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Error Calculation: No statistical difference between source dilutions

	S3C-A	S3C-B	S3C-C
Plate 1	578	536	529
Plate 2	559	577	570
Plate 3	539	609	532
Plate 4			602

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
S3C-A	3	1676	558.6667	380.3333
S3C-B	3	1722	574	1339
S3C-C	4	2233	558.25	1198.917

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	509.4833	2	254.7417	0.253459	0.782938	4.737414
Within Groups	7035.417	7	1005.06			
Total	7544.9	9				

The Analysis Toolpack in Excel was used to perform the data analysis ANOVA (analysis of variance). This analysis tool is used to test the null hypothesis Group A = Group B = Group C. The result of this operation is expressed in the value of F, which gets compared to a critical value of F (F crit) at the 0.05 level of significance. Because our computed test statistic F = 0.25 is less than F crit = 4.74, the null hypothesis is accepted. We conclude that there is no evidence of significant difference in the number of colony forming units between the three dilutions.

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APPENDIX B

Data showing plasma reduction of bacteria by 99.8% and 99.98%

Concentrations of control samples, **C (exposure crtl, plasma), CFU/ml**, and plasma-treated samples, **C (plasma), CFU/ml**, are shown in the far right column.

3/12/08 Common source dilution, CSD, used to inoculate skin, was 2µl of S5Y in 2ml of isotonic NaCl. Controls were washed in 2mL of isotonic NaCL for two 5 minute cycles in a 150khz ultrasonic bath, diluted 1:10 and 1:100, then plated. Plasma samples were washed identically to controls, and plated directly from the wash water

Test #	Plate counts (0.2 ml)	Counts/ml of final dilution					
							$C_{(exposure\ ctrl,\ plasma)}$ CFU/ml
1E-3 Ctl #1-1	7	35					
1E-3 Ctl #1-2	4	20					(51+/-9)*1000
1E-3 Ctl #1-3	6	30					
1E-3 Ctl #2-1	11	55					
1E-3 Ctl #2-2	8	40					
1E-3 Ctl #2-3	9	45					
1E-3 Ctl #3-1	13	65					
1E-3 Ctl #3-2	23	115	Average:	StDev:	StErr:		
1E-3 Ctl #3-3	10	50	51	28	9		
							$C_{(plasma)}$ CFU/ml
Pla 1 #1	35	175					
Pla 1 #2	26	130	Average:	StDev:	StErr:		65+/- 22
Pla 1 #3	28	140	148	24	14		
Pla 2 #1	18	90					
Pla 2 #2	24	120	Average:	StDev:	StErr:		
Pla 2 #3	18	90	100	17	10		
Pla 3 #1	1	5					
Pla 3 #2	2	10	Average:	StDev:	StErr:		
Pla 3 #3	2	10	8	3	1.7		
Pla 4 #1	2	10					
Pla 4 #2	0	0	Average:	StDev:	StErr:		
Pla 4 #3	1	5	5	5	3		
			Total Average:	StDev:	StErr:		
			65	65	22		%Reduction 99.8%

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3/24/08 Common source dilution, CSD, used to inoculate skin, was 2µl of S6A in 2ml of isotonic NaCl. Skin samples were kept in transwells over 7 ml of isotonic NaCl until just prior to plasma treatment. Controls were washed in 2mL of isotonic NaCL for 10 minutes at 1000rpm in a 5mL conical vial, diluted 1:10 then 1:100 (for a total dilution of 1:1000), then plated using 0.2 ml of solution. Plasma samples were washed identically to controls, and plated (with 0.2 ml) directly from the wash water.

Treated sample,plate #	Plate counts (0.2 ml)	Counts/ml of final dilution		C (plasma), CFU/ml
Pla 1	skin stuck to dielectric; sample discarded			
Pla 2 #1	67	335		189 +/- 36
Pla 2 #2	73	365	Average	
Pla 2 #3	56	280	189	
Pla 3 #1	29	145		
Pla 3 #2	28	140	St dev	
Pla 3 #3	28	140	107	
Pla 4 #1	24	120		
Pla 4 #2	17	85	St error	
Pla 4 #3	19	95	36	
Exposure controls diluted 1000:1 more than plasma-treated samples				C (exposure ctrl, plasma) CFU/ml
1E-3 Ctl #1-1	299	1495		(1680 +/- 52)*1000
1E-3 Ctl #1-2	345	1725	Average	
1E-3 Ctl #1-3	314	1570	1680	
1E-3 Ctl #2-1	316	1580		
1E-3 Ctl #2-2	311	1555	St dev	
1E-3 Ctl #2-3	328	1640	157	
1E-3 Ctl #3-1	396	1980		
1E-3 Ctl #3-2	345	1725	St error	
1E-3 Ctl #3-3	370	1850	52	

Determining conservative estimate of population reduction using $C_{ave\ (plasma)}$=189+36 and $C_{ave\ (exposure\ ctrl,\ plasma)}$ = (1680-52)*1000:

($C_{ave\ (exposure\ control,\ plasma)}$ - $C_{ave\ (plasma)}$) / $C_{ave\ (exposure\ control,\ plasma)}$ = (1628*1000-225)/(1628*1000) = 0.9998 = 99.98% reduction in bacteria CFU. Note: this is not the limit of reduction possible by plasma treatment, but only the limit of reduction shown within current experiment.

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APPENDIX C

The next several pages include runs using the strategy of bracketing low and high exposures to make sure there are bacteria to count (at low exposure) and that the bacteria are all killed (at high exposure). Concentrations of control samples, **C (exposure crtl, plasma), CFU/ml**, and plasma-treated samples, **C (plasma), CFU/ml**, are shown in the far right column. We achieved >99% reduction at the low exposure and successfully killed all bacteria at the high exposure.

5/6/2008				
Test #	Plate counts (0.2 ml of test concentrations)	Counts/ml of final dilution		C (exposure ctrl, plasma), CFU/ml
Control Samples				(2036 +/- 135)*1000
1e-3 Ctl#1-A	369	1845		
1e-3 Ctl#1-B	358	1790	Average	
1e-3 Ctl#1-C	339	1695	2036	
1e-3 Ctl#2-A	421	2105		
1e-3 Ctl#2-B	453	2265	St dev	
1e-3 Ctl#2-C	399	1995	234	
1e-3 Ctl#3-A	427	2135		
1e-3 Ctl#3-B	486	2430	St error	
1e-3 Ctl#3-C	412	2060	135	
Low Exposure Samples				C (plasma), CFU/ml
1e-1 Test #1-A	740	3700		(1743 +/- 539)*10
1e-1 Test #1-B	616	3080	Average	
1e-1 Test #1-C	569	2845	1743	
1e-1 Test #2-A	139	695		% Reduction
1e-1 Test #2-B	138	690	St dev	99.1
1e-1 Test #2-C	104	520	1078	
1e-1 Test #3-A	462	2310		
1e-1 Test #3-B	368	1840	St error	
1e-1 Test #3-C	448	2240	539	
1e-1 Test #4-A	199	995		
1e-1 Test #4-B	195	975		
1e-1 Test #4-C	205	1025		
High Exposure Samples				C (plasma), CFU/ml
Test #5				0 +/- 0
Test #6-A	0	0		
Test #6-B	0	0	Average	
Test #6-C	0	0	0	
Test #7-A	0	0		
Test #7-B	0	0	St dev	
Test #7-C	0	0	0	
Test #8-A	0	0		
Test #8-B	0	0	St error	
Test #8-C	0	0	0	

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5/7/2008				
Test #	Plate counts (0.2 ml of test concentrations)	Counts/ml of final dilution		
Control Samples				C (exposure ctrl, plasma), CFU/ml
0.2ml 1e-3 Ctl#1-A	223	1115		(1361 +/- 241)*1000
0.2ml 1e-3 Ctl#1-B	179	895	Average	
0.2ml 1e-3 Ctl#1-C	227	1135	1361	
0.2ml 1e-3 Ctl#2-A	265	1325		
0.2ml 1e-3 Ctl#2-B	220	1100	St dev	
0.2ml 1e-3 Ctl#2-C	230	1150	418	
0.2ml 1e-3 Ctl#3-A	450	2250		
0.2ml 1e-3 Ctl#3-B	340	1700	St error	
0.2ml 1e-3 Ctl#3-C	316	1580	241	
Low Exposure Samples				C (plasma), CFU/ml
0.2ml 1e-1 Test #1-A				
0.2ml 1e-1 Test #2-A	221	1105		(454 +/- 273)*10
0.2ml 1e-1 Test #2-B	217	1085	Average	
0.2ml 1e-1 Test #2-C	264	1320	454	
0.2ml 1e-1 Test #3-A	35	175		% Reduction
0.2ml 1e-1 Test #3-B	32	160	St dev	99.7
0.2ml 1e-1 Test #3-C	40	200	545	
0.2ml 1e-1 Test #4-A	6	30		
0.2ml 1e-1 Test #4-B	2	10	St error	
0.2ml 1e-1 Test #4-C	1	5	273	
High Exposure Samples				C (plasma), CFU/ml
0.2ml Test #5-A	0	0		0 +/- 0
0.2ml Test #5-B	0	0	Average	
0.2ml Test #5-C	0	0	0	
0.2ml Test #6-C				
0.2ml Test #7-A	0	0	St dev	
0.2ml Test #7-B	0	0	0	
0.2ml Test #7-C	0	0		
0.2ml Test #8-C			St error	
0.2ml Test #9-A	0	0	0	
0.2ml Test #9-B	0	0		
0.2ml Test #9-C	0	0		

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5/8/2008				
Test #	Plate counts (0.2 ml of test concentrations)	Counts/ml of final dilution		
Control Samples				C (exposure ctrl, plasma), CFU/ml (1371 +/- 127)*1000
0.2ml 1e-3 Ctl#1-A	286	1430		
0.2ml 1e-3 Ctl#1-B	256	1280	Average	
0.2ml 1e-3 Ctl#1-C	269	1345	1371	
0.2ml 1e-3 Ctl#2-A	334	1670		
0.2ml 1e-3 Ctl#2-B	341	1705	St dev	
0.2ml 1e-3 Ctl#2-C	296	1480	220	
0.2ml 1e-3 Ctl#3-A	239	1195		
0.2ml 1e-3 Ctl#3-B	228	1140	St error	
0.2ml 1e-3 Ctl#3-C	218	1090	127	
Low Exposure Samples				C (plasma), CFU/ml (298 +/- 167)*10
0.2ml 1e-1 Test #1-A	141	705		
0.2ml 1e-1 Test #1-B	135	675	Average	
0.2ml 1e-1 Test #1-C	133	665	298	% Reduction
0.2ml 1e-1 Test #2-A	18	90		99.8
0.2ml 1e-1 Test #2-B	12	60	St dev	
0.2ml 1e-1 Test #2-C	18	90	289	
0.2ml 1e-1 Test #3-C				
0.2ml 1e-1 Test #4-A	21	105	St error	
0.2ml 1e-1 Test #4-B	32	160	167	
0.2ml 1e-1 Test #4-C	26	130		
High Exposure Samples				C (plasma), CFU/ml 0 +/- 0
0.2ml Test #5-A	0	0		
0.2ml Test #5-B	0	0	Average	
0.2ml Test #5-C	0	0	0	
0.2ml Test #6-A	0	0		
0.2ml Test #6-B	0	0	St dev	
0.2ml Test #6-C	0	0	0	
0.2ml Test #7-A	0	0		
0.2ml Test #7-B	0	0	St error	
0.2ml Test #7-C	0	0	0	
0.2ml Test #8-A	0	0		
0.2ml Test #8-B	0	0		
0.2ml Test #8-C	0	0		