Decontamination of Surfaces From Extremophile Organisms Using Nonthermal Atmospheric-Pressure Plasmas

Moogega Cooper, *Member, IEEE*, Gregory Fridman, *Member, IEEE*, David Staack, Alexander F. Gutsol, Victor N. Vasilets, Shivanthi Anandan, Young I. Cho, Alexander Fridman, and Alexandre Tsapin

***Abstract*—We showed that nonthermal dielectric barrier discharge (DBD) plasma compromises the integrity of the cell membrane of *Deinococcus radiodurans*, an extremophile organism. In samples of *D. radiodurans*, which were dried in a laminar flow hood, we observe that DBD plasma exposure resulted in a six-log reduction in CFU (colony-forming unit) count after 30 min of treatment. When the *Deinococcus radiodurans* cells were suspended in distilled water and treated, it took only 15 s to achieve a four-log reduction of CFU count.**

***Index Terms*—Atmospheric-pressure discharges, cold plasma, dielectric barrier discharges (DBDs), plasma decontamination, sterilization.**

I. Introduction

ATMOSPHERIC pressure nonequilibrium plasmas have been used, for example, to sterilize skin [1], promote healing in mammalian cells [1], and inactivate bacteria [2]. The objective of this paper is to take steps toward understanding necessary conditions for complete disintegration of microorganisms and elucidate the sterilization mechanisms in atmospheric-pressure nonthermal air plasma with potential applications to planetary protection [3].

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M. Cooper and Y. I. Cho are with the Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA 19104 USA (e-mail: moogega@drexel.edu; choyi@drexel.edu).

G. Fridman is with the School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PA 19104 USA (e-mail: greg.fridman@drexel.edu).

D. Staack is with the Department of Mechanical Engineering, Texas A&M University, College Station, TX 77843-3123 USA (e-mail: dstaack@tamu.edu).

A. F. Gutsol is with the Chevron Energy Technology Company, Chevron Corporation, Richmond, CA 94801 USA (e-mail: AlexanderGutsol@chevron.com).

V. N. Vasilets is with the Institute for Energy Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka 142432, Russia (e-mail: vnvasilets@gmail.com).

S. Anandan is with the Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104-2875 USA (e-mail: anandans@drexel.edu).

A. Fridman is with the A.J. Drexel Plasma Institute, Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA 19104 USA (e-mail: af55@drexel.edu).

A. Tsapin is with the NASA Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109 USA (e-mail: tsapin@jpl.nasa.gov).

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In the planetary-protection arena, the proliferation of terrestrial bacteria beyond Earth is known as forward contamination. It is extremely important, when sending spacecraft and probes to other planets and moons, to prevent the contamination of the environment, particularly when searching for native life. If bacteria from the location in which a sample was collected returns to Earth and proliferates, then reverse contamination has taken place. The goal of this paper is to achieve surface sterilization of spacecraft materials with complete disintegration of spores and bacteria. We have successfully demonstrated that treatment by cold ambient-air plasma results in sterilization, lysing, and disintegration of microorganisms. Preliminary experiments also demonstrate its potential to completely remove organics.

Current methods used by NASA to achieve sterilization of the exterior surface of the spacecraft include the use of wet heat, dry heat, alcohol wipes, beta radiation, and gamma radiation [4]. Methods involving liquids, such as wet heat and alcohol wipes, are not suitable for surfaces that will corrode or fail due to moisture. Furthermore, procedures which encompass dry methods to achieve sterilization have drawbacks to include electronic component failure and changes in surface and optical properties. Procedures conducted at high temperatures also negatively affect the substrate which requires sterilization. With respect to planetary protection, a serious potential problem lies in whether the procedures have the ability to remove fragments of DNA, proteins, and other organic material from the surface. Most of these problems can be eliminated by employing nonequilibrium atmospheric-pressure plasmas. The energized particles, UV radiation, and chemically active species generated in plasma can kill spores, bacteria, and other microorganisms at low temperature without thermal and oxidative damage to the treated surfaces.

Atmospheric-pressure plasmas form numerous chemical products, such as reactive oxygen species [atomic oxygen (O), superoxide anion (O_2^-), ozone (O_3), hydroxyl radical (OH), hydrogen peroxide (H_2O_2)], and other excited species (N_2^+, N_4^+, NO, etc.). The chemical species with the highest chemical activity have the shortest lifetime; therefore, treatment by indirect plasma ("afterglow" or "plasma jet") exposes the surface principally to the flux of long-living chemical species. It has also been determined that direct plasma exposure has a biocidal effect several orders of magnitude greater than an indirect exposure [5]. The difference lies in the fact that there are almost

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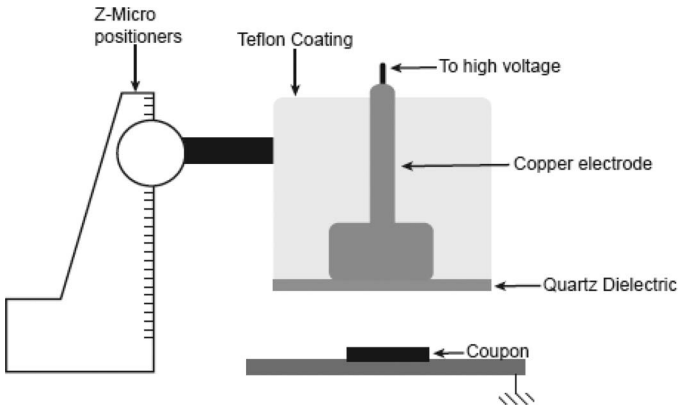


Fig. 1. DBD experimental setup.

no charged particles in afterglow plasma. For this reason, direct plasma is more effective.

We choose *D. radiodurans*, considering that if we can show that cold plasma can destroy one of the toughest microorganisms found on Earth, then we can claim that, probably, we can destroy less robust organisms as well. *D. radiodurans* is very resistive to radiation [6]–[8], temperature change [9], reactive oxygenated species [10], and vacuum [8]. It can withstand an instantaneous radiation dose of 5000 Gy with no loss of viability (60 Gy sterilizes a culture of E. coli), an instantaneous dose of up to 15 000 Gy with 37% viability loss [6], [7], and exposure to space vacuum ($\sim 10^{-6}$ Pa) for three days with decreased cell survival by four orders of magnitude [8].

II. Materials and Methods

A. DBD Experimental Setup

Dielectric barrier discharge (DBD) is a nonequilibrium and nonthermal plasma. In the presented experiments, plasma was generated using a power supply with a continuous waveform voltage characteristic [11]. The high-voltage electrode used in the DBD setup was 25.4 mm in diameter and made of copper (Fig. 1). The electrode was covered with a dielectric barrier made from fused quartz. Bacterial samples were exposed to DBD with a 1-mm gap distance between the coupon and the electrode. Plasma was formed between the electrodes with a power density of 1 $\mathrm{W/cm^2}$. The power density was previously measured electrically by taking into account the voltage across the discharge and current into it, and confirmed calorimetrically [5]. In the experimental system, the coupon to be sterilized sat above a grounded steel base (Fig. 1). This setup allowed microorganisms to be directly exposed to all plasma products at the highest intensity. Sample temperatures were measured by a noncontact infrared thermometer.

B. Deinococcus radiodurans

D. radiodurans was obtained from ATCC (#13939) and was grown in 50 mL of TGY media at slow shaking speed at 30 °C for two days. At this time, the optical density was typically 0.6–1.0. The culture was then centrifuged at 14000 r/min for 1 min and resuspended in sterile distilled water. This process was repeated three times, resulting in a suspension in distilled water.

C. Sample Preparation

Stainless steel is one example of spacecraft materials under investigation by the group. The viability measurements reported in this paper are performed on super corrosion-resistant stainless steel, type 316, with mirrorlike finish and 0.762-mm thickness. Stainless steel coupons were pretreated with ethanol and successive flaming 2 min before inoculation. The concentration of *D. radiodurans* was 10^6 colony-forming unit (CFU)/mL. When performing experiments with dry bacteria, samples were placed in a laminar flow hood for 30 min after inoculation. In the case of wet bacteria, the coupons were immediately plasma treated.

To analyze the effect of cold plasma, the inoculated coupons are then deposited in a centrifuge tube containing 5 mL of distilled water and allowed to sit for 25–30 min. The coupons were then vortexed and removed from the centrifuge tube, and the remaining solution was used for determining the bacterial viability. A standard dilution and plating technique for viable counts [5], [12] was used to follow the numbers of surviving microorganisms.

D. Scanning Electron Microscopy

Characterization of the destruction of dry *D. radiodurans* as a result of plasma treatment was performed by collecting scanning electron microscope (SEM) (FEI/Philips XL30 Field Emission Environmental SEM) images before and after plasma treatment. Relying upon the resilience of the cell wall under vacuum conditions, samples of *D. radiodurans* were imaged before plasma treatment under vacuum mode without metal coating of the sample. While taking note of the microscopic location of the bacteria, as well as the macroscopic position of the sample for future repositioning, the sample was then removed from the SEM and treated with plasma. Upon returning the sample to the SEM for posttreatment imaging, the sample was macroscopically aligned, by eye, based on indicated marks and then microscopically oriented during the imaging process. The bacteria again remained uncoated during the second imaging.

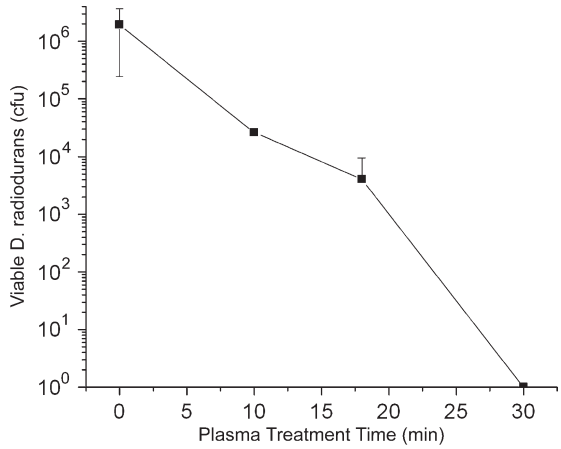


Fig. 2. Viability measurements of dry *D. radiodurans* after DBD plasma treatment.

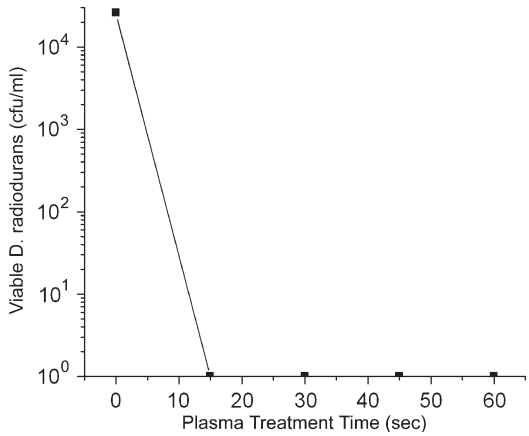


Fig. 3. Viability measurements of dry *D. radiodurans* after DBD plasma treatment.

The same bacterium was found, and the resulting damage was imaged.

III. Results

A. Sterilization Performance—Viability Measurements

Fig. 2 shows the test results obtained with *D. radiodurans* after DBD plasma treatment. At $t = 0$, the number of viable *D. radiodurans* was approximately 106 CFU. After 30 min of DBD treatment, there was a six-log reduction in the number of *D. radiodurans* cells. Temperature measurements show that during plasma treatment, the average temperature was 26 °C and the sample temperature was no higher than 30 °C.

Experiments were also performed on *D. radiodurans* which were not desiccated but treated immediately after deposition on the substrate and while encapsulated in water. Results show that an initial concentration of approximately 10^4 CFU/mL requires only 15 s of DBD treatment to result in complete inactivation (Fig. 3 and Table I). Samples treated for longer times of 30, 45, and 60 s also did not have viable bacteria. Although there is a difference in the initial concentration in Figs. 2 and 3, it is the authors' hope that emphasis can be put more on the log reduction which occurs and the amount of time it occurs in.

TABLE I
Viability Measurements of Wet *D. radiodurans* After DBD Plasma Treatment

Plasma Treatment Time (sec)	Viable *D. radiodurans* (cfu/ml)
0	2.6×10^4
15	0
30	0
45	0
60	0

After treatment of wet bacteria up to 60 s, the sample incurred little loss in water.

A detailed understanding of the increase in sterilization efficiency when water was added will be investigated in the future. One hypothesis is that the species formed by the interaction of plasma with water induces phospholipid peroxidation, thus increasing sterilization efficiency. Previous studies show the influence of hydrogen peroxide and superoxides produced in water in synergy with UV to increase sterilization efficiency [13]. There is also a mechanism of plasma-induced formation of superoxides in water through following set of chemical reactions.

1) $e^-_{(H_2O)} + O_{2(H_2O)} \rightarrow O^-_{2(H_2O)}$.
2) $2H^+ + 2O_2 \rightarrow H_2O_2 + O_2$ (dismutation reaction).
3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$ (Fenton reaction).
4) $RH + OH \rightarrow H_2O + R^*$.
5) $R^* + O_2 \rightarrow RO_2$.
6) $RO_2 + RH \rightarrow RO_2H + R^*$ (phospholipid peroxidation).
7) $RO_2H \rightarrow RO_2^- + H^+$.

This suggests that adding water enhances sterilization efficiency compared with dry conditions, and this enhancement was a result of hydrogen peroxide and superoxide production in the solution. The interaction of these species with bacteria will ultimately lead to phospholipid peroxidation, thus aiding in cell death.

On dry surfaces, radicals, such as OH, $O_2(^1\Delta_g)$, and H_2O_2, are produced by electrons ionizing molecules in air. The difference in plasma discharge formed on a dry substrate versus a wet sample is, consequently, chemistry. Furthermore, the conductivity should not vary significantly when wet samples are introduced, considering that plasma is formed above the layer of water which is on the order of 500 μm high.

Although complete sterilization of desiccated and wet *D. radiodurans* was achieved after a plasma treatment time of 30 min and 15 s, respectively, it can be inferred that, prior to complete destruction, there were lysed and partially lysed bacteria which provided naked DNA which have the potential to be transformed to other microorganisms. It was for this reason that SEM images were taken to visually determine the level of cell contents remaining.

B. Sterilization Performance—SEM Images

Imaging techniques were applied to visualize the effect of treatment with cold plasma on the morphology of microorganisms. The aforementioned viability measurements were supported by SEM images taken before and after 30 min of

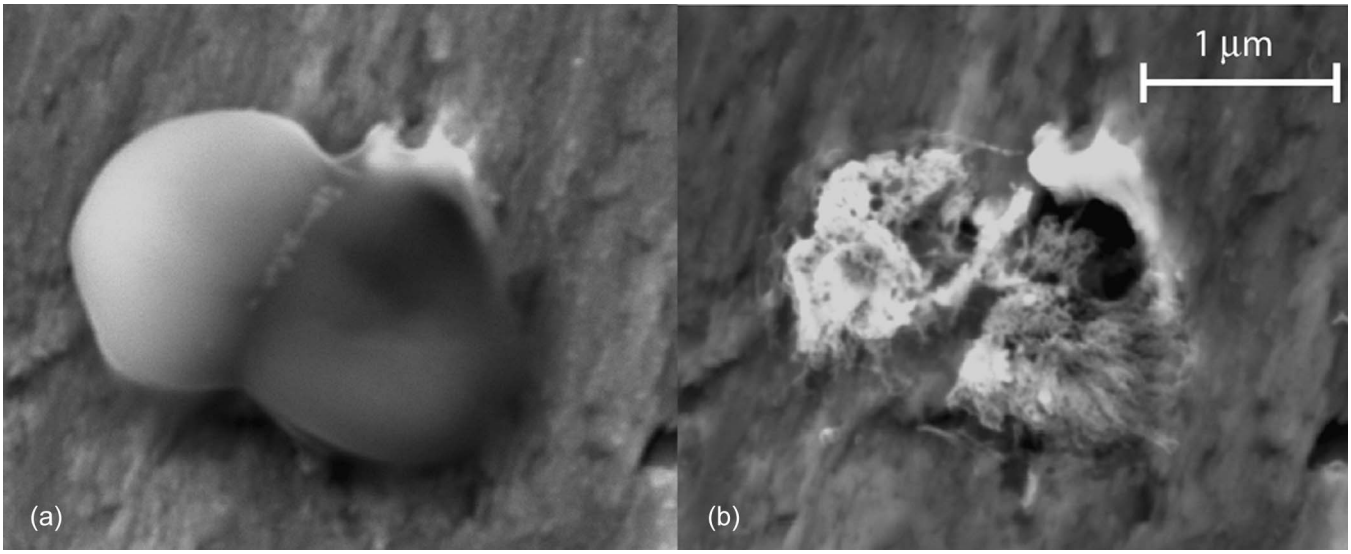


Fig. 4. SEM images of *Deinococcus radiodurans* on blue steel (a) before and (b) after 30 min of DBD plasma treatment.

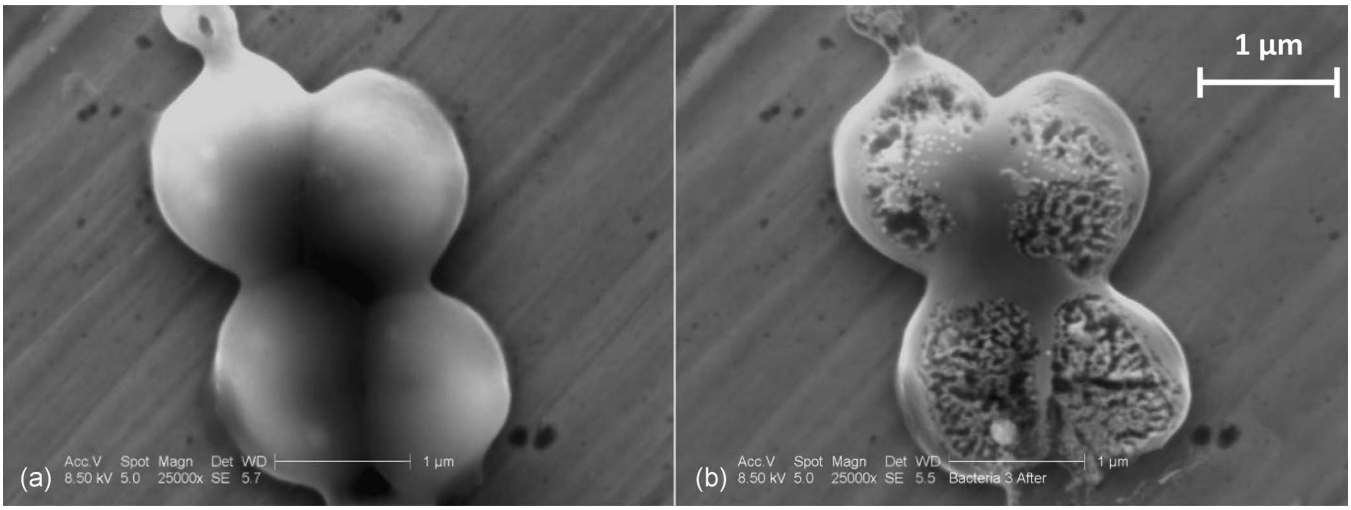


Fig. 5. SEM images of *D. radiodurans* on surgical-grade stainless steel (a) before and (b) after 20 min of DBD plasma treatment.

DBD treatment of *D. radiodurans* on blue steel at 1 $\mathrm{W/cm^2}$ (Fig. 4).

Comparison of Fig. 4(a) (before DBD plasma treatment) and Fig. 4(b) (after 30 min DBD plasma treatment) clearly shows that DBD plasma causes significant morphological changes and "physical" destruction of *D. radiodurans* on the sterilization surface at 30 °C. Although the cell was considered to be dead, its remnants continue to inhabit the surface.

SEM images were also taken of *D. radiodurans* on surgical-grade stainless steel, where the bacteria were treated for 20 min (Fig. 5). It is believed that chemical etching resulting from plasma treatment is the reason of morphological changes observed in the SEM images. To demonstrate that the damage shown in Figs. 4 and 5 was not a result of vacuum exposure and/or SEM ion beam exposure, a control experiment was also performed in the following manner. *D. radiodurans* was imaged and maintained under high vacuum for 30 min, removed from the chamber upon venting, successively replaced in the chamber, and reimaged in high vacuum for a second time. A

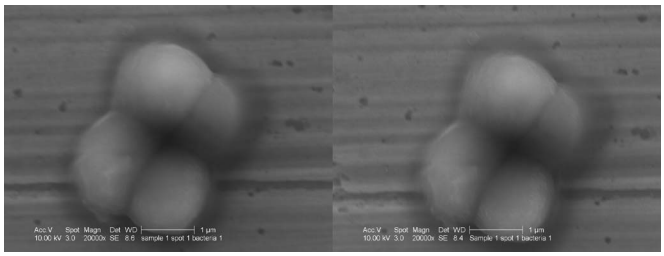


Fig. 6. Control experiments on stainless steel show only a miniscule amount of drying of the extracellular polysaccharide compounds due to (a) 30 min of vacuum exposure and desiccation in the SEM chamber and (b) successive removal, replacement, and reimaging of *D. radiodurans*.

miniscule amount of drying of the extracellular polysaccharide compounds is observed as a result of vacuum and desiccation, and Fig. 6 shows a representative sample of one set of control experiments. During the imaging process, the bacteria are in high-vacuum mode with a pressure of 1.3×10^{-4} mbar (Vac OK) to 1×10^{-5} mbar. Previous research by Saffary *et al.* [8]

has shown that the exposure of *D. radiodurans* to 1×10^{-8} mbar vacuum results in a one-log reduction and that the desiccation of *D. radiodurans* results in approximately a 0.5-log reduction. This resistance to desiccation was further proved by Mattimore and Battista [14].

IV. Conclusion

Plasma decontamination is an effective tool to eliminate the risks associated with the forward contamination of planets and moons, as well as reverse contamination by extremophile and extraterrestrial microorganisms. In this paper, a six-log reduction of dry *D. radiodurans* resulted from 30 min of DBD treatment and a four-log reduction of wet *D. radiodurans* from 15 s of DBD treatment. This enhancement phenomenon was possibly due to water chemistry which leads ultimately to phospholipid peroxidation. SEM imaging of the bacteria shows that, in the case of dry bacteria, the decrease in viability corresponds with physical damage to their structures. Coupon temperatures remain at or below 30 °C during DBD treatment, which makes this method advantageous for the treatment of spacecraft material without thermal degradation to the electronics and surfaces which are exposed to plasma. With complete removal of protein matter and DNA, the influence of inactivated bacteria will be minimized. Future efforts will expound on atmospheric-pressure microplasma glow discharge as a potential tool to achieve complete removal of all organic materials.

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Moogega Cooper (M'07) was born in Mt. Holly, NJ, in 1985. She received the B.S. degree in physics from Hampton University, Hampton, VA, in 2006. She is currently working toward the Ph.D. degree in mechanical engineering and mechanics in the Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA.

Her research focuses on sterilizing spacecraft material using nonequilibrium atmospheric-pressure plasma at the Drexel Plasma Institute. Her research interests include applications of nonthermal plasmas for sterilization of bacteria and investigation of sterilization mechanisms.



Gregory Fridman (M'05) received the B.S. degree in mathematics, statistics, and computer science from the University of Illinois, Chicago, in 2002. He is currently working toward the Ph.D. degree in the School of Biomedical Engineering, Science, and Health Systems, Drexel University, Philadelphia, PA.

His research interest includes the development of cold atmospheric-pressure plasma technologies in chemical surface processing and modification, biotechnology, and medicine.



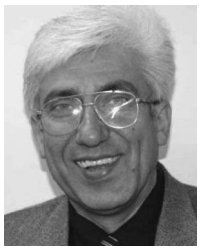
David Staack was born in New York, NY, in 1977. He received the B.S. and M.S. degrees in aerospace engineering from the University of Virginia, Charlottesville, in 2000, where his thesis research was on developing and using LIF techniques for the diagnostics of interacting rarified and continuum flows, and the Ph.D. degree from the Drexel University, Philadelphia, PA, in 2008, where his research focused on the characterization of atmospheric-pressure nonthermal plasmas and the use of such plasmas for plasma-enhanced chemical vapor deposition, biomedical applications, and fuel reforming.

From 2000 to 2004, he completed his graduate work with Princeton University, Princeton, NJ, where he was with the Princeton Plasma Physics Laboratory studying the effects of plasma surface interactions on the performance of Hall thruster rocket engines and developing plasma diagnostics. He is currently an Assistant Professor with the Department of Mechanical Engineering, Texas A&M University, College Station. His current research interests include plasma engineering applications of low-temperature plasmas at various pressures and in various media, and plasma discharge and flow diagnostics.



Alexander F. Gutsol was born in Magnitogorsk, Russia, in 1958. He received the B.S./M.S. degree in physics and engineering and the Ph.D. degree in physics and mathematics from the Moscow Institute of Physics and Technology (working for the Kurchatov Institute of Atomic Energy), Moscow, Russia, in 1982 and 1985, respectively, and the D.Sc. degree in mechanical engineering, for his achievements in plasma chemistry and technology, from the Baykov Institute of Metallurgy and Material Science, Moscow, in 2000.

From 1985 to 2000, he was with the Institute of Chemistry and Technology of Rare Elements and Minerals, Kola Science Center, Russian Academy of Sciences, Apatity, Russia. As a Visiting Researcher, he worked in different countries, including Israel in 1996, Norway in 1997, The Netherlands in 1998, and Finland during 1998–2000. Since 2000, he has been working in the U.S.; from 2000 to 2002, he was with the University of Illinois, Chicago, and from 2002 to 2008, he was with Drexel University, Philadelphia, PA, as a Research Professor with the Department of Mechanical Engineering and Mechanics and as an Associate Director of the A.J. Drexel Plasma Institute. He is currently with Chevron Energy Technology Company, Chevron Corporation, Richmond, CA. During his academic career, he was involved in physics, chemistry, and engineering of electrical discharges, fluid dynamics of swirl flows, chemistry and technology of rare metals, and powder metallurgy.



Victor N. Vasilets was born in Murmansk, Russia, in 1953. He received the B.S./M.S. degree in physics and engineering and the Ph.D. degree in physics and mathematics from the Moscow Institute of Physics and Technology, Moscow, Russia, in 1976 and 1979, respectively, and the D.Sc. degree in chemistry from the N. N. Semenov Institute of Chemical Physics, Russian Academy of Sciences, Moscow, in 2005.

He was a Research Scientist in 1979, a Senior Research Scientist in 1987, and a Principal Research Scientist in 2000 with the N. N. Semenov Institute of Chemical Physics, Russian Academy of Sciences. He was a Visiting Professor with the Center of Biomaterials, Kyoto University, Kyoto, Japan, in 1996; the Institute of Polymer Research, Dresden, Germany, in 1998–2000; and the Plasma Physics Laboratory, University of Saskatchewan, Saskatoon, SK, Canada in 2002–2005. During 2005–2007, he was with the Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA, as a Research Professor. He is currently a Principal Research Scientist with the Institute for Energy Problems of Chemical Physics, Russian Academy of Sciences, Chernogolovka, Russia. He has authored or coauthored three book chapters and more than 100 papers. His current research interests include using gas discharge plasma and vacuum ultraviolet for sterilization, wound treatment, and biological functionalization of medical polymers. He is a member of the International Advisory Board of the journal *Plasma Processes and Polymers*.



Shivanthi Anandan received the Ph.D. degree from the University of California, Los Angeles, in 1992.

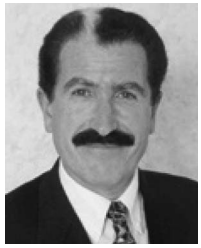
She was a Postdoctoral Research Associate, an Assistant Research Scientist, and a Lecturer with Texas A&M University, College Station, from 1992 to 1997, where her focus was mainly on cyanobacterial molecular genetics. Since 1997, she has been with the Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA, first as an Assistant Professor and then, currently, as an Associate Professor. Her research interests center on how photosynthetic organisms sense and respond to environmental cues. She is particularly interested in the molecular mechanisms that regulate gene expression in cyanobacteria and the environmental factors that cause these changes.

Dr. Anandan was the recipient of the College of Arts and Sciences Faculty Excellence Award from Drexel University in 2003. She is a member of the Education committee of the Southeast Pennsylvania branch of the American Society for Microbiology and an Ad Hoc Reviewer for the National Science Foundation.



Young I. Cho received the B.S. degree in mechanical engineering from Seoul National University, Seoul, Korea, in 1972 and the M.S. and Ph.D. degrees in mechanical engineering from the University of Illinois, Chicago, in 1977 and 1980, respectively.

He has been with the Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA, since 1985, where he has developed a non-Newtonian flow and heat transfer laboratory and has initiated the investigation of a nonchemical water treatment technology to prevent fouling from various heat exchangers. Currently, he is developing methods of applying low-temperature plasma technology to mineral- and biofouling problems caused by hard water.



Alexander Fridman received the B.S./M.S. and Ph.D. degrees in physics and mathematics from the Moscow Institute of Physics and Technology, Moscow, Russia, in 1976 and 1979, respectively, and the D.Sc. degree in mathematics from the Kurchatov Institute of Atomic Energy, Moscow, in 1987.

He is currently the Nyheim Chair Professor of Drexel University, Philadelphia, PA, and the Director of the A.J. Drexel Plasma Institute, Department of Mechanical Engineering and Mechanics, where he works on plasma approaches to material treatment, fuel conversion, and environmental control. He has more than 30 years of plasma research experience in national laboratories and universities of Russia, France, and U.S. He has authored or coauthored five books and more than 350 papers.

Prof. Fridman was the recipient of numerous awards, including the Stanley Kaplan Distinguished Professorship in Chemical Kinetics and Energy Systems, the George Soros Distinguished Professorship in Physics, and the State Prize of the USSR for the discovery of selective stimulation of chemical processes in nonthermal plasma.



Alexandre Tsapin received the B.S. degree in physics from Moscow State University, Moscow, Russia, in 1970 and the Ph.D. degree from the Academy of Science USSR, Moscow, in 1974.

He has been with Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA, since 1998, working on life-detection and astrobiological projects. He spearheaded a collaboration with Drexel Plasma Institute, Philadelphia, PA, on how to apply nonthermal plasma for spacecraft decontamination as a planetary-protection project.