Nonthermal Atmospheric Pressure Plasma Decontamination of Protein-Loaded Biodegradable Nanoparticles for Nervous Tissue Repair

Jason Coleman,[1]* Adam Yost,[2] Ross Goren,[1] Gregory Fridman,[2] & Anthony Lowman[1]

[1]Department of Chemical and Biological Engineering, Drexel University, Philadelphia, Pennsylvania; [2]A. J. Drexel Plasma Institute, Drexel University, Camden, New Jersey

*Address all correspondence to: Jason Coleman, Department of Chemical and Biological Engineering, Drexel University, 3141 Chestnut St., Philadelphia, PA 19104; Tel.: 1-610-574-6177; jason_coleman76@yahoo.com.

ABSTRACT: When injected directly into spinal tissue, poly(lactide-co-glycolide) (PLGA) particles have the potential to provide sustained delivery of proteins necessary to promote nerve regeneration. To ensure the injection of sterile particles, a novel decontamination method was developed that can inactivate bacteria while maintaining particle and protein integrity. This method involves the resuspension of protein-loaded particles after preparation in phosphate-buffered saline (PBS) or deionized water that has been subjected to a nonthermal atmospheric pressure discharge. Although different treatment times were required, it was demonstrated that both plasma-treated PBS and water can completely inactivate *Escherichia coli* when added at a concentration of 10^3 colony-forming units/mL to PLGA particles with and without protein. Plasma-treated water showed no impact on particle morphology. Plasma treatment of water for 1 minute resulted in the complete sterilization of protein-loaded particles but was able to maintain only $26.6 \pm 5.0\%$ of protein activity. On the other hand, plasma-treated PBS required 2 minutes of treatment for complete sterilization of protein-loaded particles but was able to maintain $68.4 \pm 10.6\%$ of protein activity. Particles were considered sterile if no bacterial growth was observed after being plated onto bacterial growth agar. The effect of plasma-treated water on release of active protein from particles caused a substantial loss in the initial burst release of protein but did not limit the ability of the particles to provide a sustained release of active protein.

KEY WORDS: dielectric barrier discharges (DBD), nonthermal atmospheric pressure plasma, sterilization, plasma medicine, protein delivery, poly(lactide-co-glycolide) (PLGA), spinal cord injury, lysozyme, nanoparticles

I. INTRODUCTION

The regeneration and reconnection of nerves after spinal cord injury remains one of the most challenging medical problems. Regeneration and reconnection of damaged nerves is a difficult task because of both environmental (extrinsic) factors and intrinsic factors to the neuron.[1] Proteins such as neurotrophic factors (NTFs) (e.g., brain-derived NTF, glial cell line–derived NTF, and neurotrophin-3) or chondroitinase ABC can provide the extrinsic factors necessary for the regeneration of axons after spinal cord injury,

but chondroitinase ABC and NTFs are subject to enzymatic degradation in vivo.[2–12] Therefore, a long-term delivery system that can release active protein directly into the central nervous system tissue over the course of days to weeks is desirable. Poly(lactide-co-glycolide) (PLGA) particles have been studied extensively as a sustained delivery device for proteins.[12–28] Because of the difficulty of incorporating hydrophilic proteins within a hydrophobic polymer, the preferred method of entrapment is water/oil/water (w/o/w) double-emulsion solvent evaporation, which utilizes physical entrapment so that it can be applied to a variety of proteins. Recent work used this method to prepare PLGA nanoparticles (<1 mm and >100 nm) that could deliver active protein for up to 2 months.[29] The goal is to utilize protein-loaded PLGA nano-sized particles for direct microinjection into central nervous system tissue. However, because of the difficulty in preparing particles using the w/o/w double-emulsion procedure under completely sterile conditions, a novel method was developed to inactivate bacteria present on protein-loaded particles after preparation; this method could maintain particle integrity and protein activity. Particle sterilization is required to eliminate a potential negative immune response to contaminated particles. The bacterial inactivation method developed in this work involves the resuspension of protein-loaded particles in deionized water or phosphate-buffered saline (PBS) that has been subjected to a nonthermal atmospheric pressure discharge.

Current and more traditional sterilization methods involve exposure to ethylene oxide, chlorine, ozone, gamma radiation, or heat.[30,31] When certain polymers are sterilized using these methods, the results are unfavorable changes in physical, chemical, and mechanical properties.[31] One of the most promising sterilization methods for the surface treatment of polymers is nonthermal (<50ºC) atmospheric pressure plasma.[31] Although the mechanism of nonthermal plasma sterilization is not entirely known, it has been shown to be safe enough for application directly to human tissue.[30,32] Plasma discharge generates reactive species such as ozone (O_3), peroxides, and OH radicals that can react with and destroy bacteria.[30,32,33] Because the reactive species generated can react with organic materials such as protein, there has been evidence of plasma treatment causing significant loss of protein activity.[34] Therefore, a plasma treatment method was developed so that the protein-loaded particles would not have to come in to direct contact with the plasma discharge. The specific type of plasma sterilization used in this work utilizes an electrode to discharge nonthermal plasma through water or PBS contained in a grounded well. This treated water or PBS is then used as the sterilizing agent. Indirect exposure to plasma is preferred to reduce damage to the particles and the protein, but it is less effective as a sterilizing agent.[35] This decontamination method has never been attempted with either PLGA particles or protein-loaded PLGA particles, so the sterilization effectiveness and effects on both the particles and the protein are unknown. Because the particles can be prepared in a controlled laboratory setting, bacterial inactivation is considered adequate when complete inactivation is achieved after exposure to a low bacterial load of *Escherichia coli* at a concentration of 10^3 colony-forming units (CFUs) per milliliter. To assess the impact of indirect plasma sterilization on protein activity,

the model protein lysozyme was used because of the ability to quantify both the protein amount and activity. Lysozyme is also similar in both molecular weight and isoelectric point to the clinically relevant NTFs (e.g., brain-derived NTF, glial cell line–derived NTF, and neurotrophin-3).

II. METHODS

A. Materials

PLGA (average molecular weight (MW), 18 kDa; copolymer ratio, 50:50) was purchased from Lakeshore Biomaterials (Birmingham, AL). Lysozyme (MW, 14.3 kDa; activity, 63,628 Units/mg protein), bovine serum albumin (BSA; MW, 66 kDa), sucrose acetate isobutyrate (MW, 846.91 Da), sodium azide (MW, 65.01 Da), and Dulbecco's PBS (pH, 7.4; KCl 0.2 g/L, KH_2PO_4 0.2 g/L, NaCl 8.0 g/L, and Na_2HPO_4 1.15 g/L) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(vinyl alcohol) (PVA; average MW, 25 kDa; 88% hydrolyzed) was purchased from Polysciences, Inc (Warrington, PA). Lysozyme activity assay (EnzChek® assay) was purchased from Invitrogen (Grand Island, NY) and protein concentration assay (Pierce Micro BCA™ assay) was purchased from Thermo Fisher Scientific (Waltham, MA). All organic solvents were of high-performance liquid chromatography–grade and purchased from Sigma-Aldrich. *E. coli* was purchased from American Type Culture Collection (no. 25922). Miller's Luria-Bertani broth and brain-heart infusion agar were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ).

B. Dielectric Barrier Discharges Experimental Setup

Nonthermal atmospheric pressure plasma was produced by a dielectric barrier discharge following procedures previously described by Fridman et al.[36] Plasma was generated using a power supply with continuous waveform characteristics and a 2.54-cm-diameter, high-voltage electrode made of copper and covered with a dielectric barrier made from fused quartz (Fig. 1). Two milliliters of deionized water or PBS was placed directly below the electrode in a grounded well that contained a 2-ml reservoir. The 2-mL well sat on a grounded steel base. The electrode was kept approximately 1.5 mm above the top surface of the deionized water or PBS. Plasma was discharged across the water or PBS and onto the grounded well at a power of 3.2 W and a surface power density of 0.63 W/ cm^2. Once the water or PBS was subjected to the plasma discharge at various time intervals, it was used to either resuspend particles or dilute lysozyme samples. These studies are further explained later.

C. *Escherichia coli*

E. coli was grown in 10 mL of Miller's Luria-Bertani broth at a slow shaking speed of 37ºC overnight. The culture was then centrifuged and resuspended in sterile PBS. The
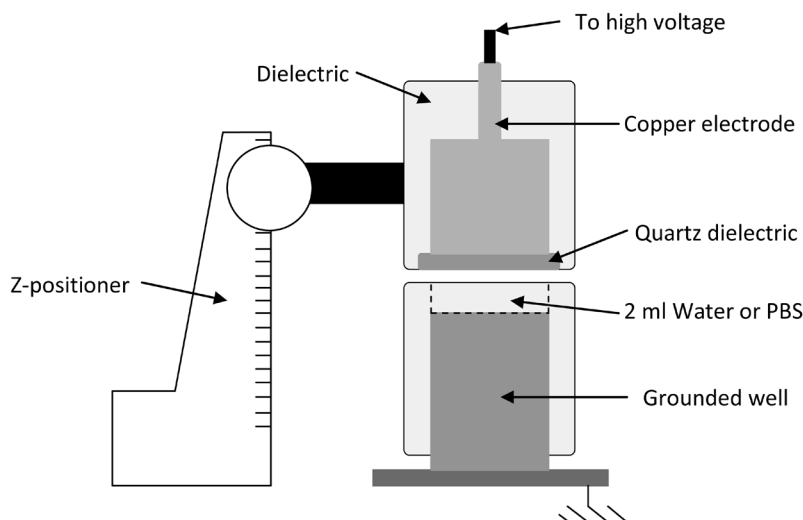
FIGURE 1. Dielectric barrier discharges experimental setup. PBS, phosphate-buffered saline.

culture was then serially diluted to a concentration of 10^3 CFU/mL for use in inactivation studies.

D. Particle Preparation

Particles were prepared using a w/o/w double-emulsion solvent evaporation procedure. Phase volume ratios and surfactant (PVA) type and concentrations established by Dziubla et al[37] were used. The first water-oil phase contained 250 mL of an aqueous solution consisting of lysozyme (5 mg/mL) or BSA (100 mg/mL) in PBS plus 2.5 mL of a dichloromethane solution consisting of PLGA (25 mg/mL). Particles were loaded with lysozyme for studies to assess the effect of plasma treatment on loading and active protein release. Particles were loaded with BSA to assess how BSA interferes with sterilization. Because of the damaging effects of the w/o/w double-emulsion procedure on protein, BSA has been used as a stabilizer.[25,38] Earlier work showed that BSA had the maximum stabilizing effectiveness at a concentration of 100 mg/mL.[29] The first water-oil phase was homogenized (Polytron® System PT3100 homogenizer with PT-DA 3007/2EC dispersing aggregate; Kinematic, Inc, Bohemia, NY) for 1 minute at 25,000 rpm. The first emulsion was then removed and slowly pipetted into 12.5 mL of 2% PVA kept at 4ºC while being homogenized at 25,000 rpm. The second emulsion was homogenized for 2 minutes. To allow for solvent removal, the second emulsion was then added to 25 mL of 2% PVA and allowed to stir overnight at 350 rpm. Particles were then collected by centrifuging at 15,000 G for 20 minutes. Particles were washed by resuspending in deionized water and centrifuging each time to collect them. Particles were collected at

various centrifugation steps for further testing.

E. Scanning Electron Microscopy

To assess the effect of plasma treatment on particle morphology, after one wash particles were resuspended in 2 mL of deionized water that had been plasma treated for 30 or 60 seconds. Particles were washed several times after plasma treatment and collected by centrifugation. After the final centrifuge, particles were resuspended in 2 mL of deionized water. A small sample of suspended particles was removed and diluted in water to an approximate concentration of 1 mg/mL. Of this diluted sample, 40 μL was pipetted onto a scanning electron microscope (SEM) stub and flash frozen in liquid nitrogen. The sample was then freeze-dried and sputter coated with a platinum/palladium mixture before imaging. Sizing of the dried, nontreated samples was determined directly using SEM (Zeiss Supra 50VP, Carl Zeiss NTS, LLC Peabody, MA) and Image J software (National Institutes of Health, Bethesda, MD). For sizing, samples were prepared in triplicate and 200 particles were counted per sample.

F. Lysozyme Loading

Lysozyme loading was determined for particles that underwent one wash step before being resuspended in plasma-treated water that had been treated for 0, 30, and 60 seconds. Glass vials used for loading studies were treated with Sigmacote® (Sigma Aldrich). Sigmacot is a thin layer of silicone deposited on the glass wall to prevent lysozyme adsorption. After plasma treatment, particles were washed twice and freeze-dried. Dry particles were resuspended in 0.5 M NaOH (10 mg/mL) and allowed to stir overnight until totally degraded. The solution was then neutralized with an equal volume of 0.5 M HCl. Samples were prepared in triplicate. Blank particles without protein also were prepared and subjected to the same degradation and assay conditions to account for any absorbance caused by the degraded polymer. Standards were subjected to the same treatment, and protein concentration was determined using the Pierce Micro BCA™ assay, a color change assay that relies on the reduction of Cu^{2+} to Cu^{1+} in an alkaline medium followed by the highly selective and sensitive colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. Encapsulation efficiency was calculated in terms of loading efficiency Eq. (1) and the protein weight percent of the particles Eq. (2).

$$\text{Encapsulation Efficiency}(\%) = 100 \times \left(\frac{\text{Mass of Lysozyme in Particles}}{\text{Total Theoretical Mass of Lysozyme in Particles}} \right) \tag{1}$$

$$\text{Weight } \% = 100 \times \left(\frac{\text{Mass of Lysozyme in Particles}}{\text{Mass of Particles} + \text{Lysozyme}} \right) \tag{2}$$

G. BSA Loading

Because BSA is not completely soluble in a basic NaOH solution, BSA loading could not be determined directly. Instead, loading was determined indirectly by measuring the amount of BSA in the supernatant after each particle centrifugation and wash step. Therefore, the weight percentage is determined according to Eq. (2), but the mass of lysozyme in particles is determined indirectly according to Eq. (3).

Mass of Lysozyme in Particles = Mass of Lysozyme Loaded –
Cumulative Mass of Lysozyme in Supernatant   (3)

H. In Vitro Lysozyme Release Studies

Lysozyme release was performed on particles that underwent one wash step before being resuspended in water that had been treated with plasma for 0, 30, and 60 second. After plasma treatment, particles were washed twice and freeze-dried. Glass vials used for release studies were treated with Sigmacote. Dry particles (30 mg) were weighed directly in vials and resuspended in 2 mL of release media (PBS [pH, 7.4] + 0.01% sodium azide). Studies were performed in a heated shaker bath maintained at 37ºC and 50 rpm. Before sampling, particles were separated from the media by centrifuging and the supernatant was collected. All but 150 mL of release media per sample was removed and an equal amount of release media was added before resuspension of the particles. Samples were performed in triplicate. Blank particles loaded without protein also were performed in triplicate. The purpose of the blank particles was to account for any fluorescence caused by degraded particles. The supernatant collected was assayed for protein activity (EnzChek assay). The EnzChek assay measures lysozyme activity on *Micrococcus lysodeikticus* cell walls, which are labeled with fluorescein such that the fluorescence is quenched. Lysozyme action relieves this quenching, resulting in an increase in fluorescence that is proportional to lysozyme activity.

I.   Effect of Plasma Treatment on Lysozyme Activity and pH

To determine the effect of plasma-treated, deionized water and PBS on protein activity, 2 mL of water or PBS was plasma treated for the desired time intervals. Fourteen microliters of a lysozyme in water solution (150 mg/mL) was added to 1666 mL of plasma-treated, deionized water or PBS. The EnzChek assay was then used to determine lysozyme activity, and the percent of activity remaining was determined by comparing the activity of treated and nontreated samples. Studies were performed in triplicate.

pH studies were performed in triplicate by subjecting 2 mL deionized water or PBS to plasma treatment at various time intervals and recording the pH using a pH meter (Accumet Basic, Denver Instruments, Göttingen, Germany).
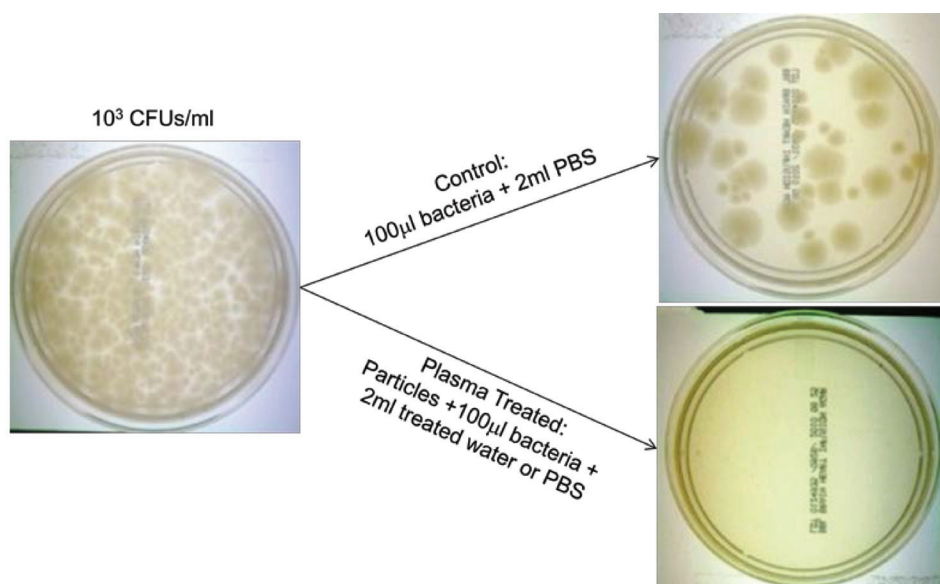
FIGURE 2. Bacteria inactivation setup.

J. Inactivation of Bacteria Using Indirect Plasma Treatment

Particles prepared for the inactivation studies underwent the same preparation conditions mentioned earlier. The primary aqueous phase contained either BSA (100 mg/mL) in PBS or only PBS. All aqueous-based solutions used to either make or wash the particles were sterile filtered using a 0.22-mm filter. After stirring overnight, the particles were collected by centrifuging. The "no wash" samples were centrifuged once, the "one wash" samples were centrifuged and then resuspended in water and centrifuged again, and the "2 wash" samples were subjected to an additional resuspension and centrifugation step. The purpose of the washes was to remove any PVA or free BSA. The remaining particle pellet was then used for inactivation studies. For the plasma treatment, 2 mL of sterile filtered deionized water or PBS was subjected to plasma discharge for 0, 30, 60, or 120 seconds. The particles were then resuspended in the plasma-treated water or PBS along with 100 mL of *E. coli* at a concentration of 10^3 CFU/mL. Samples were then vortexed and remained in the treated water or PBS for at least 30 minutes before sampling. For sampling, 100 mL of the resuspension was removed and plated to measure *E. coli* CFUs. Inactivation was measured as the percentage of CFUs remaining (Eq. [4]) by comparing CFUs of treated samples (particles plus bacteria resuspended in 2 mL of water or PBS) to the CFUs of the control (bacteria plus 2 mL of PBS). Examples of treated and control samples are shown in Fig. 2. Studies were performed in triplicate.

$$\% \text{ Remaining} = 100 \text{ x} \left(\frac{\# \text{CFUs in Treated}}{\# \text{CFUs in Control}} \right) \tag{4}$$
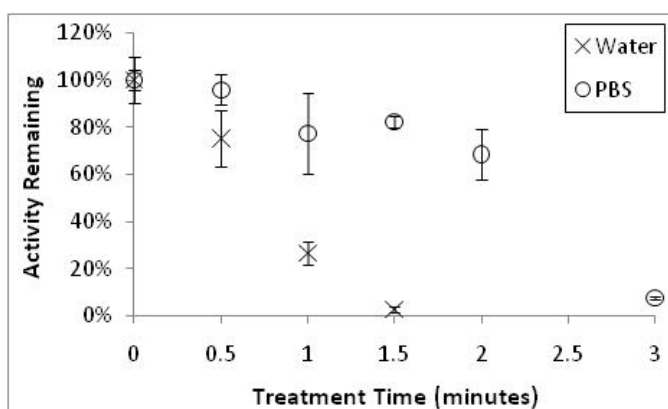
FIGURE 3. Effect of plasma treatment on lysozyme activity. Error bars indicate standard deviation (n = 3). PBS, phosphate-buffered saline.

III. RESULTS AND DISCUSSION

A. Effect of Plasma Treatment on Particle Morphology and Protein Activity

Because of the sensitivity of both nanoparticles and proteins to current sterilization techniques, a novel method that maintained protein activity and particle integrity had to be developed. For nonthermal plasma decontamination to be considered as a potential option, the effects on protein activity, protein release, and particle morphology had to be assessed. When lysozyme is diluted with plasma-treated water, there is a substantial loss of protein activity, with only $26.6 \pm 5.0\%$ and $2.8 \pm 1.4\%$ of activity remaining after treatment times of 60 and 90 seconds, respectively (Fig. 3). To maintain protein activity, lysozyme was diluted with plasma-treated PBS. The results indicated that plasma-treated PBS is less detrimental to lysozyme activity. When lysozyme was diluted with PBS that was plasma treated for 2 minutes, $68.4 \pm 10.6\%$ of activity remained. Because lysozyme is most active over a pH range of 6 to 9, one of the main differences in the damaging effects of plasma-treated water versus plasma-treated PBS is the drop in pH. The differences in pH of plasma-treated water versus plasma-treated PBS are illustrated in Fig. 4. It can be seen that the drop of pH out of the lysozyme-working pH range in plasma-treated water is almost immediate, dropping to a pH of 3.26 ± 0.12 after only 15 seconds of treatment time. However, after 2 minutes of plasma treatment, PBS drops to a pH of only 5.94 ± 0.06, just below the ideal activity range of lysozyme. It is not until after 3 minutes of treatment for PBS that there is a large drop in pH (3.46 ± 0.12), which seems to correlate with a large drop in lysozyme activity ($7.6 \pm 0.5\%$ activity remaining). Although the exact mechanism by which plasma treatment decreases protein activity is not known, the drop in pH seems to play a prominent role.

Despite losses in activity when lysozyme was resuspended directly in plasma-treated water, there remains the potential that protein trapped within particles can be
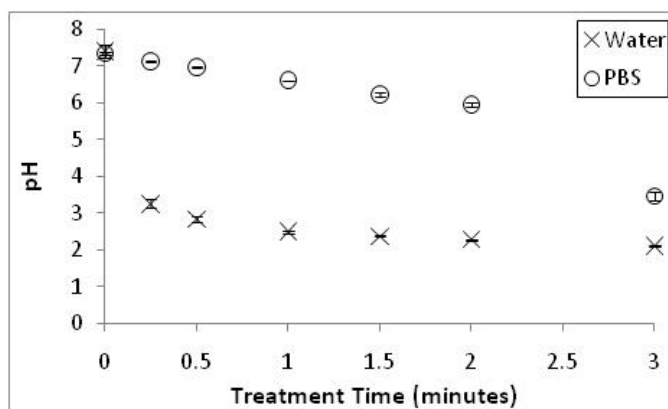
FIGURE 4. Effect of plasma treatment on pH. Error bars indicate standard deviation (n = 3). PBS, phosphate-buffered saline.

shielded from the damaging effects of plasma treatment. The average diameter of particles used for these studies was 535.1 ± 124.2 nm; an SEM image of untreated particles is shown in Fig. 5. Studies were performed on plasma-treated particles to determine the effects on loading and active protein release. Plasma treatment does result in a loss of the amount of protein loaded (Table 1) and in the amount of active protein released (Fig. 6). The drop in loading may result from the effects on surface proteins. Plasma treatment may cause the surface associated proteins to disassociate from the particles
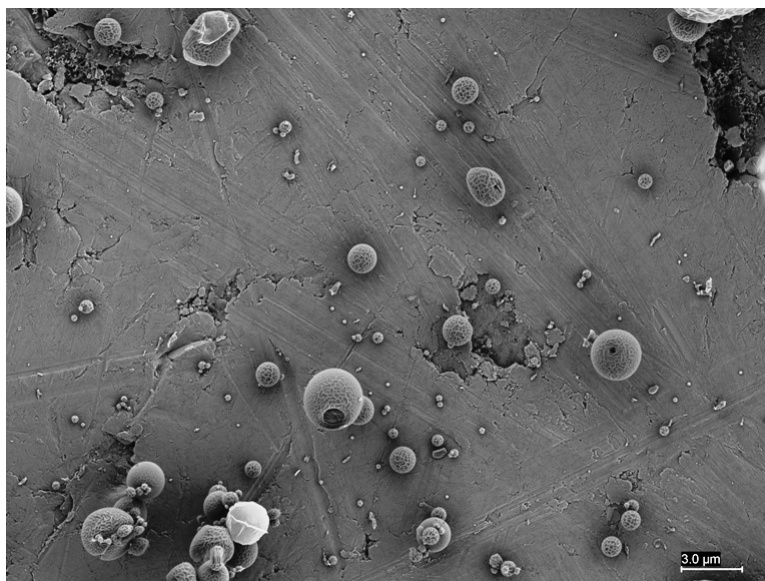

FIGURE 5. Scanning electron microscopy image of poly(lactide-co-glycolide)particles without plasma treatment; 3000× magnification, scale bar = 3 mm.

TABLE 1. Loading Results		
Treatment Time (s)	**Encapsulation Efficiency (%)***	**Lysozyme Weight Percentage (%)***
0	59.6 ± 3.4	1.19 ± 0.07
30	53.5 ± 1.2	1.07 ± 0.02
60	44.4 ± 2.2	0.89 ± 0.04

*Values are listed as mean ± standard deviation (n=3).

and be washed away in the wash steps after plasma treatment, or plasma treatment may result in insoluble protein aggregates. The effects on surface-associated protein may also explain the activity loss primarily seen during the burst-released protein at the start of the release study. Despite this, all 3 samples are able to deliver sustained amounts of active protein. The initial burst release protein is primarily the result of the surface-bound proteins immediately diffusing into the release media.[39] Therefore, it is possible that the plasma treatment is most damaging to the surface-bound protein, but the trapped protein is shielded from the damaging effects, resulting in a sustained release profile of active protein. Also, because plasma-treated water is more harmful to lysozyme activity then plasma-treated PBS, one method to limit the loss of active protein initially released from particles is to resuspend particles in plasma-treated PBS instead of plasma-treated water. However, for resuspension of protein-loaded particles into plasma-treated PBS as opposed to plasma-treated water, the plasma-treated PBS must be shown to be capable of completely sterilizing the particles both with and without protein. Although plasma-treated water showed significant detrimental effect on protein activity, SEM imaging showed no morphologic effects of particles resuspended in water treated for 30 and 60
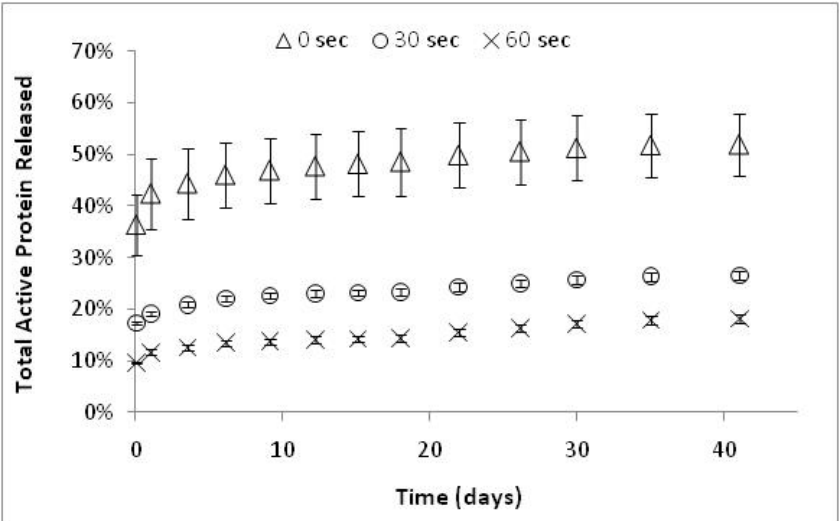

FIGURE 6. Particle release data. Error bars indicate standard deviation (n = 3).
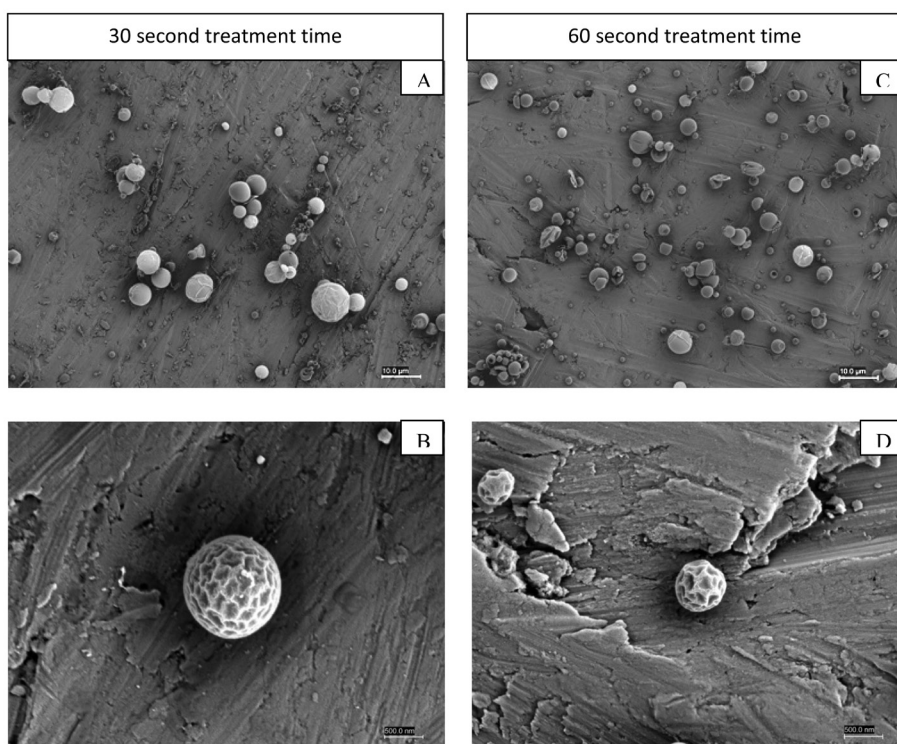
FIGURE 7. Scanning electron microscopy images of particles exposed to plasma-treated water. (a, b) 30-second treatment time; (c, d) 60-second treatment time; (a, c) 1000× magnification, scale bar = 10 mm; (b, d) 20,000× magnification, scale bar = 500 nm.

seconds (Fig. 7).

B. Inactivation of Bacteria Using Indirect Plasma Treatment

The difficulty with sterilizing protein-loaded nanoparticles is providing adequate plasma treatment to allow for complete bacteria inactivation, but not providing excessive treatment that results in particle damage and protein activity loss. The purpose of the inactivation studies was to assess the sterilization effectiveness of treatment times that did not result in complete protein activity loss. Thus, the treatment times chosen were 30 and 60 seconds for resuspension in plasma-treated water and 60 seconds and 2 minutes for resuspension in plasma-treated PBS. Also, because BSA has been used as an additive to help limit protein damage, particles loaded with and without BSA were treated.[25,29,38] The potential is that particles, PVA, and BSA could interfere with bacteria inactivation and reduce the effectiveness. Particles made with and without BSA were resuspended in plasma-treated water and a known concentration of bacteria was added. The controls consisted of non–plasma-treated PBS with the same concentration of bacteria added.

TABLE 2. Bacteria Inactivation with Plasma Treatment of Water

Substance sterilized	Water treatment time (sec)	CFUs remaining (%)*	
		No wash	One wash
Water	30	0.0 ± 0.0	—
Particles without BSA	30	21.7 ± 21.0	0.0 ± 0.0
	60	1.8 ± 3.1	0.0 ± 0.0
Particles with BSA	30	47.8 ± 13.6	10.9 ± 7.2
	60	25.0 ± 18.8	0.0 ± 0.0

*Initial bacterial load of 10^3 CFU/mL.
CFU, colony-forming unit; BSA, bovine serum albumin.

The control sample did not contain particles because the purpose of the control was to assess only the amount of bacteria that is present when the sample is not treated with plasma. The particles were included in the treated samples because the particles interfere with the ability of plasma-treated water or PBS to decontaminate. Therefore, because the control was not treated with plasma, there was no need to include particles because there was no treatment for the particles to interfere with. The bacterial load used for treatment (10^3 CFU/mL) was well below the bacterial amounts present in particles that did not undergo plasma treatment (data not shown).

The effectiveness of indirect plasma inactivation is listed in terms of percentage of CFUs remaining in Tables 1 and 3. Zero percent CFUs remaining is considered complete sterilization. Results of plasma-treated water are shown in Table 2. Without one wash step, none of the particles were completely sterilized. This indicates that residual PVA interferes with bacteria inactivation. With the wash step, particles without BSA were completely sterilized at 30- and 60-second treatment times; however, the full 60 seconds of treatment was required for particles with BSA. This indicates that residual BSA remaining in solution and on the particle surface potentially interferes with sterilization. Although a low bacterial load was used (10^3 CFU/mL), complete sterilization is considered because plating the treated particles onto bacteria growth agar resulted in no bacterial growth.

As indicated in Fig. 3, plasma-treated PBS is not as damaging to protein activity as plasma-treated water. However, because PBS buffers against the negative effects of plasma treatment on protein activity, it is critical to test whether PBS also buffers against the antibacterial effects of plasma treatment. Results of plasma-treated PBS on particle decontamination are shown in Table 3. Particles without BSA that undergo one wash are completely sterilized after 60 seconds of PBS treatment; however, particles with BSA are not sterilized with 84.5 ± 4.7% CFUs remaining. This indicates that the presence of BSA greatly interferes with the sterilizing effectiveness of plasma-treated PBS. An additional wash step (2 washes) was performed to help remove more of the surface-associated BSA, but this provided little benefit to inactivation effectiveness, potentially because most of the unbound protein had already been removed in the initial wash steps. Table 4 indicates the weight percentage of BSA in particles at each wash step. To get complete particle sterilization, the plasma treatment time of PBS had to be increased

TABLE 3. Bacteria Inactivation with Plasma Treatment of Phosphate-Buffered Saline (PBS)

Substance sterilized	PBS treatment time (sec)	CFUs remaining (%)*	
		One wash	Two washes
Particles without BSA	60	0.0 ± 0.0	—
Particles with BSA	60	84.5 ± 4.7	84.6 ± 3.9
	120	—	0.0 ± 0.0

*Initial bacterial load of 10^3 CFU/mL.
CFU, colony-forming unit; BSA, bovine serum albumin.

to 2 minutes. This is an indication that the indirect plasma treatment of PBS is not as effective as the indirect plasma treatment of water. However, even with the additional treatment time required for complete sterilization (i.e., 2 minutes), plasma treatment of PBS is not as detrimental to protein activity. Under the conditions tested, and taking into consideration the effect of plasma treatment on protein activity and bacteria inactivation, the preferred treatment method to allow for complete inactivation while maintaining protein activity is resuspending washed particles in 2 mL of PBS that has been treated with plasma for 2 minutes.

IV. CONCLUSION

Sterilization of protein-loaded PLGA nanoparticles is difficult because of the need to maintain particle integrity and protein activity while inactivating bacteria. This work demonstrated that indirect plasma treatment of water and PBS was effective at decontaminating protein-loaded particles when inoculated with 10^3 CFU/mL of *E. Coli*. Plasma-treated water showed no morphological effect on particles but did result in a significant loss in lysozyme activity. When exposed to plasma-treated water, particles loaded with lysozyme demonstrated a diminished burst release of active protein but were still able to deliver active protein over several weeks, similar to the particles that were not treated. Exposure of lysozyme to plasma-treated PBS resulted in improved retention of activity, potentially because of the pH buffering of PBS. Plasma treatment of PBS did require additional treatment time to inactivate a bacterial load of 10^3 CFU/mL, but the additional time still allowed for adequate retention of protein activity. Ultimately, resuspension of particles in plasma-treated PBS seems to be an effective way to both decontaminate particles and maintain protein activity.

TABLE 4. Weight Percentage of Bovine Serum Albumin (BSA) in Particles

Washes, n	BSA weight percentage
0	15.8 ± 0.7
1	15.4 ± 0.7
2	15.1 ± 0.6

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