Nitric oxide-releasing antibacterial albumin plastic for biomedical applications

Alexander Jones¹, Jitendra Pant², Eliza Lee¹, Marcus J. Goudie², Alexey Gruzd¹, Joel Mansfield², Abhyuday Mandal³, Suraj Sharma¹, Hitesh Handa²
¹Department of Textiles, Merchandising and Interiors, University of Georgia, Athens, Georgia
²School of Chemical, Materials and Biomedical Engineering, University of Georgia, Athens, Georgia
³Department of Statistics, University of Georgia, Athens, Georgia

Abstract

Designing innovative materials for biomedical applications is desired to prevent surface fouling and risk of associated infections arising in the surgical care patient. In the present study, albumin plastic was fabricated and nitric oxide (NO) donor, S-nitroso-N-acetylpenicillamine (SNAP), was incorporated through a solvent swelling process. The albumin-SNAP plastic was evaluated in terms of mechanical and thermal properties, and bacterial adhesion to the plastic surface. Thermal and viscoelastic analyses showed no significant difference between albumin-SNAP plastics and pure, water-plasticized albumin samples. Bacteria adhesion tests revealed that albumin-SNAP plastic can significantly reduce the surface-bound viable gram-positive Staphylococcus aureus and gram-negative Pseudomonas aeruginosa bacterial cells by 98.7 and 98.5%, respectively, when compared with the traditional polyvinyl chloride medical grade tubing material. The results from this study demonstrate NO-releasing albumin plastic’s potential as a material for biomedical device applications.

Keywords

albumin plastic; S-nitroso-N-acetylpenicillamine; nitric oxide; device-associated infections

INTRODUCTION

Biomedical devices such as catheters, wound dressings, and in vivo sensors are frequently used in a hospital setting as a part of common medical practices. While their use is inevitable in medical practices, they can also be a potential cause of nosocomial or hospital-acquired infections (HAIs) because they introduce a route of bacterial infection through contact with blood, fluids, and tissues. Between the years of 1990 and 2002, the Centers for Disease Control and Prevention estimated ~1.7 million HAIs occurred per year in the United States, contributing to 99,000 death.¹ Of the 150 million intravascular devices used annually, 200,000–400,000 result in nosocomial bloodstream infections each year in the U.S alone.²
Not surprisingly, the occurrence of nosocomial infections proportionally increases the time of hospitalization and healthcare cost. In fact, up to $11 billion is spent every year for treatment of HAIs in the United States, while hospital treatment time is increased fourfold as compared to hospitalization without infection. Preventing nosocomial infection is necessary not only to reduce the suffering of the patient but also to cut down huge medical costs.

Traditional polymers, such as polyethylene, polypropylene, polyvinylchloride (PVC), and polyethylene terephthalate, used for biomedical devices lack the physical and chemical properties that could prevent bacterial growth. Colonization of bacteria over the polymer surface can lead to matrix deposition and biofilm formation, which can ultimately lead to infection. Biofilms are bacteria’s defense mechanism that guards them against being scoured or washed away; they render bacteria ineffective to the application of toxins, detergents, and antimicrobial agents.

Since common petroleum-based plastics are susceptible to biofilm formation, alternative materials should be examined for their use in medical applications. In this regard, protein-based plastics have been investigated as promising alternative materials for implant fabrication. Albumin protein from the hen’s egg white contains the enzyme lysozyme, which is of particular interest in medical devices because of its inherent antibacterial nature. With the incorporation of plasticizers, such as water and glycerol, albumin protein plastic has the potential to be utilized in medical applications. When exposed to bacteria (Escherichia coli and Bacillus subtilis), albumin-based plastics reduced growth on their surface. Additives, such as nitric oxide (NO) donors, could be used in tandem with the albumin to enhance its potential effectiveness in medical applications.

Sustained release of NO from an exogenous NO donor may offer a safer but effective method for preventing microbial adhesion on biomedical device surfaces. NO is a cellular signaling molecule naturally secreted by many cells within the human body, including vascular endothelial cells and macrophages. NO plays a crucial role in many physiological and pathological processes, including platelet activation and adhesion, vasodilation, wound healing, and inflammation. Because NO is a natural antimicrobial agent, it has been incorporated into different medical grade polymers to not only improve the hemocompatibility but also prevent infection in blood-contacting devices such as sensors, catheters, vascular grafts, extracorporeal circuits and in medical device coatings. Moreover, NO releasing polymers have not been reported to cause any hemocompatibility, biocompatibility, or long-term storage stability issues.

In future, researchers could take polymers incorporated with a NO donor a step further by seeking to improve the antimicrobial activity of plastics. Albumin protein plastics, given their antibacterial properties, provide a promising platform to produce biomaterials that can resist bacterial adhesion and protein adsorption. Combining albumin plastics with an NO donor such as S-nitroso-N-acetyl-penicillamine (SNAP) could be a synergistic approach to preventing infection. In this study, the incorporation of SNAP into an albumin-based bioplastic matrix is done via a swelling method. The thermal and mechanical properties
and antibacterial potential of albumin-SNAP plastic are studied to propose it as a promising material for biomedical applications.

**MATERIALS AND METHODS**

Albumin (purity ≥99%) was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). N-Acetyl-d-penicillamine (NAP), ethylenediaminetetraacetic acid (EDTA), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO). Methanol, hydrochloric acid, and sulfuric acid were obtained from Fisher Scientific (Pittsburgh, PA). Tygon™ PVC #3603 was obtained from Fisher Scientific. Phosphate buffer saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, was used for all in vitro experiments wherever applicable. The bacterial strains *Pseudomonas aeruginosa* (American Type Culture Collection, ATCC, 27853) and *Staphylococcus aureus* (ATCC 6538) were used for the microbicidal characterization.

**Preparation of compression molded samples**

Compression molding of albumin plastic was performed on a 24-ton bench-top press (Carver Model 3850, Wabash, IN). Seventy-five percent albumin (w/w) was mixed with deionized water, filled into stainless steel molds, and then compressed between the press’s electrically heated and water-cooled platens (Fig. 1). Data presented in this study was generated from albumin plastic samples, compression molded for a 5-min heating time at 120°C, followed by a 10-min cooling period for setting the structure. Plastic samples were produced under a pressure of at least 40 MPa since a certain minimum amount of pressure must be applied to the protein for it to become a plastic. After cooling, the pressure was released and the samples were removed. The samples were prepared in batches of <6 g for albumin dog bone plastics and 2 g for DMA flex bars. For this study, albumin-water plastics were compared with albumin-water plastics loaded with SNAP (albumin-SNAP). The process for incorporating SNAP into albumin-water plastics is detailed below. The samples were then placed in a conditioning chamber for at least 24 h, and the conditioning chamber was set to 70°F (21.1°C) and 65% relative humidity.

**SNAP synthesis**

SNAP was synthesized following the method of Chipinda and Simoyi. Equimolar ratios of sodium nitrite and NAP were added to a 1:1 mixture of water and methanol containing 2M HCl and 2M H₂SO₄. After 30 min of stirring, the reaction vessel was cooled in an ice bath to precipitate the SNAP crystals. The crystals were collected by filtration, washed with water, and allowed to air dry. The reaction and crystals were protected from light at all times.

**Incorporating SNAP into albumin plastic**

Albumin-water plastic was submerged in methanol that contained 100 mg/mL of dissolved SNAP. The selected concentration was near the solubility of SNAP in methanol, providing an upper limit of NO-release potential from the albumin plastic. When placed in methanol bath, the albumin plastic was found to increase in mass by 20% (data not shown). The albumin plastic was allowed to swell in the SNAP-methanol solution for 24 hours at room
temperature. After 24 hours, the albumin plastic was removed from the SNAP-methanol solution, rinsed with methanol to remove excess SNAP, and dried at room temperature under vacuum. During the drying process, the plastic returned to its original dimensions. After loading SNAP into the albumin plastic, the samples were stored at −4°C before use to prevent NO release. The samples were protected from light (due to the sensitivity of SNAP to light) by placing them in containers wrapped in aluminum foil. Albumin-water plastic was also swelled with methanol for 24 hours and dried at room temperature to study effects of methanol on albumin-water plastic.

**Thermal analysis of samples**

Thermal Gravimetric Analysis (TGA) was performed using a Mettler Toledo TGA/SDTA851e. Samples were tested at 25–500°C under a N\(_2\) atmosphere with a heating rate of 10°C min\(^{-1}\). Differential Scanning Calorimetry (DSC) was performed using a Mettler Toledo DSC821e, with materials examined from −20 to 250°C under a N\(_2\) atmosphere with a heating rate of 10°C min\(^{-1}\). For all sample testing, each sample was cut and tested at a weight of 2.0–4.0 mg to ensure consistent results.

**Dynamic mechanical analysis (DMA)**

The DMA flex bars of the albumin plastics were analyzed for their viscoelastic properties through the use of DMA by using a DMA 8000 Dynamic Mechanical Analyzer from PerkinElmer.\(^{33}\) DMA experiments were conducted from 25 to 120°C, with a temperature ramp of 2°C min\(^{-1}\). The settings of the analyzer were set to dimensions of 9 × 2.5 × 12.5 mm\(^3\) using a dual-cantilever setup at a frequency of 1 Hz with a displacement of 0.05 mm. Each sample type was analyzed in duplicate.

**NO release measurements**

NO released from the albumin-SNAP plastic was measured using a Sievers Chemiluminescence NO Analyzer (NOA) 280 (Boulder, CO) for 3 h continuously. This method is a gold standard for the measurement of NO.\(^{17,34}\) The detection of NO is achieved through the reaction of NO with ozone to form excited NO\(_2\) in a localized reaction chamber. As the excited nitrates go back to their ground state, photons are released and captured by the photomultiplier tube and are then converted into molar release rates via a calibration curve. This approach allows for high selectivity to NO, where other methods can be influenced by nitrates and nitrites present. Samples were placed in the sample vessel immersed in PBS (pH 7.4) containing 100 mM EDTA. NO was continuously purged from the buffer and swept from the headspace using an N\(_2\) sweep gas and bubbles into the chemiluminescence detection chamber. Films were submerged in PBS with EDTA and maintained at 37°C for the duration of the measurement. The surface area of the albumin-SNAP plastic samples was used to normalize NO release between samples. LOWESS smoothing was applied to the data with a span of 0.01.

**Morphological properties of albumin, albumin-SNAP, and PVC plastics**

The surface morphology of albumin, and albumin-SNAP, was evaluated using optical microscopy and atomic force microscopy. Samples were viewed using an Olympus BX50
optical microscope with a camera at 20× magnification. Samples were also measured by a Bruker atomic force microscope (Dimension Icon or Multimode) with ScanAsyst at 40 μm scan size.

**Gram-positive and gram-negative viable bacteria adhesion studies**

The bacterial adhesion assay developed by Torres et al. was used to compare the antimicrobial properties of commercially available Tygon PVC plastic, albumin plastic, and NO releasing albumin-SNAP plastic. Viable colony counting of adhered bacteria was tested using two bacterial strains, gram-negative *P. aeruginosa* and the gram-positive *S. aureus*. Each strain was grown individually at 37°C overnight in LB broth medium. The optical density of bacterial culture was measured at the wavelength of 600 nm (O.D\(_{600}\)) using UV-VIS spectrophotometer (Genesis 10 S, Thermo Scientific). The culture was centrifuged at 2500 rpm for 10 min; the supernatant was discarded and cells were resuspended in a fresh PBS. To remove traces of the medium, the resulting solution was centrifuged again at 2500 rpm for 10 min. The supernatant was discarded, and fresh PBS was added to resuspend the cells for further use in the bacterial adhesion study. The O.D\(_{600}\) of the cell suspension in PBS was measured and adjusted to ensure that the colony forming units (CFUs) per mL of PBS (CFU/mL) is in the range of 10\(^6\)–10\(^8\). To verify the concentration of viable cells between experiments, serial dilutions of both bacterial strains were prepared and plated on LB agar Petri dishes. Per each strain, triplicates of all samples (PVC, albumin, and albumin-SNAP) were surface sterilized with 70% ethanol and UV-treated for 15 min. Each sample was placed in a 15 mL tube and exposed to 2 mL of the bacterial suspension (CFU: 10\(^6\)–10\(^9\)) at 37°C for 3 h at rocking speed of 24 rpm. After three hours, the samples were removed from the bacterial suspension and rinsed with fresh PBS to remove any unbound/loosely bound bacterial cells. Afterward, each sample was transferred to a new tube containing fresh 2 mL PBS and sonicated for 45 s to detach the bound bacteria from the plastic. The resulting solution, which has bacterial cells was then vortexed for 20 s, serial dilutions were prepared, and cells were plated on LB agar and incubated overnight at 37°C. The CFUs were visually counted and CFUs per square centimeter of the test sample (CFU/cm\(^2\)) was calculated.

**RESULTS AND DISCUSSION**

**Thermal properties of albumin-based plastics**

For all albumin-based plastics, the initial degradation peak between 220 and 230°C indicates bound moisture loss within the protein (Fig. 2), and the much larger degradation peak at 300–315°C indicates amino acid degradation. When the albumin-SNAP plastic is analyzed further, it appeared to have similar thermal properties to pure albumin powder. The albumin-swell samples exhibited endothermic peaks that were more similar to albumin-water plastics than albumin-SNAP samples. Albumin-SWELL samples do not contain any SNAP, which can explain this difference. Methanol does not appear to drastically change the properties of albumin-water. The thermogravimetric analysis of SNAP showed two degradation peaks, one at 155°C and one at 201°C. These results correspond to a previous study where results showed that when SNAP is subjected to DSC analysis, it decomposed at 148°C while TGA indicated NO loss at 148°C. The TGA of the albumin-SNAP plastic...
gives no indication that either peak is present, probably because the amount of SNAP present within the albumin plastic is too small for TGA instrument to detect. There is a possibility that the peak occurring between 200 and 225°C is a result of an overlap of SNAP degradation peak around 200°C and the bound water loss peak at 220 and 230°C.

When analyzed using DSC, significant endothermic peak at 75°C for pure albumin powder can be witnessed, but more broad peaks for albumin-water plastic. Albumin-SNAP demonstrated a similar endothermic peak (75°C) as pure albumin. The similar trends of thermal degradation were observed in the albumin-swell samples as albumin-SNAP samples. While the albumin-swell plastics exhibit a lower magnitude peak around 230–240°C, they will also exhibit a broader degradation peak around 275–350°C. Soaking the albumin-water samples in methanol does not appear to drastically change its properties.

On comparing the pure protein powder to the bioplastics using DSC (Fig. 3), the protein powder degraded at a lower temperature (210–215°C) than the protein bioplastics (220–225°C). This increase in degradation temperature could be caused by the more thermally stable structure formation in the bioplastics because of heat, pressure, and cooling conditions during processing. Adding water increased the molecular interactions within protein bioplastic.

Viscoelastic properties of albumin-based plastics

DMA was performed on the plastic samples to determine their viscoelastic properties (Fig. 4). With this analysis, albumin plastic containing no plasticizer results in a brittle material that exhibits high initial modulus (3.479 E9 Pa) and low initial tan delta (0.057). In contrast, when plasticized with water the resulting albumin-water plastic is more flexible as evidenced by a lower initial modulus (4.128 E8 Pa) and a higher initial tan delta value (0.204). The addition of plasticizer into the protein matrix forms a more heterogeneous plastic since the water embeds itself between protein chains. The DMA results showed that the albumin-water plastics containing SNAP display viscoelastic properties that are more similar to pure albumin protein plastics than plasticized albumin-water plastics. Therefore, the albumin-SNAP plastics will be more brittle than albumin-water plastics, with high initial modulus (1.532 E9 Pa) and low initial tan δ value (0.081). When SNAP is added to albumin-water via methanol, albumin-SNAP becomes stiffer than albumin-water, possibly due to the alcohol-water exchange with bound water being displaced.

Morphological properties of albumin, albumin-SNAP, and PVC plastics

The surface of a material plays a crucial role in affecting the degree of its interaction with blood proteins and bacteria. Therefore, it is important to assure that the antibacterial material added to the material does not alter its surface properties. The morphologies and surface roughness of the albumin plastic was compared before and after addition of SNAP. The optical imaging of the albumin and albumin SNAP material at 20x resolution showed a similarity in surface morphologies [Fig. 5(a,b)]. This can be attributed to their similarities in processing conditions. To further validate it, sample roughness was observed under Atomic Force Microscope. Similar to optical images, no significant difference was observed between albumin and albumin-SNAP plastic [Fig. 6(a,b)]. These observations
provide supportive evidence that solvent swelling of SNAP into albumin plastic does not deteriorate its surface morphology. This is in agreement with other reports where the addition of SNAP into biomedical grade polymers have been shown to maintain the surface properties such as contact angle and smoothness.

**Antibacterial potential of NO-releasing albumin-SNAP plastic**

The NO release from samples of albumin-SNAP plastic was measured under physiological conditions (in PBS buffer at 37°C) using a chemiluminescence NO analyzer. Albumin-SNAP plastic samples showed a burst of NO release initially, followed by a sustained release. The sustained release rate of NO release during this 3 h period was $18.5 \pm 3.9 \times 10^{-10}$ mol min$^{-1}$ cm$^{-2}$. A representative release profile of NO from the albumin plastic is shown in Figure 7.

A bacterial adhesion study was conducted to test the ability of albumin-SNAP plastic to prevent the adhesion of bacteria on its surface as compared to albumin-water plastic and traditionally used PVC plastic. Albumin from hen egg white has been found in a previous study to prevent bacterial growth. For both gram-positive and gram-negative bacteria, our ANOVA (Analysis of Variance) concludes that all the three factors (PVC, albumin-water, and albumin-SNAP) are not the same. The multiple comparisons conclusively prove that each pair is different at 99% level of confidence.

Albumin-water plastic by itself (without SNAP) significantly reduced the number of adhered viable *S. aureus* cells by 86.6% when compared to commercially available PVC, as shown in Figure 8. It also significantly inhibited *P. aeruginosa* by 80.2% when compared with PVC (Figure 9). SNAP was incorporated into the albumin-water plastic to increase albumin’s ability to inhibit bacteria by releasing NO. NO’s antibacterial properties are due to its ability to cause protein denaturation, DNA breakage and lipid peroxidation in bacterial cells. Adding SNAP to albumin-water plastic reduced the adhered viable bacteria by 90.2 and 92.6% for *S. aureus* and *P. aeruginosa*, respectively when compared to albumin plastic alone. However, the NO releasing albumin plastic, possessing an average NO flux of $18.5 \pm 3.9 \times 10^{-10}$ mol min$^{-1}$ cm$^{-2}$, resulted in a statistically significant decrease in *S. aureus* and *P. aeruginosa* bacterial adhesion as compared with traditionally used PVC plastic. At the reported NO flux, the adhesion of *S. aureus* was reduced by 98.7% and *P. aeruginosa* by 98.5% (Figs. 8 and 9). The results of this study are in agreement with a previous study, which showed NO’s bactericidal effect against gram-positive and gram-negative membrane via slow and sustained release from natural polymer membrane.

If albumin-SNAP plastic can prevent the adhesion of bacteria on its surface, it might also be able to prevent biofilm formation and resulting infections, especially in situations where complications due to infection from biomedical device implantation or surgery are foreseeable. The NO releasing strategy is not supposed to cause the development of bacterial resistance strain due to its rapid and non-specific killing mechanism load. It has also been shown that the NO donor, SNAP works equally well in combination with other antibacterial material such as quaternary ammonium ions which thus expands its application. The results from this study suggest that imparting NO releasing attribute to albumin plastics could expand their utility in biomedical applications.
CONCLUSIONS

Infections resulting from microbial biofilm formation remain a serious threat to patients worldwide. The use of albumin-SNAP plastic presents an opportunity to prevent the spread of diseases in hospitals from biomedical devices since this material has been found to prevent bacterial growth on its surface. In the present study, SNAP molecules (NO donor) were successfully integrated into the albumin plastic through swelling in methanol. The addition of SNAP does not significantly affect the properties of the material since the thermal and mechanical properties of the material were similar to albumin materials without SNAP. The release of NO from the albumin plastic was measured and showed a sustained release of $18.5 \pm 3.9 \times 10^{-10} \text{mol min}^{-1} \text{cm}^{-2}$ after 3 h. The sustained release of NO complemented the antibacterial albumin bioplastic and ultimately resulted in inhibiting the growth of both gram-positive *S. aureus* and gram-negative *P. aeruginosa* on a plastic surface. Thus, the incorporation of NO donor in albumin plastic offers a great potential for the utilization and development of a class of biomedical devices that could prevent biofilm formation and nosocomial infections, and reduce associated treatment cost. NO-releasing albumin plastics may offer a new paradigm for addressing the infection caused by biofilm deposition over biomedical devices surfaces, such as catheters, stents, sutures, and *in vivo* sensors.

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FIGURE 1.
Compression molding schematics of plastic samples. The mold was inserted into the mold and then covered with the Plunger. The mold was then inserted in the Compression Molder. Finally, 40 MPa pressure was applied for 5 min at 250°F followed by cooling for 10 min at 40 MPa.
FIGURE 2.
Thermogravimetric analysis of albumin-based plastics.
FIGURE 3.
DSC of albumin-based plastics.
FIGURE 4.
DMA of albumin-based plastics to measure its viscoelastic properties.
FIGURE 5.
Optical Microscopy Images (20×) of (a) albumin and (b) albumin- SNAP. Scale bar = 200 μm.
FIGURE 6.
Atomic Force Microscopy images of (a) albumin and (b) albumin-SNAP. Scale bar = 20 μm.
FIGURE 7.
Real-time NO release from albumin-SNAP plastic at physiological conditions (pH 7.4, 37°C) using chemiluminescence.
FIGURE 8.
Graphical representation showing a reduction in the viable CFU of *S. aureus* using commercially available PVC as the reference. Albumin is significantly different than PVC ($p < 0.01$) and albumin is significantly different than albumin-SNAP ($p < 0.01$).
FIGURE 9.
Graphical representation showing a reduction in the viable CFU of *P. aeruginosa* using commercially available PVC as the reference. Albumin is significantly different than PVC ($p < 0.01$) and albumin is significantly different than albumin-SNAP ($p < 0.01$).