Nanoengineered Eggshell—Silver Tailored Copolyester Polymer Blend Film with Antimicrobial Properties

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ABSTRACT: In this study, the reinforcement effect of different proportions of eggshell/silver (ES-Ag) nanomaterial on the structural and antimicrobial properties of 70/30 poly(butylene-co-adipate terephthalate)/polylactic acid (PBAT/PLA) immiscible blends was investigated. The ES-Ag was synthesized using a single step ball milling process and characterized with X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), and transmission electron microscopy (TEM). These results confirmed the existence of silver nanoparticles (Ag NPs) in the interstitial spaces of the eggshell particles. The thin films in this study were prepared using hot melt extrusion and 3D printing for mechanical and antimicrobial testing, respectively. These films were also characterized by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), XRD, tensile testing, and antimicrobial analysis. It was found that the incorporation of ES-Ag (0.5–2.0% content) compromised the tensile properties of the blend, due to poor interaction between the matrix and the ES-Ag in the ternary systems, but thermal analysis revealed improvement in the onset of degradation temperature and char yield at 500 °C. Though film toughness was better than that of PLA, the strength was lower, yet synergistic to those of PBAT and PLA. In general, the PBAT/PLA/ES-Ag ternary system had properties intermediate to those of the pure polymers. In vitro assessment of the antimicrobial activity of these films conducted on Listeria monocytogenes and Salmonella Enteritidis bacteria revealed that the blend composite films possessed bacteriostatic effects, due to the immobilized ES-Ag nanomaterials in the blend matrix. Atomic absorption spectroscopy (AAS) analysis of water and food samples exposed to the films showed that Ag NPs were not released in distilled water and chicken breast after 72 and 168 h, respectively.

KEYWORDS: antimicrobial films, flexible polymer blends, biocide, 3D-printing, extrusion

INTRODUCTION

In recent years, consumer interest in high quality and safe food products, coupled with environmental concerns, drives the development and study of antimicrobial biodegradable coatings, fillers, and films. Edible coatings, obtained from generally recognized as safe (GRAS) materials, can potentially improve food freshness, appearance, and integrity. They can be used in combination with other food preservation techniques to enhance the effectiveness of the food preservation chain. Antimicrobial films and coatings have emerged as new concepts of active packaging and have been developed to reduce, inhibit, or delay the growth of microorganisms on the surface of food in contact with the package. The use of antimicrobial packaging films to control the growth of microorganisms in food has an impact on shelf life extension and food safety.

Many organic antimicrobial agents are available and can impart antimicrobial activity in active packaging materials. Examples are chitosan, nisin, rhodamine, proallium, and cinnamaldehyde. Also, some inorganic nanomaterials incorporated in polymers are able to alter their microstructures to improve mechanical properties while adding antimicrobial benefits to the polymer systems.

The barrier to oxygen is critical in food packaging materials, due to the high sensitivity of many food products to oxidative degradation, moisture dependent microbial growth, and the need for aroma retention. The improvement in barrier properties to gases, vapors, and aromas in biopolymers makes them attractive for food protection. Hence, antioxidant nanobiocomposites based on polycaprolactone (PCL) have been prepared by incorporating hydroxytyrosol (HT) (natural antioxidant) and nanoclay (cloisite 30B (C30B)) to help improve the poor PCL intrinsic barrier properties. A significant improvement in oxygen barrier was realized in ternary nanobiocomposites containing C30B and 10 wt % HT. In contrast, the generation of singlet oxygen by fullerene revealed some antimicrobial potential in packaging materials. Blends of Sc3N@Ih-C80 metallic nitride fullerene (MNF) and C60 fullerene with polystyrene-block-polysoprene-block-polystyrene (SIS) were evaluated for antimicrobial activity on Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli). The films provided 1–2 log kill of both bacteria, with C60 blend film showing more antimicrobial activity than Sc3N@Ih-C80.

Antimicrobial activity in this material attributed to the in situ generation of reactive oxygen species (ROS) under white light induces oxidative damage on the cell membranes of bacteria.

Silver is a known antimicrobial agent. However, this antimicrobial activity can vary based on the interactions...
between silver and its host materials. Hence, establishing the antimicrobial activity of silver-based packaging materials is very important. One study investigated the antimicrobial activity of silver (Ag) in a film by incorporating 0.001–10 wt % Ag ions into an ethylene-vinyl alcohol (EVOH) copolymer matrix. The antibacterial activity of these films assessed on contact with apple peels and chicken breast showed low inhibition on chicken breast (<1 log reduction), while on the apple peels, apple peels and chicken breast showed low inhibition on (ATCC), Manassas, VA, USA.

Synthesis of Eggshell/Ag Nanomaterial. The eggshell/Ag (ES-Ag) nanomaterial was synthesized by ball milling 10.5 wt%/wt% of eggshell and silver nitrate (AgNO3) in 10 mL of 1:2 v/v distilled water/ethanol. The water/ethanol (5 mL) was added to each canister containing the precursors and mixed with a spatula for 5 min. About 0.2 mL of 0.2 M aqueous solution of potassium hydroxide (KOH) was dispersed dropwise into each canister while mixing for another 2 min. About 5 mL of deflocculating agent (PPG) and 8 pieces of 6 mm steel balls were added to each canister and ball milled for a minimum of 5 h. This was washed with 25 mL of ethanol (for at least 4 times), centrifuged at 1500 rpm for 10 min at 5 °C, and decanted after each wash before drying the final product in a vacuum environment for at least 8 h.

Extrusion of Polymer Blends. About 150 g of each of the PBAT/PLA 70/30 precipitated blends containing (0.5–2.0%) the dispersed ES-Ag was dried for 12 h at 60 °C in a hopper (DRI-AIR Industries Inc., model RHS). This was then fed into a 19 mm (diameter) tabletop single screw extruder (Wayne SN: 8001) which is driven by a 2 hp motor. Thermostat controlled five heating zones and screw rotational speed facilitated the melting, mixing, and formation of a continuous viscous melt of the extrudate. Three heating zones are located in the barrel while two are in the die zone. The optimum working temperatures were maintained at 160, 160, 157, and 156 °C, for the barrel and die zones, respectively. About 40 mm (w) × 0.3 mm (t) blend composite specimens were obtained at a screw speed of 20 rpm and a feed rate of 4.4 g/min and collected at the die orifice. The PD-mini extrusion die (SN # 13-33386), which is 6-in. deep, 3-in. tall, and 6.5-in. wide with a 4-in. lip opening, was purchased from Premier Die Corp. Chippewa Falls, WI. The continuous hot molten films were passed through water stationed at the orifice of the die for quenching. These blends were stored in a high vacuum desiccator (JEOL, EMDSC-U10A) and only removed during characterization.

Three Dimensional (3D) Printing of Polymer Blends. Three dimensional (3D) printing of the polymer blend composites became necessary for the fabrication of thinner films to avoid the physical inhibitory effect encountered with the hot melt extruded films. Hence, 3D printed films were only used for antimicrobial studies. About 50 g of each blend was dissolved in 100 mL of CHCl3 and the desired amount of each antimicrobial agent was added and magnetically dispersed in solution for at least 12 h to make 0.5, 1.0, 1.5, and 2.0 wt % content of the ES-Ag in each blend. A g-code was created to print a set of five (5) films with 200 mm (l) × 25 mm (w) × 100 μm (t) in each run. About 25 mL of the blended solution was drawn into a 30 mL metallic syringe tool and loaded onto the motorized printer head compartment. Using Repetil software, the films were printed at a feed rate of 6000 rpm on a flat clean glass plate mounted on the motorized stage at room temperature with a Hyrel 30 M printer. The printed films were removed 15 min after completion and further dried at room temperature before storing in sterile plastic zip-lock bags for antimicrobial testing.

X-ray Diffraction (XRD). XRD analysis was performed on all specimens, using a Rigaku diffractometer (DMAX 2100) equipped with Cu Kα radiation, operated at a step size of 0.02°, scan rate of 1°/min, 3° to 80° of two theta Bragg’s angle of diffraction, 40 kV, and 30 mA.

Transmission Electron Microscopy (TEM). A JEOL 2010 high resolution TEM was used to determine the particle size and morphologies of the prepared ES-Ag NPs. One milligram (mg) of the ES-Ag specimen was dispersed in 5 mL of CH3CH2OH for 10 min at a constant temperature for 2.0 min to erase previous thermal history. It was then centrifuged at 1500 rpm for 10 min at 5 °C, and decanted after each wash before drying the final product in a vacuum environment for at least 8 h.
was again cooled from 200 °C to −40 °C at 20 °C/min before finally scanning at 5 °C/min from −40 to 200 °C to determine the various heat transitions in each specimen.

Thermogravimetric Analysis (TGA). TGA was carried out with TA Q 500 equipment. Samples of 14 ± 0.2 mg were placed in platinum pans. An empty platinum pan was used as a reference. Each sample was heated from 30 to 600 °C in a 50 mL/min flow of N2. A heating rate of 5 °C/min was used, and the continuous records of sample temperature and mass were taken.

Tensile Testing. Measurement of tensile properties was done using a Zwick Roell Z2.O mechanical testing system in accordance with ASTM D 882 using a crosshead speed of 500 mm/min and 2 kN load cells and wedge grips. Specimens were cut from the extruded sheets of polymer systems to 19 mm × 0.3 mm × 120 mm dimensions. The test was conducted at 20 mm gauge length with TestXpert data acquisition and analysis software. At least 15 specimens were tested and averaged into the reported mechanical properties.

Scanning Electron Microscopy (SEM). Microstructure and blend morphologies were probed using JEOL JSM-5800 SEM. Film samples were cut and placed on a carbon tape on the 4-in. wide sample holder of the SEM. This was then sputter coated with gold palladium for 5 s, operated at 20 mTorr, 5 V, and 15 milliamps. Crossed sections of fractured surfaces of failed specimens from the tensile test were examined using Hitachi S-3400N SEM. Fractured surfaces of failed specimens from the tensile test were examined using Hitachi S-3400N SEM. The SEM was operated at 20,000× magnification with an accelerating voltage of 15 keV. The SEM images were post-processed using Adobe Photoshop (Adobe Systems, San Jose, California) for the quantification of the blend morphology.

Statistical Analysis. The statistical significance of differences in thermal properties was determined with a one-way analysis of variance (ANOVA) and Tukey’s multiple-comparison tests. In all cases, a value of p < 0.05 was considered to be significant.

Film Preparation for Antimicrobial Study. The extruded and 3D printed films were cut (2 × 2 cm²) with sterile scissors and exposed to UV light for about 30 min in a PCR Workstation (Air Clean 600, Serial # 42991). These were then stored in sterile resealable bags (Nasco WHIRL-PAK) for later use in antimicrobial testing. Extensive antimicrobial testing was done with 3D printed films, since pilot tests with the extruded thick (300 μm) films suggested physical inhibitory effects of the neat films on bacterial growth through interference with nutrient exchange. Hence, we resorted to 100 μm thick specimens obtained by 3D printing for the antimicrobial studies.

Antimicrobial Testing. Mueller Hinton Agar (MHA), Tryptic Soy Agar (TSA), and Tryptic Soy Broth (TSB) were used in the antimicrobial study. The antimicrobial activities of the films were tested qualitatively by direct placement of the films on spots of known concentrations of bacteria. Two organisms, S. Enteritidis and L. monocytogenes, were chosen based on common prevalence of these organisms as foodborne pathogens, their ability to survive and multiply in most food storage temperatures (± 4 °C), and finally as representative pathogens of Gram-negative and Gram-positive organisms, respectively. Tests were done in triplicates for these experiments on S. Enteritidis, incubated at 37 °C, and L. monocytogenes, incubated at 30 °C, each for 24 h. In order to assess the antimicrobial activities of the films, bacterial isolates were streaked onto TSA plates and incubated at their appropriate temperatures to obtain single colonies. About 3 to 5 isolated colonies were transferred from each plate into 1.5 mL sterile tubes containing 1 mL of TSB using sterile disposable inoculating loops. The bacterial culture was vortexed and incubated at 37 °C on a shaker at 350 rpm for 2–4 h. Sterile TSB was used to adjust the turbidity of L. monocytogenes and S. Enteritidis suspensions to obtain approximately equal optical density (OD) to that of 0.5 McFarland Standard (~1–2.0 × 10² CFU/mL of E. coli ATCC 25922). The OD of each bacterial suspension was measured at 600 nm (OD₆₀₀) using a UV–vis spectrophotometer (Nanodrop 2000c, Wilmington, DE, USA). The starting bacterial concentrations measured at OD₆₀₀ ranged between 0.098 and 0.112 for both S. Enteritidis and L. monocytogenes.

Antimicrobial activity of the films (bacteriostatic or bactericidal) was tested on different inoculated agar surfaces. Approximately 100 μL of the cell suspensions containing the stock (full OD₆₀₀ nm) and 10⁵ CFU/mL were dispensed at the center of the agar plates and allowed to air-dry under a biosafety hood. Films with dimensions of about 2 × 2 cm² were then placed directly on the surface of agar where the inoculum was added and incubated at their appropriate temperatures. After 24 h, films were carefully removed with sterile forceps and discarded and the plates reincubated for another 24 h. All plates were visually inspected for any colony growth after 24 h of incubation. Control plates were inoculated with sterile TSB, and growth control plates were inoculated with each of the two bacterial species.

Sample Preparation for Silver Release Study. A silver release study was conducted on films treated with distilled water (DW) and fresh chicken breast (CB) samples. About 0.4 g of the 0.5% and 2% ES-Ag incorporated PBAT/PLA 100 μm thick films were immersed in 15 mL of DW contained in 25 mL storage bottles. The specimens were stored at 4 and 50 °C and the films removed at 24 and 72 h and the water analyzed for any released silver. A release study on CB was conducted by wrapping ~1.0 g of the CB with 2 × 2 cm² of the PBAT/PLA films with 0.5% and 2% content of ES-Ag and storing the specimens at 4 °C for 24 h, 72 h, and 164 h. Each sample was removed from the refrigerator after the stipulated time and processed by removing the film and dissolving two CB samples (2 g) in a 50 mL tube using ATL tissue lysing buffer and proteinase K enzyme solution. About 360 μL of ATL buffer was added to the tube containing the CB and vortexed (Phenix Benchmix 1000) for 30 s. Approximately 10 μL of the proteinase K solution was added and vortexed for another 30 s before incubating in a water bath (ISOTEMP 220, Fisher Scientific) at 56 °C for at least 4 h. After complete lysis of the CB, 15 mL of DW was added, vortexed for 60 s, and filtered with Whatman 110 mm paper into a 25 mL storage bottle. Control CB sample was prepared following the same procedure except wrapping with the film. The
amount of silver in each film was determined by cutting into smaller pieces 30 g of the film sample and subsequent ball milling in 15 mL of DW using two 12.7 mm zirconia ceramic balls and 45 mL grinding vials for 1 h in a high energy ball mill (Spex Sample Prep 8000D). The slurry was allowed to stay for 24 h before filtering into 25 mL storage bottles. Also, in determining the amount of silver in the synthesized ES-Ag, 3 g of ES-Ag was mixed with 15 mL of DW and filtered after 24 h. Nitric acid was added to each sample to a concentration of 0.1 M before performing silver analysis using atomic absorption spectrosco-
py.

Atomic Absorption Spectroscopy (AAS). The concentration of silver in the release study was determined using a Varian AAS 240. Silver standards (0, 2.5, 4, 6, 8, and 10 ppm) were prepared from acidified (2% HNO₃) 1000 mg/L Ag AAS standard solution and run as calibration standards before the specimens were analyzed. A calibration curve was plotted with data from the standards and used to determine the silver concentration in each sample. The AAS was set to detect silver at a wavelength of 328.1 nm, slit width of 0.5 nm, and lamp current of 4.0 mA, using an air−acetylene flame. The equipment had a minimum detection limit of 0.02 ppm.

■ RESULTS AND DISCUSSION

XRD, XPS, and TEM Structural Analysis. Figure 1 shows the X-ray diffraction patterns of eggshell (a), the synthesized ES-Ag NPs (b), and pristine Ag NPs (c). The nature of the diffraction peaks suggests that the inorganic materials are highly crystalline with small crystal sizes due to the broad nature of the peaks, as observed in past studies.¹⁹ The diffraction pattern for the engineered ES-Ag NPs (b) shows that the material is highly crystalline and reveals peaks due to both eggshell and silver, when compared to the patterns in (a) and (c) for eggshell and silver, respectively. The characteristic peaks of eggshell appear at 2θ° = 23.0, 29.4, 31.5, 36.0, 39.4, 43.2, 48.5, 56.6, 57.4, and 60.8, corresponding to the kkl crystal planes of (012), (104), (110), (113), (202), (016), (018), and (222) for calcite standard with JCPDS Ppdf # 47-1743.

Also, the distinct peaks of Ag NPs appear at 2θ° = 38.1, 44.2, 64.4, and 77.3, corresponding to the (111), (200), (220), and (311) crystal planes of standard silver with JCPDS Ppdf # 040783. This confirmed the reduction of AgNO₃ to Ag NPs during the synthesis process.

The composition and chemical states of the ES-Ag nanomaterial are shown by the XPS spectrum in Figure 2a. This revealed that the material consists of Ca, O, C, N, and Ag. The doublet at 363−379 eV is due to Ag 3d binding of metallic silver. This has been associated with Ag 3d3/2 and Ag 3d5/2 energies, respectively.¹⁹,²² The peaks at 286, 348, 401, and 533 eV are due to the chemical states of C 1s, Ca 2p, O 1s, and N 1s, respectively. C, Ca, and O are the main constituents of eggshell crystals, while the N could be due to inherent proteins in eggshell extracellular matrices.²²

The mass percentage of the elements by XPS analysis revealed that Ag = 9.72%, O = 35.6%, C = 34.99%, Ca = 18.24%, and N = 1.45%. The theoretical yield of Ag from 5 g of AgNO₃ precursor is 3.18 g, the mass of Ag NPs (%) out of 15 g of starting material of AgNO₃ (5 g) and eggshell (10 g) was estimated to be 21.2%, assuming that no change in amount of the eggshell occurred during the synthesis, because of its chemical stability. This yield (21.2%) is more than the 9.72% obtained in the XPS analysis, probably because some Ag NPs may be absorbed in the interstitial space of the porous eggshell crystal beyond the reach of the XPS beam, since the depth of analysis in this technique is only 100 Å maximum.¹⁹ Although this is consistent with our past finding on the yield of Ag NPs, which revealed the theoretical yield of Ag was much more than those quantified by XPS and energy dispersive spectroscopy,¹⁹ it is possible that some of the Ag NPs were washed out of the material during the post-synthesis cleaning of the PPG, hence reducing the concentration of Ag NPs in ES-Ag.

The TEM micrograph in Figure 2b shows dark spheres of 10−15 nm Ag NPs anchored on the eggshell. The micrograph shows that the material is very crystalline and porous, suggesting that it can facilitate interactions with other materials through infiltration. The adsorption of silver into pieces of the eggshell imparts antimicrobial properties to the material and opens avenues for applications in antimicrobial paint...
This also suggests that the PLA remained amorphous during a 20 °C/min cooling rate, as observed by Jiang et al.53 Also, immiscibility in the blend and composites was confirmed by the presence of distinct melting transitions attributed to each polymer, as observed in other studies.52,53 It was evident that high amount of PBAT led to a significant change in the PLA melting peaks, suggesting the possible existence of new crystalline structure induced by PBAT. This double melting peak for PLA is the result of less perfect crystals which had enough time to melt and reorganize into crystals with higher structural perfection to melt at higher temperature.35,36

**Thermogravimetric Analysis.** The thermal stability of PLA, PBAT, and their blend systems is shown in Supporting Information Figure S3 and Table 1. The amount of remaining material during the thermal treatment of these polymer systems determines their thermal stabilities. It can be seen from the plot in Supporting Information Figure S3 that the aliphatic PLA has lower thermal stability than aliphatic–aromatic PBAT, and the thermal stability of the PBAT/PLA 70/30 blend is synergistic to those of PLA and PBAT. The incorporation of ES-Ag in the 70/30 blend led to significant improvement in the thermal stability. The following improvements were observed: onset of degradation, 7–16 °C, \(T_{\text{dmax}}\), 10–14 °C, \(T_{\text{dmax}}\), and residual yield of about 3–4% from the PBAT/PLA 70/30 blend. Similarly, a study of the PVDF/PMMA blend revealed improved \(T_{\text{dmax}}\) and \(T_{50}\) by 10.5 and 30 °C, respectively, due to the addition of 6% crystalline nanocellulose crystals.53 Also, nanoclay led to significant improvement in the thermal stability of the PLA/PHB blend, due to interruption of oxygen permeation by the filler in the blend composite, delaying the degradation of volatile components which otherwise could accelerate combustion. Inorganic fillers have also been found to improve the char yield by about 4% because of their high thermal stabilities.56 This supports our finding from the small amount of ES-Ag material included in the 70/30 blend as depicted in the residual yields in Table 1. The degradation temperature of the PLA (\(T_{\text{dmax}}\)) in the blend was observed to have aggravated due to the inclusion of ES-Ag. This is probably due to the amorphous nature of the PLA domains serving as paths for permeation of volatile combustion products, and since there is no order in the PLA structure, the inclusion of ES-Ag increased the disorder in its domain, accelerating combustion.

**Tensile Analysis.** Tensile analysis of the neat polymers, binary blend, and ternary composite systems is shown in Figure 3. Figure 3a is the full stress–strain curve representing the general mechanical behavior of the polymeric systems under 2.0 kN tensile load, while Figure 3b is the magnified portion of (a) showing the stress strain behavior of the blend composites. The plot (Figure 3) shows that PBAT (1) is extremely tough while PLA (2) is very brittle. Pure PBAT/PLA 70/30 blend (3) showed synergistic improvement in ductility (526%) and...
strength (~7 MPa). The curve (3) shows distinct yielding followed by considerable cold drawing during tensile loading, indicating the transformation of the microstructure to favor ductile fracture in the blend. The incorporation of 0.5−2.0% of the ES-Ag into the matrix of the PBAT/PLA 70/30 blend revealed that the ES-Ag weakened the structure of the blend. Though the ductility of the blend composites was better than that of PLA, both tensile strength and toughness of the ES-Ag modified 70/30 blends reduced substantially, compared to those of the pure PBAT/PLA 70/30 blend. The tensile strength did not differ much between 0.5, 1.0, and 1.5% loading of ES-Ag, as evident in Figure 3b (curves 4, 5, and 6), but at 2.0% ES-Ag loading, the tensile strength (Figure 3b curve 7) increased to nearly equal that of the neat 70/30 blend. Hence, 2.5 and 3.0% loading of ES-Ag were tried to see if tensile properties could be further improved, but these concentrations were too high such that no continuous films were obtained in the hot melt extrusion process. The same concentrations formed agglomerates which blocked the needle orifice in the 3D printing process used as an alternative processing technique for

Figure 3. Tensile analysis of PBAT/PLA/eggshell-silver blend composites: (a) stress vs strain curves, (b) magnified portion of stress vs strain curves in (a).

Figure 4. Antimicrobial effect of PBAT/PLA/eggshell-silver blend composite films: (a1−a3) pristine film against S. Enteritidis, (b1−b3) active films place on agar with tryptic soy broth, (c1−c3) active films against S. Enteritidis (inoculated at low concentration), (d1−d3) active films against S. Enteritidis (inoculated at high concentration), (e1−e3) pristine film against L. monocytogenes, (f1−f3) active films place on agar with tryptic soy broth, (g1−g3) active films against L. monocytogenes (inoculated at low concentration), (h1−h3) active films against L. monocytogenes (inoculated at high concentration).
thinner films, hindering further experiments with films containing ES-Ag percentages higher than 2.0.

This suggests that the ES-Ag could not offset the interfacial chemical distinction causing phase segregation between the two immiscible polymers. This outcome contrasts the finding in past studies where improvements in interfacial properties were reported due to inclusion of different types of nanomaterials and compatibilization in immiscible blends and in composites. This is probably so because ES-Ag does not have the texture and ability to interact well with the phase segregated blend. Particularly, the Ag NPs are free and may have poor compatibility with the blend matrix, leading to compromise in tensile properties.

Fracture Surface Analysis. Fractured surface analysis after tensile tests on the polymer systems is shown in the SEM micrographs in Supporting Information Figure S4. The fractured surfaces of the individual polymers (Figure S4a,b) for PBAT and PLA, respectively, reveal that PBAT was very much resistant to the tensile force before it finally failed, due to the presence of fibers on the fractured surface, which portrays necking. On the other hand, PLA (Figure S4b) showed brittle failure with evidence of smooth morphologies on the fractured surface which are propagated without interruptions.

The microstructure of the binary 70/30 blend presented in Figure S4c reveals a phase segregated morphology which suggests immiscibility of the two polymers. The fractured surface of this blend shows a pull-out of one phase from another at the interface (Figure S4d), indicating poor interfacial interaction between the PBAT and PLA in the binary blend. In this work, we sought to tune the interfacial boundary to minimize the weakness. Hence, eggshell/silver (ES-Ag) nanomaterial was introduced into the blend. In similar studies, other nanomaterials succeeded in tuning the morphologies to improve mechanical properties. Here, the fractured surface of the PBAT/PLA/ES-Ag ternary composite (Figure S4e) revealed an altered morphology on the fractured surface which is tortuous with loose polymer fibrils. This surface actually revealed a shattered “spaghetti-like” structure with holes in between the lose chains. This morphology suggests an obvious weakness in the material, different from other findings where rough morphologies led to toughened blends as in PLA/PEBA, PLA/PBAT, PLLA/PCL, PLA/castor oil, PLA/PCL, and PLA/PHB, because, in such cases, tortuous morphologies favored improvement in mechanical properties. The weakness can be attributed to poor interaction caused by free metallic silver in the ES-Ag nanomaterial embedded in the PBAT/PLA blend.

Antimicrobial Studies. The antimicrobial effects of the 100 μm thick 3D-printed films were evaluated on S. Enteritidis and L. monocytogenes at two bacterial concentrations, at 10^5 CFU/mL and at full optical density (FOD), as in the McFarland Standard. Prescreening of extruded films revealed that films with no additives displayed some physical microbial inhibition, possibly due to the thickness (300 μm) of the films interfering with nutrient and gaseous exchange. This necessitated the fabrication of very thin films (100 μm) with a 3D printer, which did not show the same physical inhibitory effects. The extruder could not fabricate such thin films due to die limitation. The results on antimicrobial study are from the 3D printed films.

The effect on the growth of S. Enteritidis isolates covered with 2 × 2 cm^2 PBAT/PLA/ES-Ag active films is shown in Figure 4. In the control specimen (Figure 4a1), a pristine PBAT/PLA 70/30 blend film was used to cover 100 μL of bacterial concentration (FOD) of the specimen dispensed in the middle of the plate. In another control, 100 μL of tryptic soy broth was dispensed and covered with PBAT/PLA/ES-Ag films with 0.5−2.0% of ES-Ag content as in Figure 4b1. The control films revealed that S. Enteritidis grew in the presence of the neat blend film. This is clearly seen after the film was discarded following 24 h of incubation (Figure 4a2); and the bacterial growth increased significantly during 24 h of incubation following film removal (Figure 4a3). This suggests that the neat blend had no antimicrobial activity. Also, no bacterial growth was evident on the TSB control plates, indicating the specific films used were not contaminated.

When the bioactive films were placed on a dilute concentration, 10^5 (equivalent to 5.9 × 10^6 CFU/mL), and high concentration (FOD) of S. Enteritidis, they prevented the bacteria from growing, as shown in Figure 4c1−c2,d1−d2, respectively. But some growth of the FOD specimens after 24 h incubation suggested that the bacteriostatic effect of the film could be compromised by very high concentrations of the bacteria. Also, the evidence of increased bacterial growth during the extended 24 h incubation after film removal (Figure 4c3,d3) means that the effects of the films are most likely bacteriostatic than bactericidal.

Similarly, the effect on the growth of L. monocytogenes isolates covered with 2 × 2 cm^2 PBAT/PLA/ES-Ag active films is shown in Figure 4e−h. As a negative control, pristine PBAT/PLA 70/30 blend film was used to cover 100 μL of L. monocytogenes concentration (FOD) of the specimen dispensed in the middle of the plate (Figure 4e). A second control of 100 μL of TSB was dispensed and covered with PBAT/PLA/ES-Ag films with 0.5−2.0% of ES-Ag contents as in Figure 4f. Some growth is evident on the two TSB controls (Figure 4f1−f2, 0.5 and 1.5% ES-Ag films) after film removal and 24 h extended incubation; this is probably due to contamination during the film removal, because the TSB control should not show growth. The pristine film control revealed that L. monocytogenes grew under the neat PBAT/PLA 70/30 film. This is clearly seen after the 24 h of incubation (Figure 4e1−e2). The bacterial growth increased markedly during the additional 24 h incubation period following film removal (Figure 4e3). This further suggests that the neat blend had no inhibitory activity, since a similar outcome was obtained with S. Enteritidis. When the bioactive films were placed on a dilute concentration 10^5 (equivalent to 2.34 × 10^7 CFU/mL) and a high concentration (FOD) of L. monocytogenes, they proved effective by significantly inhibiting the bacterial growth, as shown in Figure 4g1−g2,h1−h2, respectively. But evidence of some growth after the 24 h incubation period in Figure 4 panels g1 and h1, suggests that the effects of the films were also bacteriostatic, not bactericidal. The observed bacteriostatic activity instead of bactericidal activity could be due to the Ag NPs being entrapped on the surface of the thin layers of the films.

Thus, these bacteriostatic effects appear to rely on direct contact of films with bacteria. The advantage of such a bacteriostatic property is the ability to inhibit microbial growth on contact while avoiding issues related to contamination due to lacking of toxic antimicrobial agent. In turn, this indicates the potential of these films in addressing the three interrelated stages that lead to contamination of food by components of plastic packaging systems such as diffusion occurring within the polymer, solvation of the migrant at the food and polymer
interface, and the dispersion of the migrant into the bulk of the food product.46

Comparatively, the bacteriostatic effects appear to be stronger on *L. monocytogenes* than in *S. Enteritidis* in which growth was more evident in Figures 4, panels d1 and h2, respectively, when the films were removed after 24 h of incubation. Bacteriostatic effects were not concentration dependent, probably due to immobilized Ag in the blend matrix or to a trace amount of silver which may have been released.

An investigation of the antimicrobial activity of an ethylene—vinyl alcohol film fabricated with 0.001–10 wt % Ag showed that the minimal inhibitory concentration of Ag ranged between 0.01 and 0.1 ppm. Furthermore, the inhibitory effect on high proteinaceous food samples was low (<1 log reduction), while growth in low protein foods was completely inhibited by films with 1 and 10 wt % Ag.47,48 Also, the Ag-NPs/starch and Ag-NPs/nanoclay/starch composites showed inhibition in the film contact areas at 0.3–1.0 mM concentrations of silver on *S. aureus* and *E. coli*, due to interactions between Ag+ and the mercapto groups of the bacterial protein. It was concluded that the nanocomposite films with C30B and Ag NPs led to a microbistatic effect against all the tested bacteria.48 This is similar to our findings with PBAT/PLA/ES-Ag films on *L. monocytogenes* and *S. Enteritidis*. Also, other inorganic NPs have shown the potential of imparting antimicrobial activity to polymer films. A study on the antimicrobial activity of 2%, 4%, and 8% TiO2 NPs incorporated in chitosan/poly(vinyl alcohol) revealed significant antimicrobial activity against *S. aureus*, *P. aeruginosa*, *E. coli* bacteria and yeast, *C. albicans*, in a soft white cheese specimen.49 Similarly, LDPE packaging films hosting 1% CuO NPs reduced the coliform counts of ultrafiltrated cheese by 4-fold after 4 weeks compared to reduction by pure LDPE films.46 The mechanism of antibacterial activity of TiO2 has been attributed to photocatalytic effects of reactive oxygen species (ROS) and oxidative stress under nonphotocatalytic conditions.48

**Silver Release Studies.** Table 2 shows the AAS results for the Ag NPs release study. These results were calculated from the equation of the calibration curve presented in Figure S5 in the Supporting Information. The AAS revealed that 0.2 g/mL of the synthesized ES-Ag NPs contained 33.92 ppm of Ag, suggesting that 0.5 and 2 g ideally contains 82.38 and 329.50 ppm Ag, respectively, as the baseline concentrations in 50 g of the polymer blends. Also, 2 g/mL of PBAT/PLA/ES-Ag 70/30/0.5 and PBAT/PLA/ES-Ag 70/30/2.0 films ball milled in DW revealed 2.25 and 11.52 ppm, respectively. This means that the 2 × 2 cm2 films of PBAT/PLA/ES-Ag 70/30/0.5 and PBAT/PLA/ES-Ag 70/30/2.0, which weighed ~0.2 g each, proportionately contain 0.23 and 1.15 ppm, respectively.

The release study revealed that no silver was released from the film after 24 and 72 h of immersion in DW at both 4 and 50 °C. This is probably due to the relatively high hydrophobic nature of the polymer system, which results in insignificant amount of water absorption, preventing the release of embedded Ag NPs into the water. This is different from the report of a study which showed the release of silver into water due to water absorption by an EVOH/Ag polymer system.19 Alternatively, the amount of Ag NPs in 0.4 g of the two 2 × 2 cm2 films we used may be very small (−0.46–2.30 ppm), hence, trace amounts of free Ag NPs may be released into the water below the detection limit of the AAS. Similar results were observed when chicken breast (CB) samples were packaged in films and stored at 4 °C for 24, 72, and 168 h as shown in Table 2. This is probably because of the small amount of Ag NPs in these films. Compared to other reports where Ag was released into apple peels4 from EVOH/Ag films, the differences in hydrophilicity of the polymer systems and the procedures used in our study may explain the different results. Our findings with PBAT/PLA/ES-Ag films are encouraging for potential active food packaging applications devoid of Ag leaching, but prolonged release studies are needed to show if these results hold over an extended storage period.

In summary, we investigated ES-Ag NPs prepared by single step ball milling and studied the effect of the NPs on the microstructure, thermal, tensile, and antimicrobial properties of a poly(butylene adipate-co-terephthalate) (PBAT)/agro-based polyactic acid (PLA) blend. The release kinetics of Ag NPs from the films was also studied. The nanostructure of the ES-Ag was determined by TEM, XPS, and XRD analysis. The pure blend and composites with 0.5–2.0% content of ES-Ag were characterized by DSC, TGA, XRD, SEM, tensile, and antimicrobial testing. The DSC and SEM results revealed that the two polymers are immiscible, as seen by the presence of distinct melting points and phase segregated morphologies in the blend and composite structures. X-ray diffraction revealed that the PLA is amorphous while PBAT is semicrystalline, resulting in a semicrystalline immiscible blend. The tensile test showed that ES-Ag compromised the tensile properties of the blend, due to less interaction between the matrix and the ES-Ag in the ternary composite systems, as evident in the SEM microstructure of fractured surfaces of the specimens. Though toughness is better than that of PLA, the strength was lower but in between those of PBAT and PLA. The results suggest that the PBAT/PLA/ES-Ag ternary composite possessed properties intermediate to those of the two pure polymers. In *vitro* assessment of the antimicrobial activity of the film was conducted on *L. monocytogenes* and *S. Enteritidis* isolates. This test revealed that the films possess bacteriostatic effects, due to the immobilized ES-Ag nanomaterials in the blend matrix. The release study showed that Ag NPs were not released into either distilled water or chicken breast after 72 and 168 h of exposure, respectively.

<table>
<thead>
<tr>
<th>specimen</th>
<th>Ag concentration (ppm)</th>
<th>24 h</th>
<th>72 h</th>
<th>164 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES-Ag (0.2 g/mL) in DW</td>
<td>33.92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBAT/PLA/ES-Ag 70/30/0.5 (2g/mL) ball milled in DW</td>
<td>2.25</td>
<td></td>
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<tr>
<td>PBAT/PLA/ES-Ag 70/30/2.0 (2g/mL) ball milled in DW</td>
<td>11.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBAT/PLA/ES-Ag 70/30/0.5 film in DW@ 4 °C</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBAT/PLA/ES-Ag 70/30/0.5 film in DW@ 4 °C</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBAT/PLA/ES-Ag 70/30/0.5 film in DW@ 50 °C</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBAT/PLA/ES-Ag 70/30/2.0 film in DW@ 50 °C</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB Control</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>PBAT/PLA/ES-Ag 70/30/0.5 film on CB @ 4 °C</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>PBAT/PLA/ES-Ag 70/30/2.0 film on CB @ 4 °C</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>
X-ray diffraction patterns, differential scanning calorimetry curves, thermal degradation of pristine and PBAT/PLA/ES-Ag blend composites, scanning electron microscopy analysis of morphology and fractured surfaces, and calibration curve of silver standard solutions from atomic absorption spectroscopy (PDF).

**REFERENCES**


**ABBREVIATIONS USED**

3D, three-dimensional; ES-Ag, eggshell-silver; PBAT, poly(butylene-co-adipate terephthalate); PLA, polyactic acid; XRD, X-ray diffraction; XPS, X-ray photoelectron spectroscopy; TEM, transmission electron microscopy; Ag NPs, silver nanoparticles; TGA, thermogravimetric analysis; DSC, differential scanning calorimetry; SEM, scanning electron microscopy; AAS, atomic absorption spectroscopy; GRAS, generally recognized as safe; PCL, polycaprolactone; HT, hydroxytroisol; C30B, Cloisite 30B; Sc3N@Ih-C80, scandium nitride cluster fullerene; MNF, metallic nitride fullerene; SIL, polysiloxane-—polysiloxane-block-polystyrene; ROS, reactive oxygen species; EVOH, ethylene-vinyl alcohol; LDPE, low-density polyethylene; UF, ultrafiltered; CFU, coliform forming unit; ST, starch; HPLC, high performance liquid chromatography; PPG, polypropylene glycol; ATL, animal tissue lysis; MHA, Mueller Hinton agar; TSA, tryptic soy agar; ATCC, American Type Culture Collection; PD, premier die; JSM, JEOL, Japan electron optics laboratory; ASTM, American society for testing and materials; JSM, JEOL, scanning microscope; ANOVA, analysis of variance; UV, ultraviolet; PCR, polymerase chain reaction; TSB, tryptic soy broth; OD, optical density; DW, distilled water; CB, chicken breast; Td50, temperature at 50% degradation; Tdmax, temperature at maximum degradation; Td onset, temperature at maximum degradation; PVDF, polyvinylidene difluoride; PMMA, poly(methyl methacrylate); PHB, polyhydroxybutyrate; PEBA, poly(ether-amide); PLLA, poly(ε-caprolactone)-Based Nanocomposites Containing Hydroxytyrosol for Active Food Packaging. *J. Agric. Food Chem.* 2014, 62, 2244–2252.

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