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Physical properties and antibacterial activity of polylactic acid reinforced by halloysite nanotubes and zirconium dioxide nanoparticles against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

***Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) are the most common bacteria responsible for causing implant infections. We propose that antimicrobial bionanocomposites are a rational solution for the prevention of infection. Polylactic acid (PLA) reinforced by nanoclay is one of the promising polymeric materials possess required physico-chemical properties to meet demands of various applications, especially the food packaging industry. The properties of PLA nanocomposites can be tuned for desired properties by mixing with metals, metal oxides and other materials. In this research, neat PLA, PLA/HNTs and PLA/HNTs-ZrO_2 bionanocomposites and their properties have been investigated. PLA samples and its nanocomposites are prepared using solution casting method. Physical properties such as water uptake and ion exchange capacity were tested. The antimicrobial activity of different concentrations of the synthesized PLA, PLA/HNTs and PLA/HNTs-ZrO_2 bionanocomposites were examined against gram positive bacteria (*S. aureus*), gram negative bacteria (E. coli). Antimicrobial tests revealed that PLA/HNTs-ZrO_2 can act as a promising antimicrobial agent against bacteria, for E. coli and S. aureus where the bacteria count reduced by more than 99%.**

Keywords: Polylactic Acid, Halloysite Nanotubes, Zirconium Dioxide nanoparticles, Bionanocomposites, Antibacterial, Physical Properties.

Introduction

Many industries rely on the use of petrochemical-based plastics for their normal activities, but these materials have attracted much criticism due to the associated environmental concerns; these concerns have driven the fabrication of bio-based and biodegradable alternative packaging materials[1,2] such as polyglycolic acid, poly (lactic acid) (PLA)), and polyvinyl alcohol which can be fabricated in several ways. Among these alternatives, PLA has been considered the most suitable alternative to the traditional petrochemical-based packaging materials owing to its special properties. [3] PLA can be fabricated from numerous renewable resources via a simple fermentation process; the source material for the fermentation process is mostly corn sugar that is processed via either condensation polymerization or ring-opening polymerization. PLA can be easily degraded by enzymes because it is a linear aliphatic thermoplastic polyester [2-4]. However, the thermal stability and resistance of PLA are low, paving the way for the fabrication of bio-NCs as a way of improving the properties of biopolymers[5]. The fabrication of bio-NCs normally involves the combination of a nanofiller, and a biopolymer and the properties of the fabricated bio-NC is determined by the extent of distribution of the nanofiller in the biopolymer matrix; a god distribution of the nanofillers in the biopolymer improves the thermal, barrier, and physicochemical properties of the resulting bio-NC[6]. PLA bio-NCs have been prepared using numerous types of nanofillers, such as CNTs, organoclay, and graphite[7, 8, 9].

Naturally occurring tubular halloysite clay (HNTs) has received much research attention due to its multi-purpose features. HNTs can be used as a nano-filler for the fabrication of biopolymers. Different scholars have investigated the fabrication of thermoplastics matrices with HNTs. The use of HNTs to improve the mechanical strength, crystalline behaviour, and thermal stability of polymers[10].

The basal spacing of HNTs can be modified via surface functionalization/modifications of the HNTs via intercalation of organic and inorganic additives either on their interlayers or the outer surface; this can aid the production of homogeneous HNT mixtures using polymers during the preparation process. The bonding of HNTs with polymers can also be improved by precipitating suitable nanoparticles on the surface of the HNTs[11].

One of the important metal oxide nano-particles that has been widely used in various applications is zirconium dioxide (ZrO_2); this material is attractive due to its chemical, dielectric, physical, and optical properties; it is also highly biocompatible[12]. Zirconia is also a cheap transition metal oxide that shares similarities with Titania in many ways, making it a suitable alternative to titania in the preparation of dental crown and implant biomaterials[13].

This study reports the fabrication of ZrO_2 deposited Hal incorporated PLA NCs as multifunctional active films with improved physical and antimicrobial properties. First, ZrO_2 was deposited on HNTs outer surface, followed by a two-step solvothermal process to encapsulate them inside the lumen space; this process differs from the existing methodologies. ZrO_2 deposited HNTs were also used to fabricate thin PLA/HNTs- ZrO_2 films using the solution casting method at varying concentrations of 0, 1, and 3 wt%. The fabricated PLA/HNTs-ZrO_2 were also investigated for physical and antibacterial activities using standard techniques.

Experimental

Materials

PLA (95.7% L-isomer) was procured from Nature Works LLC (Blair, NE, USA) while the HNTs, isopropyl alcohol, zirconium isopropoxide, chloroform, glacial acetic acid, and nitric acid were supplied by Sigma–Aldrich (St. Louis, USA).

Preparation of HNTs–ZrO_2

HNTs–ZrO2 were made as earlier reported by [21]. A concentrated transparent solution was first made by mixing 0.5 mL zirconium isopropoxide with 22.5 mL HNO3 (2 mol/L) and 7.5 mL isopropyl alcohol in a jar for 60 minutes at room temperature. After that, pure water was introduced to the solution until it reached a final volume of 125 mL. Then, 0.5 g of HNTs was introduced into the mixture and agitated for 2 hours. To 65°C for 24 hours with constant stirring. The heterogeneous HNTs–ZrO2 precipitate was centrifuged for 10 minutes, separated, and rinsed with pure water, followed by drying for 1 day at 120°C.

Fabrication of NCs samples.

Solution casting was used to make PLA NC films; 5% w/v% was prepared from 5 g PLA and 100 mL chloroform. The equivalent HNTs–ZrO2 percentage was added to the resulting PLA solution and aggressively agitated for 15 hours using a magnetic stirrer at 750 rpm. The solution was then sonicated for 30 minutes before casting. The resulting solution (30 g) was put onto glass Petri dishes and dried in the air for 24 hours at ambient temperature before being vacuum dried for 12 hours to remove excess solvents. After submerging the Petri dishes in DW for 5 minutes, the NC films were peeled. The PLA NC films were made with varying HNTs–ZrO2 contents (2.5, 5, 7.5, and 10 wt%). For the sake of comparison, pure PLA films (with no nanofillers), PLA with different contents of ZrO_2 (0–10 wt.%), and PLA with 5 wt. % of HNTs were also fabricated. Table 1 presents the sample codes and their compositions.

Table 1. PLA sample codes and their composition

	Composition (wt. %)		
Sample code	**PLA**	**ZrO_2**	**HNTs–ZrO_2**
PLA	100.00	0.00	0.00
PLA/ZrO_2-2.5	97.50	2.50	0.00
PLA/ZrO_2-5	95.00	5.00	0.0
PLA/ZrO_2-7.5	92.50	7.50	0.00
PLA/ZrO_2-10	90.00	10.00	0.0
PLA/HNTs-ZrO_2-2.5	92.50	0.00	2.50
PLA/HNTs-ZrO_2-5	90.00	0.0	5.00
PLA/HNTs-ZrO_2-7.5	87.50	0.00	7.50
PLA/HNTs-ZrO_2-10	85.00	0.00	10.00

Characterization of the samples

Tensile properties

A tensile test was carried out on film strips of 63.5 x 10 x 0.15 mm3 on a Lloyd LR 10K tensile bench at a 40 mm distance between grips and a speed of 0.5 mm/min. The tests were performed on pre-conditioned (for at least 48 h at 20 ± 2°C; RH of 50 ± 3%) samples and the results were taken as the average of at least 5 measurements for each sample code.

Antimicrobial test

E. coli and *S. aureus* maintained in LB medium at 37°C for 12 hours were used for the antibacterial assay. The broth culture was centrifuged at 3500 g for 10 minutes, and the sediment was resuspended at 0.005 OD600 in the presence of 1 cm2 polymeric films; each film was cut into 4 equal sections and cultured in aerobiosis at 37°C at 250 rpm. *S. aureus* was re-suspended in 50 percent v/v peptone water because it is more sensitive to nutrient deficiency, whereas *E. coli* which is more tolerant to oligotrophic conditions were re-suspended in sterile distilled water. 50 µl of various dilutions of the bacterial suspensions were plated on LB agar dishes for the determination of cell survival at the appropriate times; the plates were incubated for 24 h, followed by computation of the colony-forming units (CFU). The computed values served as the guide to calculate the antibacterial activity (A) of each agent as earlier reported by [34]. The computation was done using the following formula:

$$A = F – G \qquad (1)$$

Where F = the growth values in the presence of non-filled PLA samples (control ZrO_2 & HNTs), while G = the growth values in the presence of PLA/ZrO_2 & PLA/HNTs-ZrO_2 films. The growth values were calculated as follows: $F = \text{Log}(C_{24h/7d} - \text{Log } C_0)$ and $G = \text{Log}(T_{24h/7d} - \text{Log } T_0)$. C and T are the observed CFU for the control and filled PLA samples respectively at varying times (0, 24 h, and 7 days).

Results and Discussion

Water absorption. Water uptake of PLA, PLA/HNTs and PLA/HNTs-ZrO2 nanocomposite membranes are shown in Table 2. Water absorption capacity depends on the number of ion exchange groups and nature of nano fillers present in the membrane. The lowest water uptake values were observed for neat PLA. Halloysite nanotubes are highly hydrophilic materials due to the aluminosilicate structure and, consequently, as the HNTs loading increases, the saturation water increases as observed by Russo et al.[14]. HNTs offer a high surface area with a high number of hydroxyl groups that contribute to the water uptake rate and equilibrium water. Therefore, as the HNTs loading increases, the water uptake also increases. In addition, for the samples PLA/HNTs-ZrO_2 The water absorption of the composite membrane increased with increase in zirconia content[15]. This percentage variation may be attributed to the nature of functional group and presence of zirconia that each polymer carries. In the composite membranes, the zirconia nano fillers enhance the water absorption values due to its water retention character and hydroscopic nature of zirconia.

Table 2. Water absorption and IEC values of neat PLA and bionanocomposite membranes.

Membrane	Water absorption (%)	IEC (meq/g)
PLA	1.83	0.435
PLA/HNTs-2.5	2.25	0.676
PLA/HNTs-5	2.38	0.712
PLA/HNTs-7.5	2.54	0.763
PLA/HNTs-10	2.71	0.828
PLA/HNTs-ZrO_2-2.5	2.95	0.891
PLA/HNTs-ZrO_2-5	3.39	0.921
PLA/HNTs-ZrO_2-7.5	3.88	0.954
PLA/HNTs-ZrO_2-10	4.21	0.982

Ion exchange capacity. In principle, ion exchange capacity (IEC) is an ionic-conductive membrane usually reflects the amount of exchangeable groups in the membrane, and a relatively high IEC is normally correlative to a higher ionic conductivity [40]. Experimental IEC values of the PLA and its composite membranes were calculated by back titration method. IEC can provide information on the density of ionizable functional group in the membrane. The IEC (Table 2) displayed a linear relationship with hydroxyl ion conductivity and water absorption. The maximum IEC of the prepared composite membrane was found for PLA/HNTs-ZrO2-10.

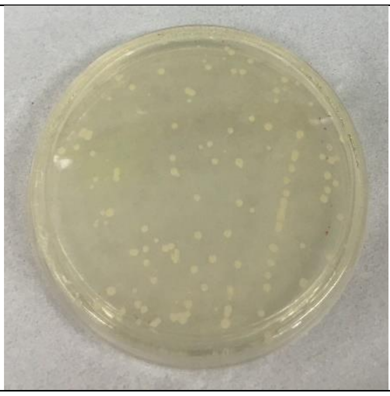
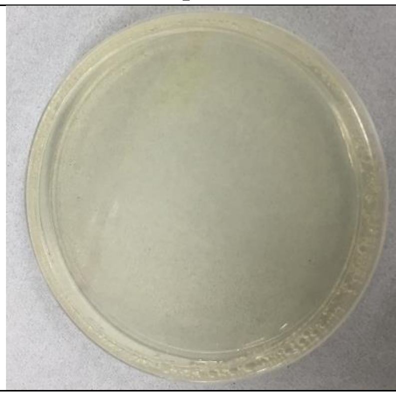
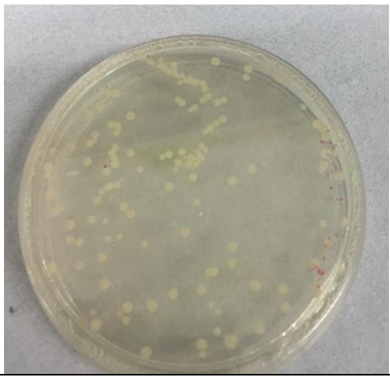
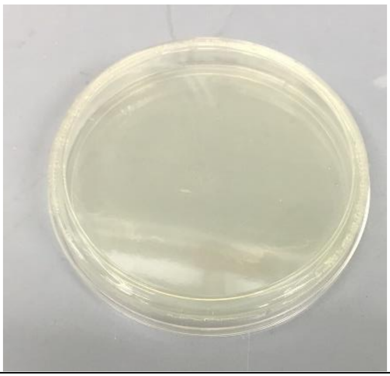
Antimicrobial property of PLA nanocomposites.

The antibacterial activity of PLA nanocomposite films was investigated for two types of bacteria, i.e., gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria as illustrated in (Fig. 1). Fig. 1 showed antibacterial activity of both PLA/HNTs and PLA/HNTs-ZrO_2 nanocomposites against E. coli. PLA/HNTs samples did not exhibit any antibacterial effect against E. coli, where the bacterial count (log CFU) wiggled within the range of 6–8 during the tested incubation time (0–2 weeks). PLA/HNTs-ZrO_2 nanocomposite samples show a somewhat good antibacterial effect against *E. coli*, particularly at high HNTs-ZrO_2 loadings. According to ANOVA, at low loadings (2.5 wt%) there is no important antibacterial effect, while the effect is considerable at high filler loading, 7.5 wt%. This could be attributed to the non-homogenous distribution of the fillers. The incorporation of HNTs-TiO_2 clearly has a significant activity against *E. coli* bacteria. For example, the incorporation of 5 wt% of HNTs-ZrO_2 decreased the bacteria count by 97%, within 24 h of incubation time and it further decreased after 2 weeks (98%). significant antibacterial effect was shown with 7.5 wt% of HNTs-ZrO_2 fillers after 1 incubation week, and with 10 wt% of HNTs-ZrO_2 after 24 h, where all the bacteria were completely eliminated (no CFU were recorded in the plates).

Figure 1 shows the antibacterial effect of PLA nanocomposite films against the gram- positive bacteria, (*S. aureus*). PLA/HNTs samples did not demonstrate any antibacterial activities within the studied incubation time and the difference is statistically not considerable (according to ANOVA, P N 0.05). In the opposite to E. coli, antibacterial effect of PLA nanocomposite samples against *S. aureus* is more effective, particularly after 1 and 2 weeks of incubation time. PLA//HNTs-ZrO_2 nanocomposite samples presented an important antibacterial effect with *S. aureus*. This important effect could be assigned to the exist of ZrO_2 comparing with PLA/HNTs. Bacteria count decreased in all the compositions of HNTs-ZrO_2 inside all tested incubation times. For example, the incorporation of 5 wt% of HNTs-ZrO_2 decreased the bacteria count by 98%, during 24 h of incubation time and it further decreased after 2 weeks (99% for all compositions). Outstanding performances were registered with high filler concentrations, which no bacteria colonies were seen even after 24 h.

Remarkably, the antibacterial impact of PLA nanocomposite samples against *E. coli* and *S. aureus* follows both of time and filler amount dependant process.

The reason why the Gram-negative bacteria (E. coli) need a higher concentration of antibacterial agent than the Gram-positive bacteria (S. aureus) is that these two kinds of bacteria have different cell wall structures. For the Gram-positive bacteria, there is a membrane formed by a single bilayer, and thus they are very sensitive to the antimicrobial activity of the nanocomposites. In contrast, the Gram-negative bacteria are composed of two bilayer membranes, making these bacteria more resistant to the external attack of the anti-microbial agents [19].

Bacteria	PLA/HNTs-10	PLA/HNTs-ZrO_2-10
E. coli		
S. aureus		

Conclusions

In summary, the physical properties and antibacterial effects of neat PLA, PLA/HNTs and PLA/HNTs-ZrO2 bionanocomposites have been studied in this work. The results were as follows: The lowest water uptake values were observed for neat PLA. With the increasing of both HNTs and ZrO2 concentrations, the water uptake values increased, this result could be assigned to the polar nature of both of them. The same results were observed with the ion exchange capacity test, which the highest value of IEC of the prepared composite membrane was found for PLA/HNTs-ZrO2-10. The result was explained by the increasing of OH^- concentration. The antibacterial test results revealed effective inhibition of *E. coli* and *S. aureus* bacteria. The incorporation of ZrO_2 nanoparticles on the PLA/HNTs improved its antibacterial activity.

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