Chapter II

Materials And Experimental Techniques

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2.1. Materials

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer containing 5.4 mole % 3-hydroxyvalerate, was purchased from Copersucar, Piracicaba, Brazil. The sample was purified by dissolution in $CHCl_3$, filtration to remove any insoluble matter, and reprecipitation in diethyl ether. N-Vinylpyrrolidone (VP) was supplied from Aldrich and distilled under vacuum before use. N-Isopropylacrylamide (IPA) was supplied from Aldrich, was purified by recrystallization from (methanol). 2, 2`-Azobisisobutyronitrile, AIBN (BDH Chemicals Ltd.), was purified by recrystallization from methanol before use. Benzoyl peroxide (BPO) was provided by Hayashi Chemical Co. Japan and was crystallized from methanol just before use. All other reagents were used as received.

2.2. Synthesis of poly (N-vinylpyrrolidone) homopolymer

3 ml of N-vinylpyrrolidone monomer (VP) and the initiator (AIBN) were placed in flask (and the flask was sealed) in microwave for 5 minutes and the resulted polymer sample was purified by dissolving in methyl alcohol, filtration to remove any insoluble matter, and reprecipitation in petroleum ether.

2.3. PHBV graft-poly (N-vinylpyrrolidone)

1 gram of PHBV was first dissolved into 25 ml of methylene chloride under reflux in a flat bottomed three necked flask. The monomer (VP) and the initiator (AIBN) were added under nitrogen atmosphere and the reaction was carried out under reflux and

stirring. The resultant solution was precipitated into methanol with vigorous stirring. The product was removed, washed and extracted extensively with distill water in a Soxhlet for 48 h to remove the unreacted monomer and poly (N-vinylpyrrolidone) homopolymer followed by drying at 80°C under vacuum. The grafting percentage (% G) and grafting efficiency (GE) were calculated as follows:

$$G\% = \frac{(W - W_o)}{W_o} x100 \quad (1)$$

$$GE\ (\text{efficiency}\ \%) = \frac{W}{\text{total weight of polymer}} x100 \quad (2)$$

where W is the weight in grams of the grafted PHBV and W_o is the weight of the native PHBV.

2.4. Synthesis of poly(N-Isopropylacrylamide) homopolymer

2 grams of N-Isopropylacrylamide monomer (IPAM) and the potassium persulfate/potassium sulfite (1/0.5) dissolved in 30 ml distill water and the reaction was carried out in water bath at 60°C for 24 hr. The resultant solution was precipitated into methanol with vigorous stirring, filtered and dried in oven at 80°C till constant weight.

2.5. PHBV graft-poly (N-Isopropylacrylamide)

1 gram of PHBV was first dissolved into 25 ml of chlorobenzene under reflux in a flat bottomed three necked flask. The monomer (IPA) and the initiator (BPO) were added under nitrogen atmosphere and the reaction was carried out under reflux and stirring. The resultant solution was precipitated into petroleum ether with vigorous stirring. The product was removed, washed and extracted extensively with distill water in a Soxhlet

for 48 h to remove the unreacted monomer and poly (N-Isopropylacrylamide) homopolymer followed by drying at 80°C under vacuum. The grafting percentage (% G) was calculated using equation (1).

2.6. Preparation of polymer films

The films of PHBV and different grafted PHBV samples of different grafting percent (0.10–0.12 mm thickness) were prepared by solvent–casting technique from 4 wt% chloroform solution in Teflon Petri dishes of known constant dimensions as casting surface. The films produced were dried under vacuum to constant weight and then left to stand for at least two weeks at room temperature to attain equilibrium crystallinity prior any measurements.

2.7. Antimicrobial activity

2.7.1. Sources and cultures of microorganisms

Different microorganisms including Gram-positive and gram negative bacteria, yeasts and fungi were kindly provided by the Microbial center, Ain-Shams University, Faculty of Agricultural, Egypt (CAIM, Cairo Mircen) and form the Bacteriological Department Laboratory of National Organization for Drug Control and Research (NODCAR), Egypt.

These involved *Bacillus subtilis* (CAIM-1007), *Staphylococcus aureus* (CAIM-1352), and *Micrococcus luteus* (CAIM-1246), from Gram positive bacteria; *Escherichia coli* (CAIM-1357), *Pseudomonas aeruginosae*, *Shigella spp.* (NMRO), *Salmonella typhimurium* (CAIM-1350), and *Klebsiella pneumonia* from gram negative bacteria; *Saccharomyces cerevisiae* (CAIM-14), *Candidia albicans* (CAIM-22), *Candida tropicalis*, *Candida parapsilosis* and *Candida nonalbicans* from Yeasts, *Fusarium oxysporum* (CAIM-123), *Aspergillus niger* (CAIM-147), and *Aspergillus flavus* (CAIM-127), from Filameutous fungi.

The cultures maintained on slants of appropriate medium, where the bacteria kept on slants of nutrient agar medium, and yeast kept on slants of Sabouraud's dextrose medium, and fungi kept on slants of Czapek-dox agar medium.

2.7.2. Screening of the bioactive compound for antimicrobial activities

2.7.2.1. Agar well method

The isolated bioactive were tested for the antagonistic activity against the selected microorganisms by the diffusion agar method according to British Pharmacopoeia (1968 and 2000).

The seeded plates with the targets organism were cut by sterile cork borer to make holes (5 mm in diameter). Only 200 µl of the investigated PHBV and PHBV-g-PVP grafted copolymers were transferred into each hole under aseptic conditions. Then the plates were kept in a refrigerator for 1 hr before incubation to permit the diffusion of the antimicrobial substances and the diameter of the inhibition zone were measured after incubation for 48 and 72 hr at 28°C for yeasts and fungi, and 24 hr at 37°C for bacteria. Cultures were examined for zone of inhibition, which indicate positive reactions.

2.7.2.2. Minimum Inhibition Concentration (MIC_S)

The minimum inhibition concentrations (MIC_S) of the PHBV and PHBV-g-PVP grafted samples upon the tested fungal and bacterial species were determined by the dilution method described by Nair et al.[124]

2.7.3. Statistics

All measurements of the antifungal and antibacterial activities are the means of three replicates; the results were processed by analysis of variance, and the significance was determined as the significant difference (LSD) levels of 1 and 5 %[125]

2.8. Biodegradation

Film samples of the base polymer and some graft copolymers (20 mm × 20 mm; 80 + 5 mg total weight) were buried in covered trays containing a standard soil mixture (1: 1: 1 w/w/w mix of top-soil, sand and composted manure) maintained at a moisture content of 22 to 26%, pH range of 7.5 to 8.5 and temperature range 25–30 °C. Film samples were recovered at different time intervals, washed several times with distilled water and then dried to constant weight under vacuum. The degradation of the film was conventionally evaluated in terms of weight loss%.

$$\text{Weight loss \%} = \frac{(W_o - W)}{W_o} x100 \qquad (3)$$

where Wo is the weight of initial film and W is the weight of degraded film

2.9. Viscosity Measurements

The reduced viscosity, η_{sp}/C, of the base polymer and some graft copolymers was measured in chloroform at 30°C using an Ubbelhode suspended level viscometer. The concentrations of polymer solution in $CHCl_3$ were ranged from 1% – 0.6%. The time of flow of chloroform was 54 seconds at room temperature. The intrinsic viscosity [η] was calculated by plotting η_{sp}/C versus several concentrations (C) and extrapolate to zero concentration. The intercept is [η].

2.10. Swelling determination

The swelling behavior of grafted PHBV was studied at 30°C as a function of time in distilled water (pH =6.5). The well known tea-bag method was used. An exact amount of pre-dried sample was placed in tea-bag made of 200 mesh nylon screen and then immersed in distilled water. After the excess water was removed with filter paper, the

weight of the swollen sample was measured. The degree of swelling at a specific time was calculated using the relation

$$\text{Swelling}\% = \frac{W_s - W_o}{W_o} x100 \qquad (4)$$

where W_s and W_o are the weights of swollen and dry copolymer, respectively

2.11. Instrumentation

FTIR Infrared analysis was carried out using a Perkin–Elmer 398 FTIR spectrophotometer between 400- 4000 cm^{-1}.

^{1}H-NMR analysis of samples was carried out on a Bruker AC-400 at 20°C in $CDCl_3$ solution.

Differential Scanning calorimetry (DSC) was performed on a PL–DSC (Polymer Laboratories England). The calorimeter was calibrated with ultra – pure indium. Samples (10-12 mg) were first heated from -40 to 190°C with a heating rate of 10oC/min (Run I). After keeping them at 190°C for 2 min, samples were rapidly cooled to -100°C at a rate of 80°C/min to obtain specimen with very low crystallinity or totally amorphous, and then heated again with a heating rate of 10°C/min to 180°C (Run II). The melting temperature (Tm) and the cold crystallization temperature (Tc) were taken as the peak values of the respective endothermal and exothermal processes in DSC curves. The apparent melting enthalpy (ΔHm) was determined from the area of the endothermic peaks, while the enthalpy of cold crystallization (ΔHc) was determined from exothermic peaks in Run (II). The glass transition temperature (Tg) was taken as the midpoint of the specific heat capacity.

Thermal degradation studies were conducted in air under dynamic heating rate of 10°C /min using a Shimadzu TGA-50H Thermal analyzer. All experiments were

conducted from room temperature to 600°C and the reference material was α–alumina. The sample weights for all the experiments were taken in the range of 7– 10 mg.

WAXD measurements were performed with a Philips PW 1700 X-ray diffractometer in a 2 Θ range of 5-45° at scanning speeds of 2°/min. Cu–Kα X-ray radiation at a voltage 30 kV and a current of 20 mA was used as the source.