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MECHANICAL, THERMAL, AND TOXICOLOGICAL EVALUATION OF POROUS NANOCOMPOSITES PROJECTED FOR BONE GRAFTING

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Abstract
Cellulose/hydroxyapatite/Poly L(Lactide)acid nanocomposites with different weight ratios were prepared using solvent-sublimation method. The properties of nanocomposites were characterized by means of Scanning Electron Microscopy (SEM), Diffractional Scanning Calorimetry (DSC), and mechanical testing machine. Additionally, in vitro test were conducted in order to investigate the toxicity and bioactivity of the novel nanocomposites. SEM images showed no sign of agglomeration of reinforcing agents. Due to uniform dispersion and molecular interactions between constituents, crystallinity of the composites increased from 10% to 38%, which yield to improving of compressive strength up to 90%. In addition, fabricated nanocomposites displayed excellent biological activity, demonstrating their potential for bone grafting.

1. Introduction
When the bone self-healing mechanism fails as a result of defect size, infection or other causes, bone grafting has been shown to be a highly successful treatment. Preferably, graft is the patient's own bone (*autograft*). *Autografts* are considered the gold standard for this purpose, but their usage is limited by chronic pain at the donor-site, and the lack of bone supply [1,2]. So far, an appropriate alternative for missing bone that has all the advantages of the *autologous* bone without any disadvantages does not exist. Since, bone itself is a true nanocomposite, the combination of synthetic materials to introduce novel nanocomposites to mimic artificial bone has been attracted much interest in recent years. So, attempts have been made to develop polymeric/ceramic composites to improve the mechanical as well as biological properties of the final product. Such materials not only have light weight, but they also have stiffness matching to real bone. Polymers are being used as a structural host matrix for nanoparticles. Poly L-lactic acid (PLLA) is a recognized biocompatible polymer that has been used for manufacturing of the orthopedic biomaterial due to its relative high stiffness, biodegradability as well as biocompatibility. However, physical, thermal, and mechanical properties of the PLLA are still not adequate for load-bearing applications [3,4]. On the other side, biodegradation

by-products of PLLA decrease the PH of implantation site *in vivo*, which cause necrosis of the surrounding tissue [5]. Therefore, solution is to strengthen the polymeric matrix by implication of reinforcing agents such as hydroxyapatite nanoparticles (HA). HA is the most important bioceramics due to its bioactivity and similarity to natural minerals of bone. It is not only thermodynamically stable at physiological PH, but it also encourages bone-bonding process [6]. According to literature review, it is found that reinforcing power of the HA nanoparticles is not as much as enough for bone grafting applications due to lack of interfacial bonding with polymeric matrix. Also, because of its brittle nature, elongation at break of the nanocomposites decreases after incorporation of HA. In order to overcome these shortcomings, cotton sourced cellulose crystals has been introduced to the composition of the PLLA/HA nanocomposite [7,8,9]. Cellulose is an appropriate reinforcing constituent with relative high strength and stiffness and low density due to its extended chain structure [9,10,11]. But when it comes to preparation of the nanocomposites, the main challenge is homogeneous dispersion of various reinforcing agents in the matrix and adequate interfacial bonding between the constituents to achieve targeted goals. To tackle this challenge sodium-dodecyl Sulphate (SDS) has been recruited as surfactant to isolate cellulose crystals [12]. The hydrophilic head of surfactant absorbs on the cellulose surface whereas its hydrophobic tail attaches to the PLLA. The objective of this research is in depth evaluation of mechanical, thermal and toxicity of the novel PLLA/MCC/HA/SDS nanocomposites designed for bone grafting applications.

2. Material and Methods

2.1. Material

Poly L-Lactide acid (PLLA) was purchased from Sigma-Aldrich (Mw≥85KDalton, Lactel®, USA) and directly used as matrix for production of composites. PLLA when purchased had an average molecular weight (Mw) of 85000 g/mol and 37% of crystallinity and has a glass transition temperature (Tg) ranging 60 to 65 °C and degradation time beyond 24 months. Cotton source microcrystalline cellulose (MCC), with a mean particle size of 20μm and an aspect ratio of 2-4, from Sigma-Aldrich (USA) was used as reinforcing agent. Hydroxyapatite nanoparticles (HA) was obtained from Sigma-Aldrich (USA), with a mean particle size <200 nm and surface area 14.3 (m2/g). The Sodium Dodyl Sulphate (SDS) used as a coupling agent was purchased from (Fluka, Canada). The amount of the SDS, HA and MCC used is reported in Table 1. We also used various solvents during the processing of the nanocomposite such as 1,4-Dioxane, from (Sigma-Aldrich, ACS Reagent, with purity ≥99%) ethanol (VWR, Canada, purity ≥99%) were used as solvents. All materials were used as received without further purification.

2.2. Methods

2.2.1. Preparation of PLLA-HA-MCC nanocomposites

The preparation of the PLLA-HA-MCC nanocomposites involves two main steps: First step is the pre-treatment of reinforcing agents (MCC and HA) and the second step is the fabrication of nanocomposites. The objective of the pretreatment of the reinforcing agents is to disperse them homogenously in the polymer solution (PLLA in dioxane), prevent them from agglomeration. The pre-treatment of MCC and HA was done using a

combination of two physical and mechanical methods. HA nanoparticles was dispersed in water when purchased. Solvent-extraction method used to remove water from nanoparticles. Then, MCC and HA nanoparticles were then ultrasonicated in an ice-bath for 30 min at 4 mv with the presence of coupling agent (SDS) to decrease the size of the MCC crystals and get homogeneous colloidal dispersion. In order to fabricate the nanocomposites, 0.5 g of PLLA was dissolved in 5cc of 1,4-Dioxane solvent with the aid of vortex shaker and water bath at 60°C. In all the nanocomposites with variable compositions, the concentration of the PLLA in the solvent was kept constant which is equal to 0.1 g/cc. The pristine specimen prepared without incorporation of reinforcing agents to be considered as reference. Then the polymer solution mixed with the mixture of pre-treated particles in dioxane. The result was a stable, homogenous, colloidal suspension of the nanoparticles in polymer solution. Afterword, the suspension was molded then frozen at -20°C, and transferred to freeze-dryer at -54°C under vacuum to sublimate the solvent. This procedure yielded to a porous composite nanostructure. The composite samples were placed in flask containing PBS (phosphate buffer saline) and shacked gently at 100 rpm for 12h to remove the excess of SDS from the composite. This step was repeated four times. Then sample was washed with distilled water using the same precedent procedure. Finally the samples were dried in a vacuum oven for 6 hours at 50 °C.

Table1. Specimen nomenclature and composition in weight percentage.

Specimen Designation	PLLA (%)	MCC (%)	HA (%)	SDS (%)
Pristine	100	-	-	-
MA1010	76.9	7.7	7.7	7.7
MA3030	52.6	15.8	15.8	15.8
MA4040	45.45	18.18	18.18	18.18
MA5050	40	20	20	20

2.2.2. Description of analyses

Scanning Electron Microscopy

The microstructure of the nanocomposite was examined using a Hitachi 2500 scanning electron microscope. All specimens were coated with a conductive layer of sputtered gold. The micrographs were taken at an accelerating voltage of 15kV in secondary electron mode to ensure a suitable image resolution. The analyzed SEM images were taken at X200 magnification. The SEM micrographs at 200 times magnification were analyzed after adjusting the contrast and applying a threshold of the level of dark. The images were analyzed after adjusting the contrast and applying a threshold level of dark. The pore area in all the manufactured composites investigated using image analysis software (image J) developed by the National Institutes of Health-US.

Mechanical testing

The compressive strength and modulus of the porous nanocomposite with different compositions were measured using an electronic universal testing instrument (United, USA). The tests were performed using a 500N load cell and a crosshead speed of

1mm/min. The tested specimens were cylindrical with a 13mm diameter and 20mm height. The specimens' surfaces were prepared using sandpapers with super fin grit size (P1200) in order to insure the contact with the top and bottom compression jigs. The compressive yield strength was defined as the cross point of the two tangent in stress-strain curve around the yield point. The compressive modulus was determined from the initial linear stress versus strain plot at strain <2%. Four samples were tested for each type of composite.

Differential scanning calorimetry (DSC)

To investigate the effect of MCC and HA on thermal behaviors of PLLA matrix, DSC study was performed on the pure PLLA and PLLA/HA/MCC composites with different weight ratio of MCC and HA. The crystallization and melting behavior of the prepared nanocomposites and neat PLLA were investigated using DSC (PerkinElmers, USA) in nitrogen atmosphere A sample mass of approximately 6 mg was used for each sample. A heat-cool-heat regime was applied in this measurement and the second heating curve was used for thermal analysis. The sample was heated from 0°C to 200°C at a heating rate of 5°C.min^{-1} before cooling it down to 0°C using at a cooling rate of 5°C.min^{-1}. The crystallinity of the prepared composites was obtained from equation (1):

$$\chi c(\%) = \frac{\Delta H_m - \Delta H_c}{\Delta H^{\circ}_m \times w} \times 100\% \qquad (1)$$

Where χc is the crystallinity, *w* is the weight fraction of PLLA in the composites, ΔH_m is the melting enthalpy calculated from heating step of DSC graph, ΔH_c is the recrystallization enthalpy in cooling step and ΔH°_m is the melting enthalpy of pure crystalline PLLA (93.7Jg^{-1}) [13]. To analyze the effect of the reinforcing agents on crystallinity of the PLLA, the area under the exothermic peak which is due to recrystallization of the amorphous regions of PLLA has been taken to account in order to calculate ΔHc and the area under the endothermic peak which is due to melting of crystalline PLLA between (160-185 °C) has been evaluated in order to calculate ΔH_m. DSC analysis have been conducted on the MCC, SDS and HA separately in order to evaluate the influence of MCC and HA on the nanocomposite. The results showed that there is no peak from these raw materials that overlaps with the PLLA peaks. In order to analyze the DSC graphs, normalization of each DSC thermogram is required to have the heat flow in Joule per gram of PLLA.

Cell culture

Rat cells line, URM-106 (Osteosarcoma, ATCC) were grown in Dulbecco Minimal Essential Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin (PS) and 2 ml-glutamine. This mixture will be referred as cell culture medium. These cells (passages 25-30) were maintained in humidified atmosphere of 5% CO_2 at 37°C. The cell culture medium was changed every 48-72 h. For subculturing, the cells were dissociated with 0.25% trypsin-EDTA (Ethylene diamine tetraacetic acid), which was neutralized with culture medium, and subcultured in 75 cm^2 flasks.

MTT reduction assay

Methyl tetrazolium (MTT) reduction assay was chosen in order to evaluate the toxicity of the fabricated nanocomposite. Selected specimens sterilized by autoclave (Heidoplph, USA) at 121 °C for 45 minutes at 2 atm. Each specimen was immersed in DMEM as an extracting media with an extraction medium volume to surface area ratio of 1.25ml/cm^2 at 5%CO_2 and 37 °C. URM-106 was detached from the culture using trypsin/EDTA and re-suspended to a concentration of 1x104 cells/ml, and 100µl of this suspension was added per well to a 96 well plate. Control samples consisted of URM-106 cells grown on tissue cultures plastic (TCP) supplemented with complete DMEM not in contact with extracts. At the end of incubation periods (24,48, and 72 h), 150µL of 0.5 mg/mL MTT solution was added to each well, followed by incubation of plates for 30 min at 37°C in a 5% CO_2 incubator. The reaction was terminated by removal of the media and addition of 150 µL of DMSO. Levels of reduced MTT were determined by measuring the absorbance at 550nm using a microtiter plate reader (PowerWaveX;Bio-Tek Instrument).Toxicity was calculated as the percentage of control cell viability [14].

3. Results and Discussion

3.1. Morphological observations

The morphology and microstructure of the nanocomposites were examined using SEM as shown in figure 1. Figure 1.a. represents an internal porous structure formed by thermally induced phase separation. The SEM micrograph presented in figure 1.a revealed that HA nanoparticles and MCC crystals are distributed in the matrix randomly and homogenously, i.e. some are embedded in pore walls and some piled between pores and there is no sign of agglomeration of MCC and HA nanoparticles.

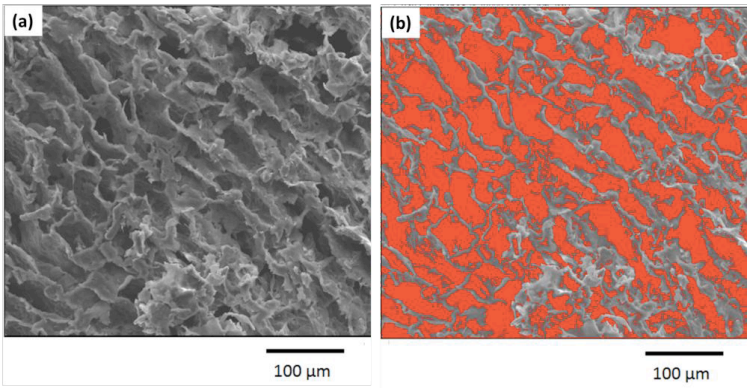

Figure 1: SEM micrographs illustrating representative cross-section of MA4040 composite image (a) shows raw micrograph and image (b) shows the estimated porous areas.

PLLA/MCC/HA nanocomposites had about 75% of the porosity, which is required porosity for simulating real bone structure. However, optimization of pore size and pore morphology is under progress in current work in our lab.

3.2. Thermal properties of nanocomposites

Figure 2 shows the typical DSC thermogram of MA5050 used to evaluate crystallinity of the nanocomposites. The area under the peak between 170-180°C in the heating curve indicates the melting of crystalline phase of PLLA (Figure2).

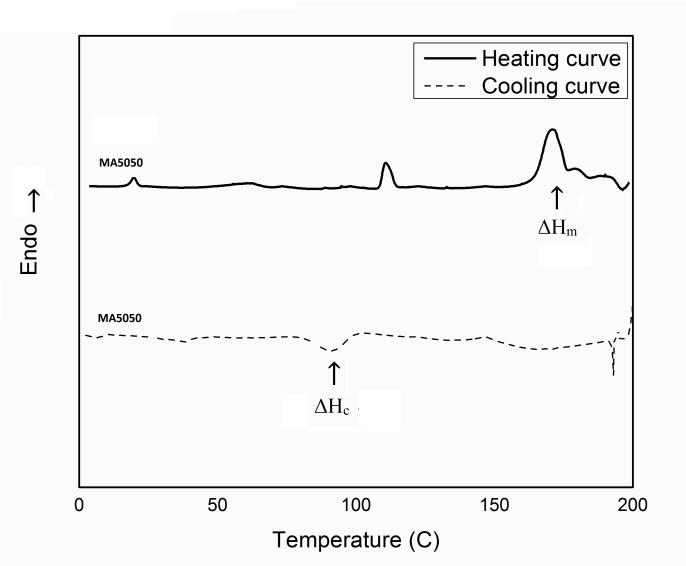

Figure 2: Typical DSC heating and cooling curve for MA5050 nanocomposite used for crystallinity calculation.

The crystallinity of the raw PLLA is about 37%, which decreased to 9% in pristine specimen (Table 2). It may attribute to the negative effect of solving the polymer in its solvent and irregular orientation of crystallites during freezing. However, by incorporation of the HA and MCC in the structure of the nanocomposite, this effect compensated.

Specimen	W_{PLLA} (%)	ΔHm (J/g)	ΔHc (J/g)	χ (%)
Raw PLLA	100	+47	-12.5	37
Pristine	100	+54	-45	9
MA3030	53	+29	-23	12
MA4040	45.45	+27	-18	21
MA5050	40	+30	-15.7	38

Table 2: Crystallinity of the PLLA and PLLA-MCC-HA composites derived from the heating DSC scans. Data corrected for the percentages of the PLLA in nanocomposites.

The area under the peak at 90-100 of the exothermic peak clearly decreased with increasing the PLLA crystallinity. Besides, the corresponding exothermic peak responsible for the amorphous PLLA and its re-crystallization phenomenon becomes smaller. Table 2 lists the calculated χ_c from the analyzed thermograms. The increase in the ΔH_m values shows the effect of increasing the weight ratio of MCC and HA on the crystallinity of the nanocomposites, which may cause higher amount of ordered bonds via hydrogen bonding. The crystallinity of PLLA increased up to 38% by increasing the content of MCC and HA.

3.3. Compressive strength of the nanocomposites

The values of the compressive strength and modulus of the composites are reported in figure 3 and figure 4. The compressive strength and modulus of the composites represented a rising trend with higher ratio of MCC and HA. Increasing of the weight

ratio of MCC and HA to 0.5 led to an improvement in the compressive yield stress from 0.127 (Pristine PLLA) to 1.2 MPa (MA5050) and the Young's modulus from 6.6 (Pristine PLLA) to 38 MPa (MA5050), respectively.

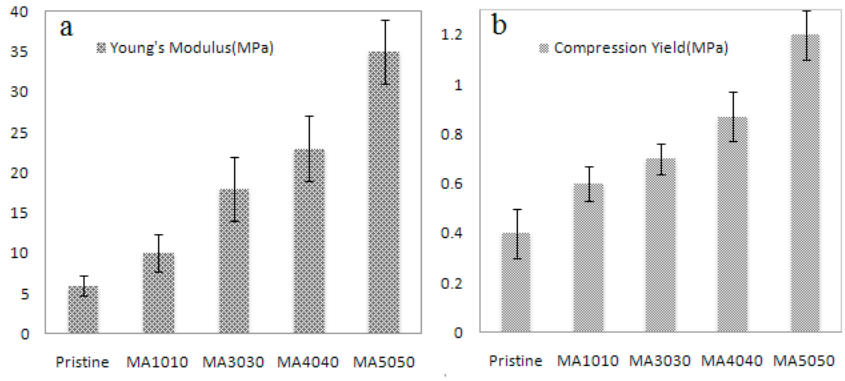

Figure 3: (a) Young's modulus (b) Compression yield of PLLA/MCC/HA nanocomposites

Thus the increase of the weight ratio of MCC and HA to 0.5 increased the compression yield stress by 90% and the Young's modulus of the nanocomposites by 82%. This significant increase may be attributed to hydrogen bonding between the reinforcing agents (MCC and HA) and the PLLA matrix.

3.4. Toxicity assessment

Viability of cells represented in figure 5 after 24, 48, and 72 h exposure of cells to three sample test groups and control sample (with no extract). Cells viability generally was not affected significantly after 24 h incubation time for all studied extracts compared to the control. For MA5050, proliferation rate seems to be the highest. Increasing the number of live cells up to 700% of the control sample, demonstrate the materials high potential to encourage cells to proliferate and growth.

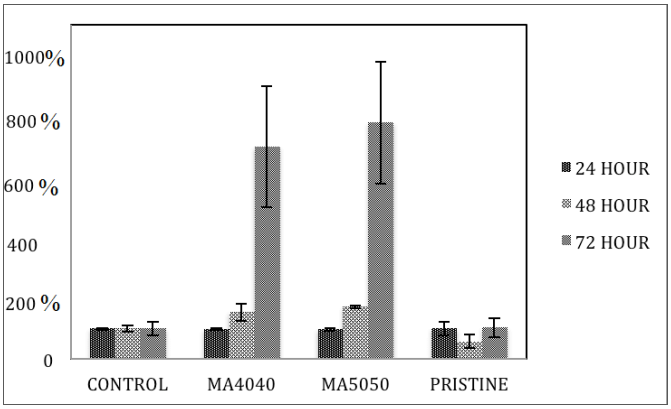

Figure 4: Cells viability of URM-106 cells after incubation in extracts of MA4040, MA5050, and pristine specimens for 24, 48, and 72h. Tissue culture plastic (TCP) with no biomaterial extract was the control group. Results are expressed as % of control for all.

Toxicity results proved not only the absence of toxicological effect of nanocomposites on cells after 48 and 72 h incubation times but also their increased population. This may attributed to the osteoconductivity effect of HA and bioactivity of cellulose.

Conclusion

Increasing of the HA and the MCC ratio led to an increase of the crystallinity of the nanocomposites. Additionally, the results of the compression tests indicated that the incorporation of MCC and HA nanoparticles improves the mechanical strength of the nanocomposites up to 90%. This improvement is due to the homogenous dispersion of HA and MCC in PLLA as a result of hydrogen bonding and increased crystallinity. Increased number of live cells in toxicity assay demonstrated the high potential of the newly developed nanocomposites for bone grafting applications.

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