Metal Chloride Induced Formation of Porous Polyhydroxybutyrate (PHB) Films: Morphology, Thermal Properties and Crystallinity

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Abstract. Polyhydroxybutyrate (PHB) films with highly porous structures were synthesized using a one phase system comprising of metal chloride/methanol/PHB/chloroform (MCl₂/CH₃OH/PHB/CHCl₃). SEM analyses confirmed that the MCl₂ (where M = Cu²⁺ or Ni²⁺) induced porous structures with pore sizes ranging from 0.3 – 2.0 μm. The average pore size increased with the increasing MCl₂ content. There existed weak physical interactions between the PHB chains and MCl₂ as revealed by FTIR and NMR spectroscopies. The residue of MCl₂ in the porous PHB film does not exert significant influence on the thermal stability of PHB. Nevertheless, the crystallinity of the prepared film is enhanced, as MCl₂ acts as the nucleation sites to promote the growth of spherulites.

1. Introduction
Nowadays, the ever increasing environmental issues and the increasing cost and limitations of petroleum resources have driven researches to focus on biopolymers. Polyhydroxybutyrate (PHB) a biopolymer belonging to the family of polyesters, polyhydroxyalkanoate (PHA). It is derived from renewable resources and shows excellent biocompatibility and biodegradability. It is also non-toxic and harmless to the environment.

The fabrication of porous PHB is of great interest among scientists as this material has shown a wide variety of applications. One of the most common applications of porous PHB is in the biomedical area. The porous structure of PHB not only acts as a support to adhere the cells, but also enables the transport of nutrient, drugs or bioactive molecules to the tissues or cells [1]. As such, it has been applied as nerve guides [2], tissue scaffolds [3] and in drug delivery [4]. Another prominent field of porous PHB is water treatment. Zhang and co-workers [5, 6] has demonstrated the removal of chlorobenzene and o-nitrochlorobenzene using porous PHB as a biomimetic adsorbent. The maximum adsorption capacity for chlorobenzene and o-nitrochlorobenzene is 125.99 and 39.56 mg g⁻¹ respectively.

Solvent casting-particulate leaching method, a conventional technique to produce scaffold/highly porous biopolymer was first developed by Mikos et al. [7]. This technique was adopted by Zubairi [8] and Misra et al. [9] to prepare PHB scaffolds using sodium salt crystals or sugar cubes as porogens. The porous structure was formed upon leaching of the porogen using deionized water. Porous PHB can also be prepared via enzymatic hydrolysis technique where PHA depolymerase enzyme from Acidovorax sp. is used to degrade the PHB film [10]. Emulsion is another method to create porous PHB [5, 6, 11]. In this method, water droplets template the porous structure of PHB during evaporation. Other techniques including freeze-drying [3], laser sintering [12] as well as electrospinning [13], have also been reported.
Most of the techniques that were used to create porous PHB are either time consuming, involves complicated procedures or needs specific instruments. Furthermore, the homogeneity as well as the inter-connectivity of the pores are hardly achieved. In this study, we report a simple and low cost method that is capable to produce porous PHB film with homogenous inter-connected pores. Unlike the emulsion and particulate-leaching techniques that employs a bi-phasic system, the method described here involves only one phase. The pore textures, thermal properties and crystallinity of the PHB film prepared in a singular phase (MCl₂/methanol/PHB/chloroform, where M = Ni²⁺ or Cu²⁺) is thus investigated. The role of metal chloride and methanol on the formation of the porous structure is also discussed. These porous PHB films may have potential applications in the field of waste water treatment as an adsorbent or separation membrane [5,6].

2. Materials and Methods

2.1 Materials
Poly(3-hydroxybutyrate) (PHB) was supplied by BIOCYCLE, Brazil and purified before used [14]. Other chemicals were commercially obtained and used without further purification: 98% nickel (II) chloride hexahydrate (NiCl₂.6H₂O) from UNIVAR, Ajax Chemicals, Australia, 99.99% copper (II) chloride dehydrate (CuCl₂.2H₂O) from Aldrich, US, chloroform from Systerm, Malaysia and methanol from QRec, Malaysia.

2.2 Preparation of porous PHB films
Three different moles of MCl₂ (where M = Cu²⁺ or Ni²⁺) which are 5.0 x 10⁻⁶, 2.5 x 10⁻⁵ and 5.0 x 10⁻⁵ mol were used against a constant mole of PHB (5 x 10⁻⁴ mol). In a typical preparation, 10 mL of 0.05 M PHB in chloroform was added to a stipulated amount of 0.05 M MCl₂ in methanol and topped up with methanol where necessary (Table 1). The mixture was stirred for 10 min. Then, the solvent was evaporated via rotary evaporator at room temperature until a viscous solution was obtained (~ 10 minutes). A porous PHB film was obtained upon further air drying the viscous solution at ambient condition. For comparison purposes, a sample with solely PHB mixed with equivalent volume of methanol was also prepared.

| MCl₂ (mol) | Methanol (mL) | MCl₂/CH₃OH (mL) |
|------------|---------------|-----------------|
| 5.0 x 10⁻⁶ | 0.1           | 0.9             |
| 2.5 x 10⁻⁵ | 0.5           | 0.5             |
| 5.0 x 10⁻⁵ | 1.0           | -               |
| NIL        | -             | 1               |

2.3 Characterizations
The possible structural changes of the samples were determined by Fourier Transform Infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The samples were scanned from 400 – 4000 cm⁻¹ using a Frontier Universal ATR Perkin Elmer FT – NIR Spectrometer. The 1H NMR and 13C NMR spectra were analyzed using a BRUKER AscendTM 500 NMR spectrometer. Samples were prepared by dissolving ~ 10 mg of the samples in deuterated chloroform. The percentage of metal content in the samples (expressed as wt % of M) was analyzed using a Perkin Elmer AAnalyst 400 atomic absorption spectrometer. The samples were digested using aqua regia solution and diluted to 50 mL with distilled water. The surface morphology of the samples were characterized by a LEO SUPRA 50 VP scanning electron microscope (SEM) operating at 15 kV. The samples were coated with a layer of gold prior to analysis. The thermal degradation of the samples were analyzed on ~7 mg
sample using a Perkin Elmer STA 6000 simultaneous thermal analyzer at a heating rate of 20 °C min⁻¹. The samples were heated from 30 °C to 920 °C under nitrogen atmosphere. The thermal behaviour of the samples were investigated using a Perkin Elmer DSC 800. Approximately 7 mg of sample were first heated from 30 °C to 190 °C at a heating rate of 20 °C min⁻¹ under nitrogen atmosphere and held for 1 min. The samples were then quenched to -40 °C at a rate of 100 °C min⁻¹ and held for another 3 min. Finally, the samples were subjected to the second heating scan where they were again heated from -40 °C to 190 °C at a rate of 20 °C min⁻¹. The growth of PHB spherulites was observed under a polarized optical microscope, Olympus EX53 equipped with Linkam THM5600 hot stage and thermal programmer. A thin layer specimen was heated from room temperature to 190 °C in the hot stage at a rate of 20 °C min⁻¹. It was annealed for 1 min before cooling to room temperature. PHB spherulites were allowed to grow during the cooling stage and the images of the growing spherulites were acquired using an Infinity Analyze software.

3. Results and Discussion
3.1 Surface Morphology and Porous Texture

The surface morphology and textural properties of the samples are studied by SEM and BET analyses. Typical SEM images of pristine PHB film, PHB film prepared in methanol and films prepared from the various concentration methanol solution of MCl₂ are shown in Figure 1. The surface of pristine PHB (Figure 1(a)) is almost smooth but rougher when methanol is employed during the film formation (Figure 1(b)). There is also no apparent porous texture observed in both samples. However, the interconnected porous structure can be observed when MCl₂/methanol was added during the preparation of PHB film (Fig. 1(c)-(h)). The pores were homogenously distributed throughout the film with size ranging from 0.3 – 2.0 μm. It is also observed that the porosity and pore size of the film increases with the increased in MCl₂ concentration. For instance, the average pore size for PHB film prepared with 5.0 x 10⁻⁶ mol CuCl₂ in methanol was 0.80 ± 0.40 μm. This was increased to 1.29 ± 0.43 μm for 2.5 x 10⁻⁵ mol CuCl₂ and continued to increase to 1.46 ± 0.65 μm for 5.0 x 10⁻⁵ mol CuCl₂. Furthermore, the pore size of the PHB prepared with NiCl₂ was generally smaller as compared to CuCl₂ analogues. This may be due to the larger size of CuCl₂ as compared to NiCl₂. It is also worth to point out that no serious aggregation of MCl₂ salt particles occurred in these samples.

| Additive     | MCl₂ (mol) | #Pore size (μm) | *MCl₂ content (% wt) Before washing | After washing |
|--------------|------------|-----------------|-------------------------------------|--------------|
| CH₃OH        | -          | N/A             |                                     |              |
| NiCl₂/CH₃OH  | 5.0 x 10⁻⁶ | 0.40 ± 0.14     | 4.86                                | 2.47         |
|              | 2.5 x 10⁻⁵ | 0.62 ± 0.24     | 12.31                               | 0.51         |
|              | 5.0 x 10⁻⁵ | 0.73 ± 0.29     | 20.02                               | 0.44         |
| CuCl₂/CH₃OH  | 5.0 x 10⁻⁶ | 0.80 ± 0.40     | 2.29                                | 1.40         |
|              | 2.5 x 10⁻⁵ | 1.29 ± 0.43     | 6.99                                | 2.43         |
|              | 5.0 x 10⁻⁵ | 1.46 ± 0.65     | 11.96                               | 1.82         |
Figure 1: Typical SEM images for films of (a) pristine PHB and PHB prepared in (b) methanol, (c) 5.0 \times 10^{-6} \text{ mol NiCl}_2/\text{CH}_3\text{OH} and (d) CuCl}_2/\text{CH}_3\text{OH}, (e) 2.5 \times 10^{-5} \text{ mol NiCl}_2/\text{CH}_3\text{OH} and (f) CuCl}_2/\text{CH}_3\text{OH}, (g) 5.0 \times 10^{-5} \text{ mol NiCl}_2/\text{CH}_3\text{OH} and (h) CuCl}_2/\text{CH}_3\text{OH solutions}
3.2 Surface interactions

NMR analyses was conducted to study the structural changes in PHB if any. Figures 2 and 3 show respectively the typical $^1$H and $^{13}$C NMR spectra of film of pristine PHB as well as porous PHB prepared in CuCl$_2$/CH$_3$OH. The $^1$H spectrum of pristine PHB shows the doublet signals at 1.25 ppm that was assigned to –CH$_3$. Mirror quartet signals appeared at 2.43 and 2.61 ppm indicate the CH$_2$ (bonded to C=O). Multiplet signals of CH (bonded to C-O) are at 5.20 – 5.27 ppm, whereas singlet signal of H$_2$O was at 1.49 – 1.56 ppm. Similar results was reported in the literature [15]. The $^{13}$C NMR spectrum of pristine PHB displayed four signals for four types of carbon. These are situated at 19.77, 40.80, 67.62 and 169.14 ppm which represents the CH$_3$, CH$_2$, C-O and C=O (due to ester group) respectively. The NMR spectrum for porous PHB prepared in MCl$_2$/CH$_3$OH solution are similar to pristine PHB. Nevertheless, the signals are broader and slightly shifted as compared to pristine PHB indicating only weak physical interaction between MCl$_2$ and PHB existed in the samples.

Figure 2: Typical $^1$H NMR spectra for films of (a) pristine PHB and (b) PHB prepared in CuCl$_2$/CH$_3$OH
Figure 3: Typical $^{13}$C NMR spectra for films of (a) pristine PHB and (b) PHB prepared in CuCl$_2$/CH$_3$OH solution.

Figure 4 shows the typical FTIR spectra for films of pristine PHB and PHB prepared in NiCl$_2$/CH$_3$OH and CuCl$_2$/CH$_3$OH solutions. Pristine PHB exhibits characteristic peaks similar to that reported in the literature [16]. The $\text{–CH}$ stretching are situated at 2976 and 2933 cm$^{-1}$ whereas the $\text{–CH}_2$ and $\text{–CH}_3$ bending are located at 1452 and 1379 cm$^{-1}$ respectively. The intense peak at ~1720 cm$^{-1}$ indicates the C=O stretching. The stretching and wagging of the C-O due to the ester group of PHB chain are at ~1276, ~1228 and 1181 cm$^{-1}$. The MCl$_2$ in PHB films showed no significant difference (i.e. the position of peaks) in the FTIR spectrum as compared to pristine PHB. Hence, it is envisaged that no chemical interactions occurred between the MCl$_2$ and PHB. The MCl$_2$ is only physically interacted with PHB. The FTIR results are in accordance to the NMR analyses as discussed above.
Some FTIR peaks in the spectrum are sensitive to the changes in the crystallinity of PHB. For instance, the peaks at 1228, 1278 as well as 1724 cm\(^{-1}\) are indicative for the crystalline phase in PHB [17]. On the other hand, the peak at ~1181 cm\(^{-1}\) is assignable to the asymmetric stretching of C-O-C backbone in the amorphous phase of PHB [17]. By monitoring the relative peak height of these peaks to a reference peak, the degree of crystallinity/amorphocity of PHB in a sample can be obtained. According to Porter and Yu [17], the C-O-C backbone stretching (\(\nu\)) at 1181 cm\(^{-1}\)) is the peak of interest while -CH\(_3\) bending (\(\nu\)) at 1379 cm\(^{-1}\)) was selected as the reference peak. In this study, the ratio of [C-O-C\(_{\text{amorphous}}\)-CH\(_3\)] for pristine PHB film is 1.20. The porous PHB film prepared in 5 \(\times\) 10\(^{-6}\) mol MCl\(_2\)/CH\(_3\)OH solution showed similar values (i.e. 1.20-1.21) to PHB. However, these are respectively 1.10 and 1.14 for the porous film samples prepared in 5 \(\times\) 10\(^{-5}\) mol NiCl\(_2\) and CuCl\(_2\)/CH\(_3\)OH solutions. Obviously, the amorphocity of PHB decreases with the increase of MCl\(_2\) in the samples. In other words, the crystalline phase increases as more MCl\(_2\) is present during the preparation of PHB porous films.

### 3.3 Thermal Properties

Figure 5 shows the typical TG and DTG curves of pristine PHB and porous PHB films prepared in MCl\(_2\)/CH\(_3\)OH. The onset of decomposition temperature (\(T_{\text{onset}}\)) and temperature at maximum decomposition rate (\(T_{\text{max}}\)) of the samples are presented in Table 2. Pristine PHB was stable up to ~280 °C, beyond which, it starts to decompose [18]. The maximum mass loss of purified PHB occurred at 292 °C. The porous PHB films prepared in MCl\(_2\)/CH\(_3\)OH exhibit a low mass loss of < 5% of the total mass loss at the initial stage (30-150 °C). This relates to the evaporation of adsorbed moisture or trapped solvent in the samples. The major mass loss that attributed to the decomposition of PHB chains is observed at ~280-300 °C. Also, the higher residue as compared to pristine PHB observed (~10 %) is due to the presence of MCl\(_2\) in the samples. As tabulated in Table 3, the values of \(T_{\text{onset}}, T_{50}\) and \(T_{\text{max}}\) for porous PHB films prepared in MCl\(_2\)/CH\(_3\)OH are comparable to that of pristine PHB. This
depicts that the presence of MCl₂ in PHB does not exert significant influence on the thermal stability of PHB.

![Figure 5: Typical (a) TG and (b) DTG curves for pristine PHB (black) and porous PHB films prepared with NiCl₂/CH₃OH (blue) or CuCl₂/CH₃OH (red)](image)

**Table 3**: Thermal data of pristine PHB film and PHB films incorporated with NiCl₂ or CuCl₂

| Sample    | MCl₂ (mol) | T onset (°C) | T 50 (°C) | T max (°C) | T c (°C) | T m (°C) | χc (%) |
|-----------|------------|--------------|-----------|------------|----------|----------|--------|
| PHB       | -          | 281          | 288       | 292        | 53       | 168      | 41.02  |
| PHB-NiCl₂ | 5.0 x 10⁻⁶ | 271          | 287       | 290        | N/D      | 168      | 39.12  |
|           | 2.5 x 10⁻⁵ | 285          | 289       | 291        | N/D      | 169      | 38.85  |
|           | 5.0 x 10⁻⁵ | 278          | 287       | 288        | N/D      | 169      | 51.12  |
| PHB-CuCl₂ | 5.0 x 10⁻⁶ | 277          | 290       | 294        | 54       | 167      | 31.63  |
|           | 2.5 x 10⁻⁵ | 281          | 293       | 289        | N/D      | 169      | 37.36  |
|           | 5.0 x 10⁻⁵ | 281          | 287       | 287        | N/D      | 169      | 43.42  |

Typical DSC thermograms for pristine PHB and porous PHB film prepared in CuCl₂/CH₃OH are depicted in Figure 2. The thermal data including the cold crystallization temperature (T c), melting temperature (T m) as well as the calculated polymer’s crystallinity (χc) for all the prepared samples are tabulated in Table 3. As can be seen in Figure 2(a), pristine PHB clearly showed three stages of thermal transitions. The glass transition of pristine PHB is found at 6 °C, whereas the cold crystallization and melting temperature are respectively located at ~53 °C and ~168 °C [18]. The appearance of cold crystallization peak in the second heating scan of pristine PHB indicates that the crystallization process was incomplete during the cooling step. Some crystallizable PHB segments are thus reorganized to form folded lamellar crystals during the second heating scan [19,20].

It is interesting to note that the crystallization peak are undetectable in all the samples prepared in MCl₂/CH₃OH. Only an endothermic peak that relates to the melting of PHB is observed in their thermograms. As much as 2-20 wt% of metal salt are present in the porous PHB samples as determined by AAS analyses (see Table 2). It is envisaged that the presence of MCl₂ interrupts the molecular attraction between PHB chains. Hence inhibits cold crystallization to occur. Similar phenomenon is reported elsewhere [19]. There are small differences in T m value (Δ T m = ± 1 °C) between the pristine PHB and porous PHB films prepared in MCl₂/CH₃OH. Nevertheless, the pristine PHB exerts a broader range of melting, with a shoulder at ~171 °C as compared to porous PHB film. The
peak is attributed to the melting of crystals formed during the cooling step. However, the shoulder observed in pristine PHB is associated to the melting of crystals formed during the second heating scan.

Figure 6: Typical DSC thermograms (second heating scan) of (a) pristine PHB and porous PHB film prepared in (b) NiCl₂ or (c) CuCl₂/CH₃OH solutions.

Degree of crystallinity ($\chi_c$) of the pristine PHB and porous PHB containing MCl₂ is calculated according to equation (1) [21]:

$$\chi_c = \frac{\Delta H_m}{\Delta H_{100}} \cdot W_{PHB} \times 100\%$$ (1)

where $\Delta H_m$ and $W_{PHB}$ respectively represents the melting enthalpy and weight fraction of PHB in the sample. The $\Delta H_{100}$ is the melting enthalpy of a 100 % crystalline PHB with the value of 146 Jg⁻¹ [18]. Pristine PHB exerts 41 % crystallinity. On the other hand, $\chi_c$ for porous PHB films falls into the range of 32-51 %. Similarly to the result obtained by FTIR, the degree of crystallinity increased with an increase in MCl₂ content in the samples regardless of the type of metal. For instance, porous PHB films with the highest salt loading (20.02 wt% Ni) displayed the highest $\chi_c$ of 51.12 %. This was decreased to 39.12 % when the sample contains only 4.86 wt% of Ni salt. It is also interesting to note that the $\chi_c$ value for sample with lower MCl₂ content (i.e. prepared with ≤ 2.5 x 10⁻⁵ mol salt) is lower than pristine PHB. This may due to the adverse effects that is discussed in the following section.

The growth of PHB spherullite is further investigated using a polarized optical microscope (POM). As shown in Figure 7(a), the POM images of purified PHB features large spherulites with closely ring bands. In the presence of MCl₂, the size of spherulites are much smaller and imperfect, especially at high MCl₂ content (Figure 7(c-d)). This indicates that MCl₂ can act as the heterogeneous nucleation sites to increase the growth rate of spherulites. Similar observation is reported elsewhere [22].
The presence of salt as an additive in PHB sample may influence the crystallinity of PHB in two adverse ways – (1) as the nucleation agent to promote the crystal growth or (2) decrease the mobility of PHB segments and retard the growth of crystal [23]. Generally, the additive with good dispersion provides heterogeneous nucleation sites to initiate the formation of polymer crystallite. In contrast, highly aggregated additive or additive that interacted strongly with polymer reduces the growth of polymer crystallite. In lower MCl2 content, the contribution of (2) maybe higher than (1), hence the $\chi_c$ is slightly lower than pristine PHB. Nevertheless, at high MCl2 content (viz. 5.0 x 10^{-5} mol MCl2), the presence of MCl2 salt encourages more reorganization of PHB chains. This can be clearly seen from POM results. Hence in this study, (1) is the dominant attributes and the crystallinity of the PHB samples increase with the increased in MCl2 content in the samples.

4. Conclusion
PHB films with porous structure have been successfully formed via simple MCl2 induced solvent casting method. The pore size of the films are fall into the range of 0.3-2.0 $\mu$m and it increases with the increased in MCl2 concentration. Generally, PHB prepared with NiCl2 gave smaller pore size as compared to CuCl2 analogues. The presence of MCl2 exerts no significant influence on the thermal stability of PHB but greatly enhance the crystallinity of PHB. FTIR and NMR analyses shown that there is only weak physical interaction occurred between the PHB chains and MCl2 salt. Thus most of
the MCl$_2$ can be easily removed by simple washing. AAS results revealed that there exist < 2.5 wt% of MCl$_2$ in the PHB after washing step.

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