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# In vitro Biostability and Biocompatibility of Ethyl Vinyl Acetate (EVA) Nanocomposites for Biomedical Applications

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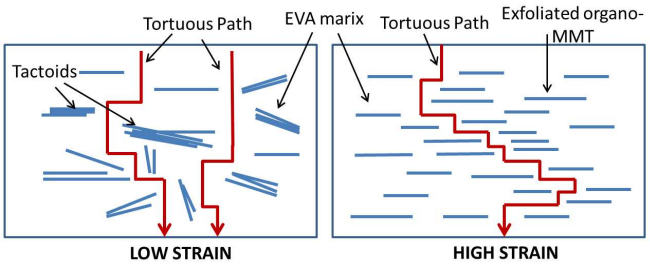



The EVA incorporating organo-MMT performs greater **toughness** and **biostability** at **high strain**, where the organo-MMTs exfoliate and introduce **more tortuous path** for the entering of permeants.

**ABSTRACT**: The in vitro biostability and biocompatibility of Ethyl Vinyl Acetate (EVA) nanocomposites incorporating organically modified montmorillonite (organo-MMT) was investigated as new candidate material for biomedical applications. The in vitro treatment on the neat EVA and EVA nanocomposites was done by immersing the materials in the oxidizing and hydrolytic agents, at temperature of 37°C for 4 weeks. The in vitro mechanical properties of the materials under these environmental challenge conditions were assessed. Based on morphology studies, the quality of MMT dispersion and exfoliation decreased as the nanofiller loading increased. The EVA containing 1 wt% organo-MMT performed the best quality of nanofiller dispersion and exfoliation. The surface features of degradation for this nanocomposite were seen to be the smoothest as compared to the neat EVA and other EVA nanocomposites. Furthermore, the EVA nanocomposites have higher mechanical properties as compared to the neat EVA and these properties were less affected by the in vitro conditions. The best in vitro mechanical properties were achieved when 1 wt% of organo-MMT was added into the EVA. It was postulated that the presence of better dispersed and exfoliated organo-MMT layered structure introduced a more tortuous path for the diffusing of oxidant and water molecules, thereby decreasing their permeability towards EVA molecular chains. Therefore, the kinetic of degradation inside the EVA molecular chains was at lower rate, and resulted in enhanced biostability. Furthermore, the toughness of the hydrated EVA was greatly enhanced when conditioned at 37°C with the presence of the 1 wt% organo-MMT. The biocompatibility assessment suggests that the EVA nanocomposites are not cytotoxic, thus have fulfilled the prerequisite to be further developed as biomedical material.

## 1. Introduction

In this new era, where disease and health problems are the main issue among the community, there is a constant demand for cost-effective and innovative biomedical materials for both medical devices and packaging purpose. However, a problematic issue in the medical field is the limited number of existing biocompatible, biostable and tough materials that offer versatility, exceptional performance, and meet industrial nature. This could probably due to the stringent requirements; properties, design, processing and availability constraints [1,2]. The increasing research and development of quality plastic materials is therefore, vital in order to fulfill the requirements of this diverse medical industry. This is believed to be an area where innovation in new medical nanocomposite materials is needed. Biocompability is a prerequisite for any materials intended for use in biomedical device applications [1,3]. The application of polymer-clay based nanocomposites as biomedical material is hampered, especially when concerning their unknown biosafety. The organic surfactants used as surface modifier could compromise the biocompatibility of these materials [4-6]. For long term safety, the materials should possess biostability to withstand repeat sterilization processes that may involve gamma irradiation, high temperatures, electron beams, and oxidative and hydrolytic treatments [3]. In addition, a material must show excellent chemical resistance, toughness, clarity, and color stability in order to be effectively applied to biomedical applications [1-3]. Meanwhile, for the medical packaging purpose, the material should withstand prolonged mechanical stress and impact forces during transportation and storage. Therefore, the recommended properties are strong, tough, durable, resist tears and ability to protect package contents from physical damage [7,8]. Lack of these properties may result in the occurrence of pinholes in the structure of the packaging materials, especially during transportation and long storage. In worst case scenario, this pinhole defect can cause the entrance and accumulation of bacteria on the medical device and bring health risk to patients. Achieving an effective biomedical nanocomposite material that fits these stringent criteria may be accomplished by carefully examining the structure, morphology and mechanical performance of this material under ambient and environmental challenge conditions.

Ethylene vinyl acetate (EVA) is the copolymer of ethylene and vinyl acetate. EVA combine the chemical and material properties of chemically cross linked elastomer with engineered plastics, which are often much easier and affordable to manufacture. It is an extremely elastic material that can be sintered to form a porous material similar to rubber, yet with excellent toughness [9]. EVA has a long and successful history of innovation in medical packaging, medical devices, and pharmaceutical applications. In fact, EVA has been an innovative material in those applications for over 35 years and its wide range properties can be tailored and manipulated to develop the novel materials with

numerous potential applications [9-11]. For biomedical applications, continuous research and innovations on this material are required for continually improving patient healthcare device and medical support.

EVA nanocomposites are a relatively new class of materials that leverage the benefits of engineered plastics, nanofiller and the properties of elastomer. The incorporation of a small amount of nanometer size particles can significantly affect the final properties of the EVA and offer tremendous improvement in its physical and mechanical performance [12,13]. A review of the current literature suggests that there is only limited research on developing EVA-nanoclay based nanocomposites for biomedical applications. Most of the work focuses on the investigation of the EVA-Nanoclay nanocomposites as general film packaging and flame retardant applications [12-17]. The next generations of medical devices and medical packaging require materials that are very biostable, easily processed and have substantially improved mechanical performance [1,3]. The well formulated EVA-MMT nanocomposites can be potential candidates for several medical/pharmaceutical device and packaging applications including thermoformed trays, containers, boxes, tubes, needle cover, bottle, blister packs, clamshells and bottle caps. Exploration and thorough study on EVA nanocomposite systems is therefore needed to develop this versatile material into innovative products. The targeted property profiles can be achieved through careful formulation of EVA nanocomposite, and optimized EVA -nanofiller interactions. In an effort to achieve this goal, we have performed investigations on structure and properties of the EVA/MMT nanocomposites under ambient and in vitro conditions. Furthermore, the preliminary biocompatibility evaluation was also done as part of assessments to determine their suitability for use in a broad range of biomedical applications. Some morphology and preliminary in vitro biostability and biocompatibility studies are reported and discussed herein.

## 2. Experimental Section

### 2.1. Materials

The matrix material used was Ethyl Vinyl Acetate (EVA), which is a thermoplastic copolymer and solid at room temperature. It is manufactured by UBE-Maruzen Polyethylene Co. Ltd., Tokyo, Japan and commercially known as UBE EVA V215. The weight percent of vinyl acetate is 15%, with the remainder being ethylene. Organically modified montmorillonite (organo-MMT), which contains 35-45 wt. % dimethyl dialkyl (C14-C18) amine as an organic surfactant was used as nanofiller. It was manufactured by Sigma-Aldrich (USA) and supplied by Zarm Scientific and Supplies Sdn. Bhd. It is a beige colour powder with chemical formula of $(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2 \bullet nH_2O$. The average particle size of this organo-clay is $\leq 20$ microns, while the bulk density ranges from 200 to 500 kg/m$^3$. The oxidizing agent used in this research was hydrogen peroxide ($H_2O_2$). $H_2O_2$, 30-32% solution

(Qrec®), and supplied by Qrec (Asia) Sdn. Bhd. Phophate buffered saline (PBS) tablets was manufactured by Sigma-Aldrich and utilized as hydrolytic agent after being dissolved in distilled water.

### 2.2. Samples preparation

The samples were prepared by melt compounding the EVA copolymer with different ratios of organo-MMT nanofiller (0, 1, 3, and 5 wt%) using brabender plasticoder machine (manufactured by Lab Tech Co. (LZ80)). Firstly, the materials (EVA and organo-MMT) were dried in the oven at temperature of 50 ºC for 24 hours. After the Brabender Plasticoder machine reached 160°C, the EVA pellets were fed into the feeder and allowed to melt in 4 minutes. Then the organo-MMT was added and compounded with the EVA melts for further 6 minutes. The resulting nanocomposite samples were cut and weighed to about 20g and then compressed into 1 mm thick sheets using the compression moulding machine model GT-7014-H30C that manufactured by GOTECH Co. The temperature of this process was set at 160ºC, The samples were compressed at 10 KPa for 3 minutes, and then cooled under pressure for another 3 minutes. The samples were then cut accordingly for testing and analysis.

### 2.3. Characterization and Mechanical Testing

#### *2.3.1. X-ray diffraction (XRD) analysis*

XRD analysis on the organo-MMT nanofiller and nanocomposites was done using XRD device model Phaser-D2 manufactured by Bruker company, to characterize basal plane spacing changes in the organo-MMT, due to insertion of EVA molecular chains between the layered structure.

#### *2.3.2. Transmission electron microscopy (TEM)*

The dispersion of the organo-MMT inside the EVA matrix was characterized using TEM. Thin sections of approximately 30-50 nm thickness were cut using a Diatome diamond knife on a Leica Ultracut microtome (Leica Biosystems GmbH) and placed on carbon copper grids. Samples were examined at low magnification (12000x) and high magnifications (93000×) on a LEO 922 A EFTEM (Carl Zeiss AG) operating at 200 kV accelaration voltage.

#### *2.3.3. Scanning electron microscopy (SEM)*

The surface degradation of the neat EVA and EVA nanocomposites was characterized using SEM (Leo 1530 FESEM Zeiss), before and after 4 weeks of immersion in $H_2O_2$ solution (at condition of 37ºC).

#### *2.3.4. Water permeability test*

Water permeability test using PBS solution was done to study the hydration characteristic of the studied materials and relate it with their *in vitro* biostability. Rectangular test samples with a dimension of L= W=3 cm were cut, oven dried for 1 hour to remove the moisture and immediately

weighed to determine the initial mass. Next, the samples were immersed in the beaker containing phosphate buffered saline (PBS) solution and conditioned at 37°C, which approximates human body temperature. The sample mass were recorded after every 7 days of immersion. The hydration characteristic of each material was determined from the percent increase in mass after each weeks of immersion, using the following calculation:

$$\text{Increase in mass (\%)} = (\text{Hydrated mass} - \text{Initial mass}) \times 100 / \text{Initial mass} \quad (1)$$

#### *2.3.5. Ambient and in vitro tensile tests*

The preliminary studies on the *in vitro* mechanical properties were carried out as a first step towards predicting the potential improvements in long-term *in vivo* performance of the EVA nanocomposites for long-term medical device applications. The tensile tests on the neat EVA and EVA nanocomposites were performed using Instron machine model-5582, before and after being immersed in PBS (hydrolytic agent) and $H_2O_2$ (oxidative agent) solutions, to study the effect of hydrolytic and oxidative exposure on tensile properties of these materials. ASTM D638 method was used with ten replicates of dumbbell sample from each material were tested. Mean values for tensile strength, elongation at break and toughness were taken for comparison between materials.

### 2.4. Biocompatibility Test

Chang liver cell line was used to observe the cytotoxicity of the neat EVA and EVA nanocomposites, due to its capability to indicate the hepatoxicity potential when incubated with the polymeric materials. Since liver is an important target of toxic leachable substances, xenobiotics and oxidative stress, this hepatoxicity assessment is considered a very sensitive means of biocompatibility and biosafety testing. Furthermore, this method was chosen due to its lower cost, has relatively well-controlled variables and quantitative results in short time periods [18].

#### *2.4.1. Cell culture*

Chang liver cells were first cultured in a culture medium containing minimum essential medium (Gibco) in Earle's BSS with non-essential amino acids and 1mM sodium pyruvate with 10% fetal bovine serum. Trypsinization and subcultivation of the cells were done by following the standard procedures.

##### *2.4.1.1. Co-cultivation of Chang liver cells on the surface of EVA and EVA nanocomposites: Direct contact toxicity assay*

Cytotoxicity assessment was done by using direct contact toxicity assay. Chang liver cells ($2 \times 10^5$) were grown on the surface of neat EVA and EVA nanocomposites (incorporating 1%, 3% and 5% of organo-MMT). As for negative control, Chang liver cells were grown on the glass cover slips. All

treatments were conducted in 6-well plate (SPL, Korea) for 96 hours and cells were incubated at 37°C in 5% $CO_2$ humidified incubator.

#### *2.4.2. Scanning electron microscopy (SEM) analysis for Chang liver cells observation*

After 96 hours of incubation time, the culture medium was replaced with warm 4% glutaraldehyde in phosphate buffered saline (PBS) and fixed for 30 minutes. The Chang liver cells attached on the surface of neat EVA, EVA nanocomposites and cover slips were then post fixed in 1% osmium tetraoxide at room temperature for 90 minutes. After post fixation, these materials and cover slips with the attached liver cells were rinsed with PBS solution twice and then continued with dehydration process using a graded series of ethanol from 10% to 100%. The specimens were rapidly transferred to critical-point drying device (BAL-TEC 030) in liquid carbon dioxide. After the critical point drying process, all the specimens were attached to 13 mm aluminium stub ducting paint and coated with gold using JEOL JFC-1600 Auto Fine Coater, ion-sputtering device prior to SEM analysis (JEOL JSM-6360LA, Analytical SEM).

## 3. Results and Discussion

### 3.1. XRD analysis on the organo-MMT and EVA nanocomposites

According to Tettenhost and Roberson [19], MMT have large repeat inter-gallery distances, especially when complexed with organic compounds and, therefore, basal spacings occur at small diffraction angles. Basal spacing shifts are relatively more pronounced at small diffraction angles [19]. Therefore, in this study, we focus on the XRD analysis between the 2θ angles of 1.5° to 12°. Figure 1 shows the XRD pattern of the organo-MMT nanofiller, neat EVA and the EVA nanocomposites containing 1, 3 and 5 wt% MMT. Basal spacings were determined using Bragg's law, which is known as $n\lambda = 2d\sin\theta$.

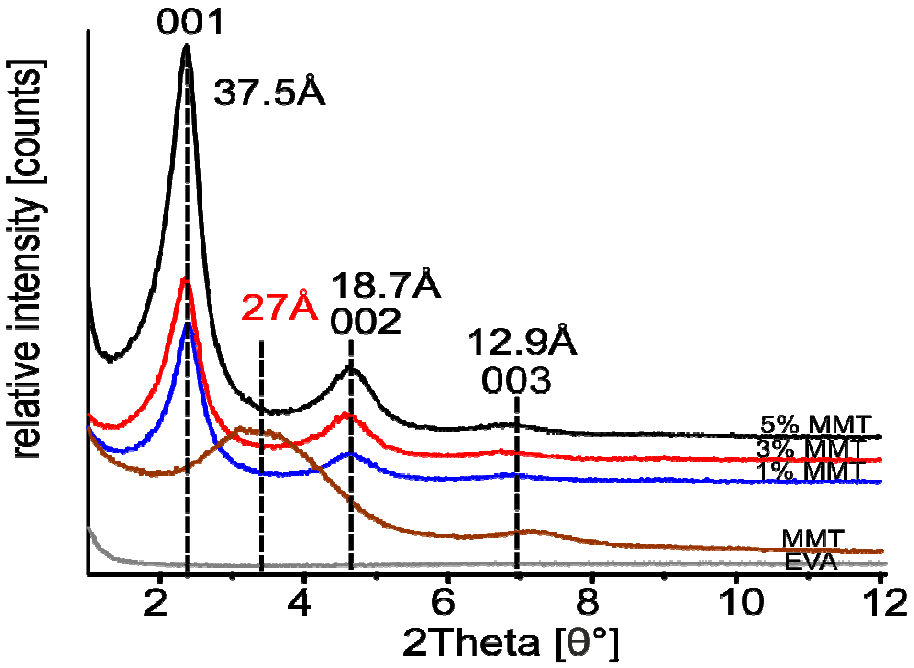



Figure 1: XRD pattern of the organo-MMT and EVA nanocomposites containing 1, 3 and 5wt% organo-MMT

XRD profile of the neat EVA shows an amorphous halo. This is expected, as this copolymer does not show any diffraction peaks between 2θ = 0.5º to 10º [19]. Thus, the diffraction peaks revealed by the EVA nanocomposites in this 2θ range can be associated with the nanofiller basal spacings. The organo-MMT nanofiller exhibits a ($d_{001}$) basal spacing of 2.7 nm. However, there were changes in basal spacing and intensity of the XRD patterns after the incorporation of the organo-MMT into the EVA matrix. These could be associated with the nanofiller loading, average platelet size, orientation, degree of inter-platelet registration, platelet/tactoid alignment, and intercalation by the host polymer [21-26].

It is observed that when the organo-MMT was dispersed in the EVA matrix in 1, 3, 5wt%, the XRD peaks were shifted to lower 2θ angles, which is an indication of increasing inter-gallery spacing between the clay layers, most probably due to EVA intercalation. All nanocomposites (with 1, 3 and 5 wt% MMT) exhibit three well-defined diffraction peaks centered at 2θ = 2.4°, 2θ = 4.7° and 2θ = 6.8°, corresponding to basal spacings of approximately 3.8 nm, 1.9 nm and 1.3 nm. However, the decreased in filler loading (from 5 to 1 wt%) resulted in the lowering of XRD peaks intensity, probably due to better EVA intercalation between the platelets, which produced smaller tactoids. For instance, the nanocomposite with 1 wt% MMT produced the weakest XRD signals, which can be associated with the best quality of MMT dispersion and intercalation in EVA matrix as compared to other systems.

The increase in filler loading resulted in more intense diffraction peaks, which can be associated with the presence of larger tactoids. Bear in mind that in some cases the XRD signature does not differentiate between increased spacing, very small platelets and platelet misalignment, thus misleading results sometimes cannot be avoided. Therefore, the XRD data need to be coupled with the TEM analysis for more complete assessment on nanofiller dispersion in the polymer matrix [21,25, 26]. In this study, the TEM analysis was used to support the XRD analysis and will be discussed in the next section.

### 3.2. TEM analysis of the EVA nanocomposites (dispersity analysis)

TEM images of the EVA nanocomposites containing the organo-MMT in 1,3 and 5wt% are displayed in Figure 2. Generally, well dispersed organo-MMT was seen to be distributed throughout the EVA matrix. The EVA nanocomposites incorporating 1 and 3 wt% MMT exhibit a mixed morphology of exfoliated/intercalated clay layers, whereas the EVA nanocomposite containing 5 wt% MMT exhibits mainly an intercalated morphology. All nanocomposites contained some large silicate tactoids (up to hundreds nm) dispersed inside the EVA matrix. This shows that the shear energy obtained from the brabender mixer was not sufficiently peel some of the platelets form the well-intercalated tactoids. The large organo-MMT stacking platelets (tactoids) with limited mobility and high spatial restrictions is also believed to experience frustrated orientational freedom in the matrix, thus making it more difficult for the intercalated polymer to delaminate them into single layers [25-27].

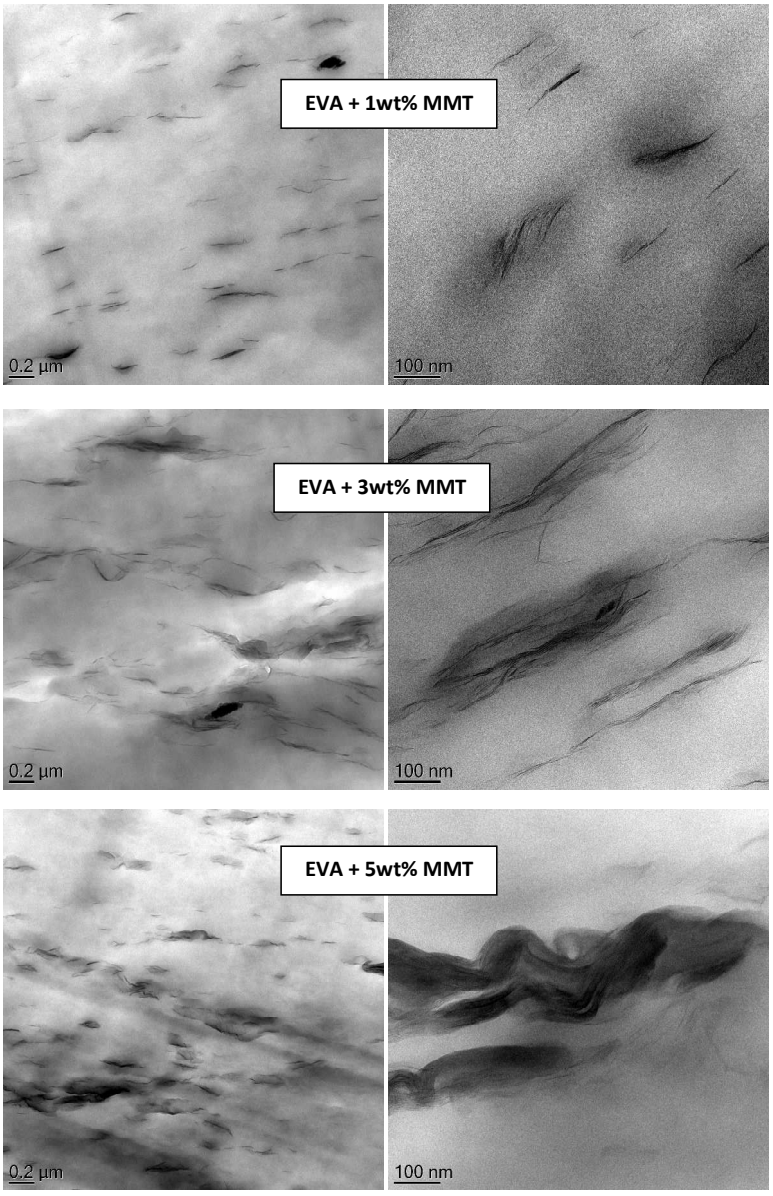



Figure 2: TEM micrographs of EVA nanocomposites containing 1,3 and 5 wt% organo-MMT loadings

It is noticeable that the extent of exfoliation/intercalation decreases as the organoclay concentration increases, which somehow in good agreement with the XRD results. Larger tactoids were observable in the EVA containing higher organo-MMT loading. This might be due to the collision between the nanoclay platelets that increases with the concentration, thus leading to platelet agglomeration [23, 28]. This suggests that, if the nanoclay loading were to be increased to a higher amount, greater degree of agglomeration would occur. As a consequence, the exfoliated structure would not be achieved, resulting in a possible reduction of the mechanical performance of the nanocomposites. This will be further discussed in the following section. It is also interesting to observe the variations in the shape, size and geometry of the nanoclay platelets that were distributed throughout the EVA matrix. These

variations could be probably due to the processes of chemical treatment of the surface, melt compounding and compression moulding that can extend the range of shapes and sizes of the organo-MMT.

### 3.3. SEM analysis on the EVA and EVA nanocomposites (surface degradation upon oxidative exposure)

SEM characterization was performed for all samples before and after four weeks of oxidative exposure. The SEM images obtained and displayed in Figure 3 show signs of surface degradation in the neat EVA and EVA nanocomposites, as a results of oxidative exposure. The surface of the neat EVA sample was smooth and featureless initially, but after being exposed with the $H_2O_2$, rough surface with the present of pits and crack was obtained. On the other hand, the surface features of degradation for EVA nanocomposites containing 1 and 3 wt% were seen smoother compared with that of neat EVA after four weeks of oxidative treatment. Among all, the EVA nanocomposite with 1wt% MMT displays the smoothest surface. Based on the XRD and TEM results, this material also exhibits the best quality of nanofiller dispersion and exfoliation. Thus, it can be said that lower degree of degradation process took place in this particular nanocomposite was resulted from well-dispersed and exfoliated organo-MMT. It was mentioned in previous researches that exfoliated and well dispersed organo-clay can create more tortuous path for the entrance of oxidants into the polymer chain, thereby slower down the kinetic of degradation [29, 30]. However, when the nanofiller concentration increased, the kinetic of degradation also increased due to poorer and poorer quality of nanofiller dispersion and exfoliation (see Figure 2). As expected, EVA nanocomposite with 5 wt% organo-MMT shows the roughest surface morphology, with larger pits and crack, due to more severe degradation.

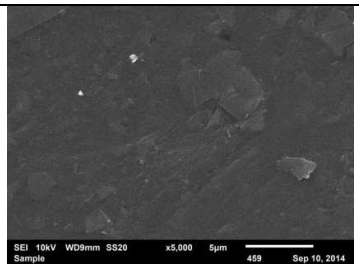
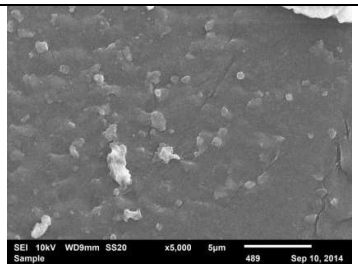
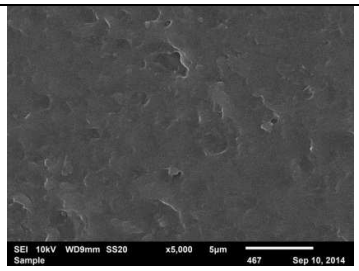
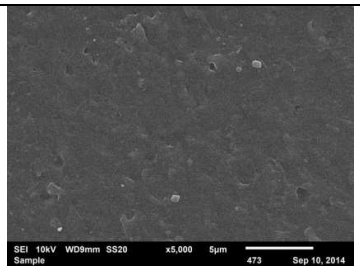
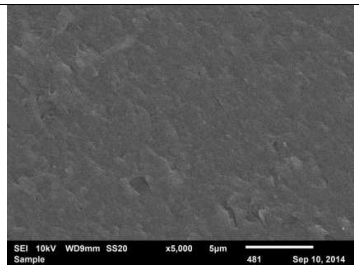
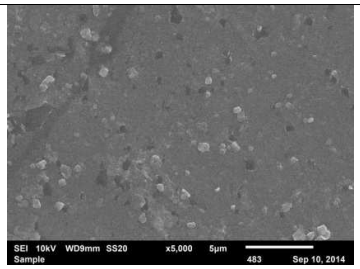
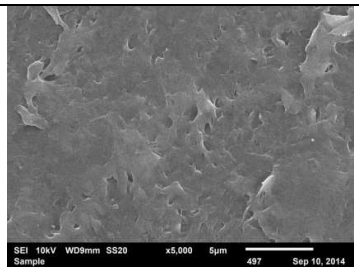
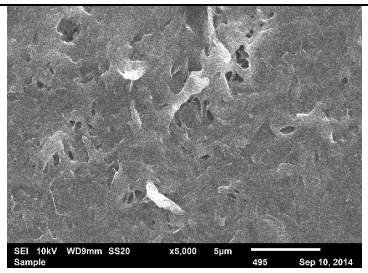
| Material | Before (Week 0) | After (Week 4) |
|---|---|---|
| Neat EVA |  |  |
| EVA+ 1wt% MMT |  |  |
| EVA+ 3wt% MMT |  |  |
| EVA+ 5wt% MMT |  |  |

Figure 3: SEM images of neat EVA and EVA nanocomposites, before and after being exposed in $H_2O_2$ solution at 37°C for 4 weeks. The magnifications are 5000x (scale bar = 5µm)

### 3.4. Ambient and *In Vitro* Mechanical Properties ($H_2O_2$ exposed, 37°C)

The tensile properties of the neat EVA and EVA nanocomposites (before and after oxidative agent ($H_2O_2$) exposure) are summarized in Table 2, while their representative stress-strain curves are displayed in Figure 4. At ambient condition, the incorporation of 1,3 and 5 wt% organo-MMT into the EVA resulted in an increase in tensile strength, elongation at break and toughness. Overall, the highest tensile strength, elongation at break and toughness was achieved when 1wt% organo-MMT was added with an increase of ~16.3 %, ~9.9 % and ~26.6 % respectively. However, increasing the nanofiller loading from 1 wt% to 5 wt% resulted in the reduction of these tensile properties, which is most likely due to poorer quality of organo-MMT dispersion and delamination as observed by TEM (Figure 2) and

XRD analysis. It is worth mentioning that, the level of nanofiller dispersion, nanofiller-polymer interaction, nanofiller orientation and nanofiller content are among the factors that determine the tensile properties of the nanocomposites produced [21-27].

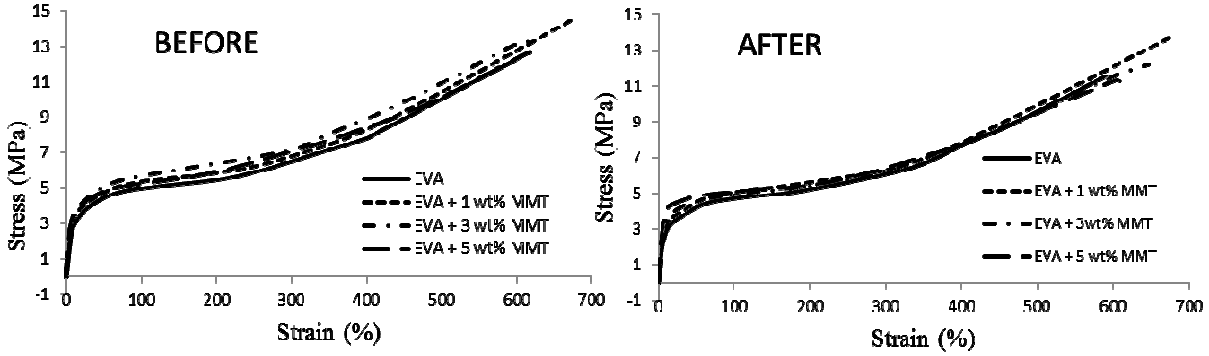



Figure 4: Representative stress-strain curves for neat EVA and EVA nanocomposites: before and after 4 weeks of in vitro exposure (in $H_2O_2$, 37°C)

Table 1: Tensile properties of neat EVA and EVA nanocomposites, before and after 4 weeks of *in vitro* treatment ($H_2O_2$ exposed, 37°C)

| Mechanical Properties | $H_2O_2$ exposure | EVA | EVA + 1wt% MMT | EVA + 3wt% MMT | EVA + 5wt% MMT |
|---|---|---|---|---|---|
| Tensile Strength (MPa) | Before | 12.3 ± 0.7 | 14.3 ± 0.6 | 13.0 ± 0.4 | 12.6 ± 0.1 |
| | After | 11.6 ± 0.7 | 13.7 ± 0.3 | 12.4 ± 0.2 | 11.5 ± 0.5 |
| Elongation at break (%) | Before | 625 ± 27 | 687 ± 25 | 647 ± 16 | 665 ± 32 |
| | After | 613 ± 11 | 687 ± 31 | 646 ± 19 | 607 ± 13 |
| Toughness (MPa) | Before | 44.0 ± 3.3 | 55.7 ± 3.0 | 49.7 ± 1.0 | 50.0 ± 1.8 |
| | After | 40.8 ± 3.1 | 53.0 ± 2.9 | 47.2 ± 0.9 | 42.6 ± 1.9 |

The enhancement in tensile strength of polymer nanocomposite is attributed to the reinforcement provided by the dispersed nanoclay platelets. Better dispersed nanoclay may promote higher tensile strength due to an increase contact surface area and interaction between the platelets and the polymer [25, 31]. The well-bonded interface may enable the load transfer and energy dissipation in an area of high stress. This is proved by our results that show the EVA performed the best improvement in tensile strength when added with the 1 wt% organo-MMT, the loading at which resulted in best quality of nanofiller dispersion. The improvement in toughness of the EVA upon the incorporation of the organo-MMT might be attributed to the plasticizing effect of organic onium ions from the organo-MMT, which effects the conformation of the EVA copolymer chains at the layered platelets-matrix interface.

These would promote relaxation at local stress regions, allowing the material to achieve a higher elongation at break [32].

Our results show that the incorporation of nanofillers provide greater influences on the stress-strain behaviour of the EVA when exposed to the oxidizing agent ($H_2O_2$) at 37°C. The nanocomposites display greater tensile properties when compared with the neat EVA, showing gains on tensile strength, elongation at break and toughness. Interestingly, the nanocomposite incorporating 1wt% organo-MMT is not only displaying the best tensile properties, but also performing the greatest retention in tensile toughness upon oxidative exposure as compared to other materials. As displays in Table 3, the toughness for EVA nanocomposite containing 1 wt% MMT was only reduced by 4.8% after 4 weeks of $H_2O_2$ exposure, while for the neat EVA, the toughness reduced more significantly (7.3%). This suggests that the presence of the organo-MMT layered structure may introduce a more tortuous path for the diffusing of oxidant molecules, thereby decreasing their permeability into the EVA molecular chains. The smaller amount of oxidants entering the polymer molecular chains resulted in greater retention of mechanical properties [29, 30]. The toughness property is very important for materials intended for biomedical devices as it will delays the crack propagation and lengthen the service of the material. Based on these tensile test results, we can suggest that, among all, the EVA nanocomposite with 1wt% MMT is the most biostable material. When increased the nanofiller content to 3 and 5wt%, the biostability decreased as a result of higher organo-MMT agglomeration and larger tactoids.

Table 2: Reduction in tensile strength and toughness of the neat EVA and EVA nanocomposites after 4 weeks of *in vitro* exposure (in $H_2O_2$, 37°C)

| Materials | % reduction of **tensile strength** after $H_2O_2$ exposure | % reduction of **toughness** after $H_2O_2$ exposure |
|---|---|---|
| 0% | 5.7 | 7.3 |
| 1% | 4.2 | 4.8 |
| 3% | 4.6 | 5.0 |
| 5% | 8.7 | 14.8 |

### 3.5. Water permeability (PBS solution exposed, 37ºC)

It is well understood that the factors that can affect the liquid water permeability towards the polymer nanocomposite include nanofiller loading, degree of dispersion, defects at the polymer-filler interface, misalignment of platelets, disrupted molecular packing and an increase in the size of free volume elements in the polymer, which can result in an increased rate of water transmission through the polymer [33-35]. In this study, the water permeability test was performed to investigate the hydration characteristics of the EVA and EVA nanocomposites and its relation with the mechanical properties.

The analysis was done by immersing the samples in the PBS solution at 37°C. The results are summarized in Table 4 and Figure 5. It is observed that the water uptake was highest in the nanocomposites with 5% organo-MMT upon 4 weeks of hydrolytic exposure. This is based on the percent increase in mass (0.34%), which was found higher than the neat EVA. However, the EVA exhibits reduction in water uptake when incorporated with 1% organo-MMT. This nanocomposite displays the lowest percentage (0.08%) of mass increase as compared to other materials. These results are corresponded with the TEM analysis, which indicated the best quality of nanofiller dispersion in EVA matrix was obtained with the sample containing 1wt% organo-MMT. The well-dispersed and exfoliated hydrophobic nanoplatelets may create more tortuous path for the water molecules diffusion, and restrict their entrance into the EVA copolymer chains.

Table 3: The percent increase in mass of EVA and EVA nanocomposites after the *in vitro* exposure (PBS solution, 37°C)

| Material | Increase in mass upon 4 weeks of in vitro exposure (%) |
|---|---|
| EVA | 0.14 |
| EVA + 1wt% MMT | 0.08 |
| EVA + 3wt% MMT | 0.18 |
| EVA + 5wt% MMT | 0.34 |

### 3.6. Ambient and *In Vitro* Mechanical Properties (immersion in PBS solution, 37°C)

The tensile properties of the neat EVA and EVA nanocomposites before and after exposed in the hydrolytic agent, PBS at 37ºC, are summarized in Table 4.6 and 4.7, the representative stress-strain curves are shown in Figure 5. As expected, the nanocomposite incorporating 1 wt% MMT exhibits the best in vitro mechanical properties, due to enhanced water impermeability. This can also be associated with the presence of well-dispersed and exfoliated organo-MMT layers. Interestingly, the effect of the hydrolytic agent on the mechanical properties was different as compared to the oxidative agent. It was found that for all the samples, the tensile strength was decreased, but the elongation at break and toughness were increased when they were in hydrated condition.

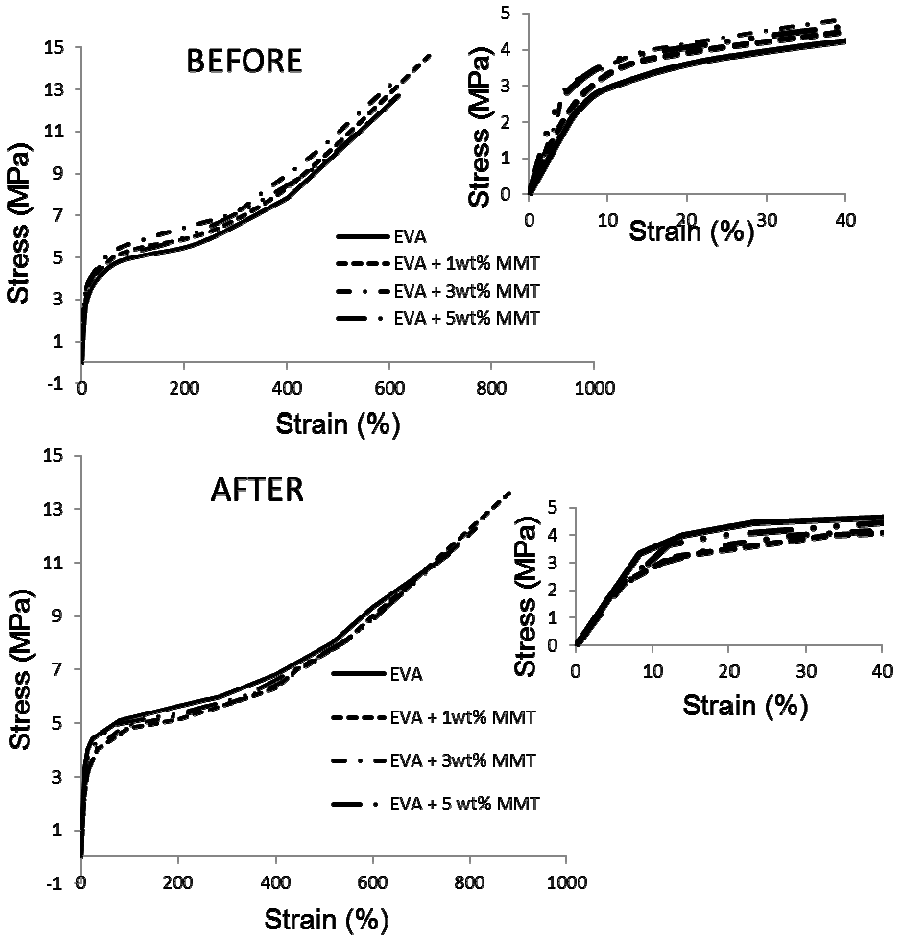



Figure 5: Representative stress-strain curves for neat EVA and EVA nanocomposites: before and after 4 weeks of in vitro exposure (in PBS solution, 37°C)

Table 4: Tensile properties of neat EVA and EVA nanocomposites before and after 4 weeks of in vitro exposure (in PBS solution, 37°C)

| Mechanical Properties | $H_2O_2$ exposure | EVA | EVA + 1wt% MMT | EVA + 3wt% MMT | EVA + 5wt% MMT |
|---|---|---|---|---|---|
| Tensile Strength (MPa) | Before | 12.3 ± 0.7 | 14.3 ± 0.6 | 13.0 ± 0.4 | 12.6 ± 0.1 |
| | After | 11.6 ± 2.0 | 13.8 ± 1.1 | 12.2 ± 0.6 | 12.0 ± 1.3 |
| Elongation at break (%) | Before | 624 ± 26 | 686 ± 24 | 647 ± 15 | 664 ± 32 |
| | After | 768 ± 32 | 875 ± 20 | 812 ± 36 | 808 ± 44 |
| Toughness (MPa) | Before | 43.9 ± 3.3 | 55.7 ± 3.0 | 49.7 ± 1.0 | 49.9 ± 1.8 |
| | After | 51.2 ± 1.6 | 66.2 ± 4.9 | 55.4 ± 4.9 | 54.7 ± 8.6 |

Table 5: Reduction of tensile strength and toughness of EVA and EVA nanocomposites after 4 weeks of *in vitro* exposure (in PBS, 37°C)

| Materials | % reduction of **tensile strength** after PBS exposure | % increasing of **toughness** after PBS exposure |
|---|---|---|
| 0% | 5.0 | 14.1 |
| 1% | 3.4 | 15.9 |
| 3% | 6.6 | 10.2 |
| 5% | 4.9 | 8.6 |

The reduction in tensile strength of the hydrated EVA is expected, as the water molecules can occupy the hydrogen bonding sites and subsequently hindering the secondary bonding between the copolymer chains. The accompanied increase of elongation at break and toughness was probably attributed to an increase in elasticity of the EVA due to plasticization of vinyl acetate upon water inclusion. Water molecules may have greater attraction to the polar vinyl acetate structure, penetrate into the molecular chains and plasticize them [36]. It was hypothesized that this interaction contributes to enhanced elasticity and toughness of the EVA when conditioned at 37°C, as it promotes higher degree of conformation and relaxation at local stress regions when compared with ambient condition (lower temperature). The toughening effect is somehow more pronounced in the EVA containing the organo-MMT (Figure 5). This suggests that the chain segment mobility is further enhanced by the presence of the organic oniom ions from the organo-MMT, especially when less amount of water was absorbed into the structure. This is because; the onium ions can better interact with the polyethylene structure and generate further conformation at the layered platelets-matrix interface. One could possibly questioned, why the stress-strain behaviour of the EVA has been moderately altered by the inclusion of the nanofiller. We provide the explanation based on illustration in Figure 6. At low strain, the presence of tactoids may introduce interference in load transfer efficiency, thus preventing the increase in Young's Modulus. However, when high strain is reached, the organo-MMT exfoliate and resulting in greater number of single nanoplatelets, which are more preferentially align in the direction of the stress and introduce more tortuous path for the diffusing of the permeant (water). We postulate that these are the reasons for the enhancement of ultimate strength, toughness and biostability of the EVA, which are shown in EVA nanocomposite with 1 wt% organo-MMT. Apparently, these molecular interactions are very interesting to be explored, therefore future studies will be done to better elucidate these scientifically.

The best retention in tensile strength and toughness upon PBS solution exposure was achieved when the EVA was added with 1 wt% organo-MMT. The reduction in tensile strength was only 3.4% as

compared to 5.0% for the neat EVA. This is in line with the water permeability data that suggests this material absorbs less water upon 4 weeks of hydrolytic exposure.

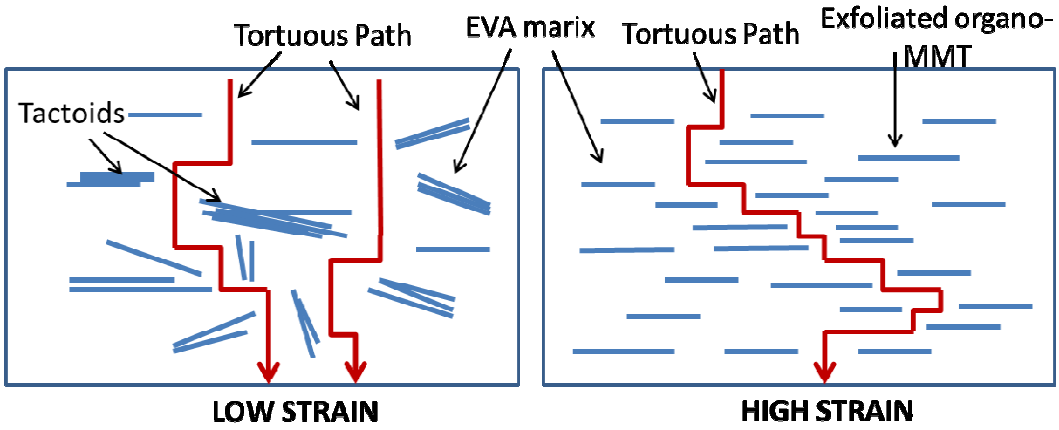



Figure 6: The morphology schematic of EVA nanocomposites at low and high strains

### 3.7. Biocompatibility assessment

The biocompatibility of the neat EVA and EVA nanocomposites was assessed by observing the cell attachment and proliferation of Chang liver cells on the surface of these materials. The interaction of cells by attaching and growing on the surface of the polymers is the initial and important indication of their biocompatibility. Without the cytotoxic substances, the cells may undergo further development such as differentiation and proliferation processes. Based on SEM analysis (Figure 7), we suggest that the EVA and EVA nanocomposites incorporating organo-MMT do not produce leachable substances, contributing for the inhibition of the Chang liver cells growth, during the applied incubation time and conditions. The similar cell morphology observed upon the incubation of the cells with the neat EVA and EVA nanocomposites implies that the biocompatibility of the EVA incorporating the organo-MMT is similar to that of neat EVA. Cells were tightly attached to the surface of the polymers even after reaching 96 hours of incubation time, indicating that no harmful substances were released and caused toxicity to the liver cells. Observation of Chang liver cells under scanning electron microscopy however indicated that cell adhesion was slightly favoured in the EVA incorporating 1 wt% organo-MMT as compared to the neat EVA and other nanocomposites (see Figure 7). Chang liver cells were seen with thicker filopodia attached onto the polymer surface as compared to other polymers with higher percentage of organo-MMT. This might be attributed by the organoclay filler dispersion and interaction with the host EVA, resulted in smoother and homogeneous surface morphology in the EVA+1 wt% organo-MMT nanocomposite sample (Figure 2 & 3), thereby allowing more preferential filopodia growth and attachment. Previous research also proved that the biocompatibility of the host polymer is greater if the dispersion and exfoliation of the organo-clay filler are better [5]. It is also

noticeable that, the cells incubated with neat EVA sample resulted in thin and short filopodia structure extended from the cell cytoplasm, and the cells became rounded in form. The enhancement of filopodia attachment with the addition of 1wt% organo-MMT into the host EVA can be hypothesized as a result of cell adherence to both the EVA and organo-MMT structure. However, the exact mechanism of this phenomenon has yet to be elucidated.

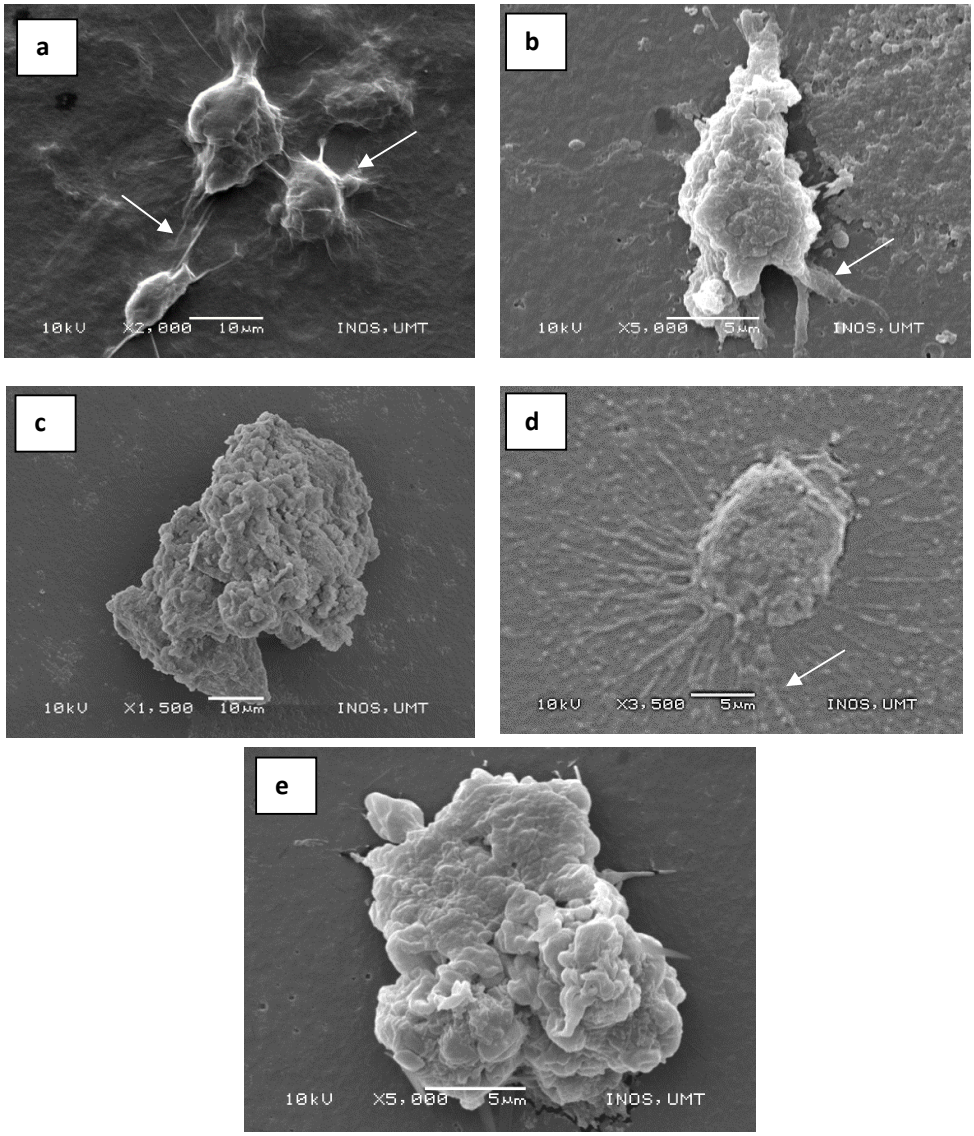



Figure 7: Scanning electron micrograph of Chang liver cells grown of glass cover slip (a), EVA+1wt% MMT (b), EVA+3wt% MMT (c), EVA+5wt% MMT (d) and neat EVA (e) after 96 hours of incubation time. Cells are seen to attach onto the glass cover slip (control) and polymer surfaces. Arrows indicate filopodia structure of Chang liver cells.

As benchmarked with the morphology of the cells attached onto the surface of the control medium (glass cover slip), we can suggest that the EVA nanocomposites do not inhibit the growth and

proliferation of cell even after 96 hours of incubation on the their surface. The ability of the cells to remain attached onto the polymer surface are due to the biocompatibility of the polymers towards the Chang liver cells and it can be concluded that the organo-MMT nanofiller used in this study did not cause hepatotoxicity during this biosafety assessment.

## 4. Conclusion

The concentration of the organo-MMT might affect the arrangement of the MMT nano-platelets, thus their exfoliation and intercalation behaviour in the EVA matrix. It was noticeable that the dispersed organo-MMTs in 1, 3 and 5 wt% have few large stacks (tactoids) that still remain throughout the EVA matrix. This suggests that the shear energy provided by the brabender mixer was not sufficiently break and peel some of the large tactoids into individual clay layers. The quality of MMT dispersion and exfoliation decreased as the nanofiller loading increased. As revealed by both XRD and TEM analysis, the EVA containing 1 wt% MMT performed the best quality of nanofiller dispersion and exfoliation among all the nanocomposites. The SEM images illustrated signs of surface degradation in the neat EVA and EVA nanocomposites upon 4 weeks of oxidative exposure. The surface features of degradation for EVA nanocomposites containing 1 wt% were seen the smoothest compared with that of neat EVA and other nanocomposites after four weeks of oxidative treatment. This can be related with the morphology of this material that shows a better dispersed and exfoliated organo-MMTs distributed throughout the EVA matrix. Lower kinetic of degradation process took place as the diffusion of the oxidant molecules was restricted by greater number of nanoplatelet layers. The tensile properties of the neat EVA and EVA nanocomposites (before and after oxidative and hydrolytic agent) were studied. The best mechanical properties were achieved from nanocomposite with 1wt% organo-MMT, and it was also less affected by the oxidative and the hydrolytic treatment when comparing with the other materials. The superior in vitro mechanical properties of EVA nanocomposite with 1 wt% MMT can be associated with its morphology as indicated by XRD, TEM, SEM and also permeability characteristic. We hypothesized that the well dispersed and exfoliated organo-MMTs (in which have been surface modified with a hydrophobic surfactant) have created more tortuous path for the diffusion of oxidant and water molecules, thereby hindering more severe degradation in the EVA molecular chains. Other than surface degradation features, the superior mechanical properties of this particular material have provided further evidence on its enhanced biostability. The results also demonstrate that the hydrated EVA (conditioned at 37°C) was further toughened upon the addition of 1 wt% organo-MMT. The biocompatibility test using co-cultivation of Chang liver cells method suggests that the EVA nanocomposites did not cause hepatoxicity when incubated with the cells. The incorporation of 1 wt% of organo-MMT into the host EVA resulted in the enhancement of filopodia growth and attachment on the surface of this copolymer. These preliminary studies revealed that the EVA-organo-

MMT nanocomposites are potential materials to be further developed for biomedical applications, thus more research and development on these materials should be proceeded.

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# In vitro Biostability of Ethyl Vinyl Acetate (EVA) Nanocomposites for Biomedical Applications

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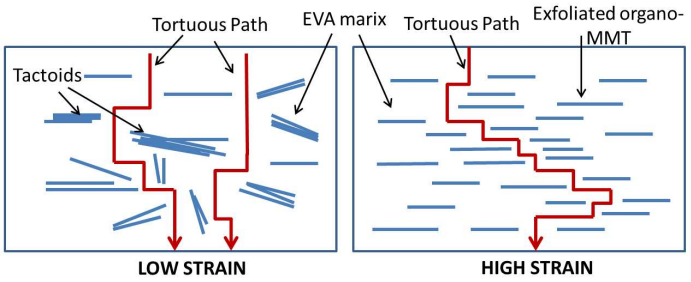


The EVA incorporating organo-MMT performs greater **toughness** and **biostability** at **high strain**, where the organo-MMTs exfoliate and introduce **more tortuous path** for the entering of permeants.