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SYNTHESIS AND CHARACTERIZATION OF CATALYTIC POLYMER-CLAY FILM FOR TREATMENT OF 17α-ETHINYLESTRADIOL

(Sintesis dan Pencirian Filem Polimer-Tanah Liat dengan Pemangkin untuk Rawatan 17α-Etinilestradiol)

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Abstract

Synthetic estrogen such as 17α-ethinylestradiol (EE_2) is considered as one of the pharmaceuticals found in waterways worldwide usually due to human utilization and excretion into wastewater treatment system. In this study, catalytic polymer-clay film was used to treat EE_2 in the contaminated water by oxidizing the pollutant. Polychloromethylstyrene was synthesized along with the clay cloisite to form films that can anchor catalyst Iron-tetra amido microcylic ligand (Fe-TAML). Fe-TAML works efficiently with hydrogen peroxide to activate the iron to higher oxidation state for a film performance. Polymer-clay was then casted, cured, cross-linked and functionalized to form a film that can bind the catalyst into it. Characterization of the films was done using Fourier Transform Infrared (FTIR) to determine the presence of the functional group; preliminary analysis of oxidation study was carried out using UV-Vis Spectroscopy. Furthermore, the toxicity of the oxidized solution of EE_2 after undergoing oxidation with catalytic polymer-clay film was investigated using *Acanthamoeba* sp. to determine its toxicity towards the environment. The results of preliminary oxidation study showed the performance of film in oxidizing the contaminant and the result of toxicity study using MTT assay shows more than 70% of viability of *Acanthamoeba* sp. which indicated that the film was less toxic towards the environment. Hence, this film has high potential for solving the problems of contaminated water.

Keywords: ethinylestradiol, polychloromethylstyrene, Fe-TAML, cloisite, catalytic polymer-clay

Abstrak

Estrogen sintetik yang dikenali sebagai 17α-etinilestradiol (EE_2) adalah salah satu farmaseutikal yang terdapat dalam saluran air di seluruh dunia kebiasaannya disebabkan penggunaan oleh manusia dan perkumuhan ke dalam sistem rawatan air sisa. Dalam kajian ini, filem polimer-tanah liat pemangkin digunakan untuk merawat EE_2 dalam air yang tercemar dengan menumpukan bahan pencemar dan mengoksida bahan tersebut. Poliklorometilsterina di sintesis bersama dengan tanah liat cloisite untuk membentuk filem-filem yang boleh melekatkan pemangkin ferum ligan tetra amido mikrosiklik (Fe-TAML). Fe-TAML berfungsi cekap dengan hidrogen peroksida untuk mengaktifkan ferum kepada keadaan pengoksidaan yang lebih tinggi untuk prestasi filem. Polimer-tanah liat kemudian diacuankan, dirawat, disilangkan dan difungsikan untuk membentuk sebuah filem yang boleh mengikat pemangkin kepadanya. Pencirian filem dilakukan dengan menggunakan spektroskopi inframerah transformasi Fourier (FTIR) untuk menentukan kehadiran kumpulan berfungsi. Tambahan lagi, kajian ketoksikan EE_2 yang teroksida setelah menjalani pengoksidaan dengan filem polimer-tanah liat pemangkin telah disiasat menggunakan *Acanthamoeba* sp. untuk menentukan ketoksikannya terhadap alam sekitar. Hasil kajian ketoksikan menggunakan pengujian MTT menunjukkan lebih dari 70% ia bertahan dan menujukkan filem tersebut kurang toksik terhadap alam sekitar. Oleh itu, filem ini mempunyai potensi yang tinggi untuk menyelesaikan masalah berkaitan air tercemar.

Kata kunci: ethinillestradiol, poliklorometilsterina, Fe-TAML, cloisite, pemangkin polimer-tanah liat

Introduction

Ethinylestradiol (EE_2), an artificial estrogen in oral contraceptives, can be found in inland waterways worldwide [1]. It mainly emerges in wastewater treatment system from human excretion [2] such as urine and through the disposal of wastes including pregnancy test [3-5]. To prevent pregnancy and stabilize menstrual cycle, most women use oral contraceptive. This is known as a huge contributor to synthetic EE_2 in the environment [6]. Based on several studies, human estrogens namely estradiol (E2) and estrone (E1) as well as the artificial estrogen (EE_2) are responsible for a significant part of the endocrine disrupting effects seen in the aquatic environment [7-9]. Compounds that adjust the endocrine system and because harmful effects are referred to as endocrine disrupting chemicals (EDCs) [10]. When EDCs pollute the aquatic environment, they will potentially disturb the endocrine system of animals by altering the natural balance of hormones [11]. EE_2 seems to be more harmful as it has a longer half-life compared to other natural estrogen [12]. Moreover, EE_2 has the possibility to accumulate in biota and sediment [13] where it has been shown that it is endocranially active [14]. The exposure effects of EE_2 in wastewater treatment bring the feminization of male fish based on most research [15]. As stated by Jobling et al. [16] and Harris et al. [17], EE_2 can reduce the reproductive success, leading to the feminization of wild male fish [18].

The method of using hydrogen peroxide with the presence of tetra amido microcyclic ligand (TAML) catalyst is one of the environmentally friendly, money saving and very effective way to help us overcome the unsuitable method of using ozone or activated carbon method to remove pharmaceutical ingredient from wastewater. Iron TAML are fully-functional, small molecule that imitate of peroxidase enzymes at nanomolar concentrations can decompose many micro pollutants. For example, in laboratory studies, TAML/peroxide has been shown to efficiently degrade the persistent drug sertraline via its oxidative metabolites [19], the recalcitrant pesticides trichlorophenol and pentachlorophenol [20], thiophosphate pesticides and many other micro pollutants [21]. TAML/peroxide has been reported to degrade a lot of pharmaceutical based on the preliminary studies on United Kingdom municipal wastewater [2].

The contraceptive pill hormone EE_2 causes serious impacts on aquatic life [22]; therefore, this study investigates the effectiveness of TAML/peroxide and polymer-clay in removing EE_2 and its biological effects. Preliminary studies at high pH (8-10) and high concentrations of hydrogen peroxide (4 mM, 136 ppm) indicate that the removal of EE_2 and other estrogens (estradiol (E2), estrone (E1) all at 80 μM) with TAML (83 nM**)** is possible [23]. In addition, *in vivo* toxicity tests using embryonic zebrafish suggest that TAML was not overtly toxic to fish [24] while *in vitro* tests also showed that TAML did not show inherent endocrine disrupting activity [25].

Taken together, these studies set the stage for the current multidisciplinary, cross-sector study to investigate the removal of EE_2 from laboratory waters and real wastewater, under more environmentally appropriate conditions. These include neutral pH, lower hydrogen peroxide concentrations and estrogen concentrations of more than a million-fold lower than previously tested, which are much more environmentally relevant and still ecotoxic to fish [2].

Organic-inorganic composite-based materials known as clay minerals have been widely used for water purification for decades. They have many characteristics such as high surface area, easily to adsorb and unique chemical composition besides being small in size (nano). However, to make the clay effective in removing the micro pollutant from water, the developments of clay-polymer nanocomposite film have been introduced. The modified polymer or known as Cloisite has been use in this study. Cloisite 10A contains dimethyl, benzyl, hydrogenated tallow and quaternary ammonium and is used with polychloromethylstyrene. Several studies have reported enhanced performance and characteristic of polymeric films by adding clay-based nanomaterials into the film matrix [26-29]. The blending of polymer with clay improved the characteristic in term of optical [30-32], mechanical [33, 34] and barrier properties [35-37].

From this study, polymerization of chloromethyl styrene was used together with clay Cloisite 10A to treat the estrogen known as EE_2. Catalyst Fe-TAML/H_2O_2 helps in the process of heterogeneous catalytic oxidation reaction. In addition, the toxicity studies using MTT assay have been conducted.

Materials and Methods

4-vinylbenzyl chloride was purchase from Sigma Aldrich. N, N-dimethylhexadecylamine, 1,4-dioxane, 1,6-diaminohexane, diethanolamine, dimethyl-formamide and 17α-ethinylestradiol were purchased from Sigma Aldrich. Buffer solution were prepared by using sodium carbonate, sodium bicarbonate and 35% hydrogen peroxide from Merck.

Polymerization of chloromethylstyrene with clay

30 mL of 0.5% NaOH was added to 30 mL chloromethylstyrene (CMS) in separating funnel to remove inhibitor from chloromethylstyrene. This step was repeated for another two times. After that, the CMS were washed by using deionized water for three times until the water turned neutral. This step was followed by adding potassium carbonate to remove excess water. An amount 4% (w/v) of cloisite clay from the volume of CMS were added into flat bottom flask containing CMS and was heated in an oil bath with temperature of under 85 ^{o}C with stirring speed of 150 rpm in nitrogen condition. The CMS was heated until completely viscous. After viscous, methyl ethyl ketone was dropped in the viscous solution and the mixture was dropped added slowly to the volume of 20 mL in the beaker that contained methanol to obtain the polymer flakes. The flakes were then filtered using Buchner funnel and dried for 10 minutes in the oven.

Casting, curing and functionalization of polymer clay film

The flakes were dissolved in the dimethylformamide (DMF) and casted on the backing membrane (polypropylene) to obtain the film. After casted on the membrane and glass, the film was cured for 5 hours at the oven at 85 ^{o}C. Next, the film was heated for 5 hours at 85 ^{o}C in N, N-dimethylhexadecylamine in 1,4-dioxane with a ratio of (6:4). After functionalization, the polymer film was heated in 1,6-diaminohexane in 1,4-dioxane with ratio (6:4) for crosslinking the polymer. End capping step involved heating the polymer film with neat diethanolamine for 5 hours at 85 ^{o}C.

Anchoring of Fe-TAML

The film was immersed in the aqueous Fe-TAML solution (20.0 mL, 50 µM, 1.0 µmole Fe-TAML) until all the absorbed. After that, the film was removed from the solution, washed and immersed on a wire support in fresh carbonate buffer solution (20.0 mL 0.01 M, pH 9.5) and stirred for 15 to 20 minutes. The procedure was repeated for three times. The measurement of amount of Fe-TAML that was not adsorbed from the original solution of Fe-TAML and the amounts that leached into solution after immersion in the buffer solution was measured. To measure this, dye orange II together with hydrogen peroxide were catalyzed by small amount of Fe-TAML and used as a standard condition.

Characterization using attenuated total reflectance-Fourier-transform infrared and scanning electron microscope

ATR-FTIR spectra of the samples were analyzed on a Nicolet 380 FT-IR. The samples were put on the stage to analyzed their functional group. Further, SEM JEOL JSM-5800 was used to analyze the morphology of the film's surface.

Oxidation of EE_2 using catalyzed polymer-clay film

The oxidation of EE_2 was done in a beaker containing carbonate/bicarbonate buffer solution (8.5 mL, 0.01 M, pH 9.5). The EE_2 solution (11.4 mL, 11.83 µM) was added followed by H_2O_2 solution (0.1 mL, 0.2 M) and buffer solution was added until the total volume of the solution became 20 mL. The solution was stirred at 750 rpm and the film was added to the solution. The ratio of H_2O_2:EE_2:Fe-TAML was 200: 1.35: 40.5, while the resulting concentration for EE_2 was 2 ppm (6.76 µM), H_2O_2 (1.0 mM). After 30 minutes, the film was removed, and 1 mg catalase was added to the solution to destroy the H_2O_2 and stirred for another 30 minutes. Peroxide test strip was used to confirm that all H_2O_2 has been destroyed. The solution was then passed through the hydrophilic-lipophilic cartridge from Waters Corporation. The solution was collected for the MTT assay study.

MTT (2 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay of oxidize solution on *Acanthamoeba* sp.

Acanthamoeba sp. (environmental isolate) used in the experiment was isolated from Setiu Wetlands, Terengganu and used in this experiment. The trophozoites were maintained in axenic conditions in 4% protease-yeast-glucose medium and were subculture for a week. MTT assay by Mossman [38] was used in this study to determine the toxicity of oxidized solution by catalytic polymer-clay film on *Acanthamoeba sp.* The first step was subculturing the cell in new flask for 4 days. After 4 days, the cells were ready to be used for the experiment. To start the experiment, all cells and media were transferred to centrifuge tube to sediment the cell. After centrifugation for 15 minutes, the media was removed and replaced with new media. The cell and media contained in the centrifuge tube were transferred in a 96-well plate, a process called cell seeding. After that, the 96-well plate was kept in the oven at 30 ^oC for 24 hours. After 24 hours, the treatment process was done. The media were removed, while oxidized solutions with different concentrations were put in the 96-well plate. There were five replicates with seven concentrations. This process was carried out for 24 hours. After that, the solutions were removed and rinsed with PBS solution. MTT was transferred into the 96-well plate and after 4 hours, MTT was suspended while DMSO was dropped into the 96-well plate. The readings of viability of the cell were analyzed by micro plate reader instrument.

Results and Discussion

Fourier-transform infrared analysis

The FTIR spectrum of polymer-clay film the characteristic absorption peaks due to stretching vibration of C–H in the region 3000-2850 cm^{-1} region which showed alkane functional group, whereas the band around 1645 cm^{-1} was due to stretching vibration of C–C stretch for alkane (Figure 1). The band around 1463 cm^{-1} showed the stretching vibration of C–C in ring or known as aromatic, 1043 cm^{-1} band was due to C–N stretching of functional group aliphatic amine and 808 cm^{-1} band was due to C–Cl stretching vibration and functional group of alkyl halide. These bands showed the stretching from the polychloromethylstyrene-clay films that were functionalized with N, N-dimethylhexadecylamine and 1,4-dioxane, 1,6-diaminohexane with 1,4-dioxane and neat diethanolamine.

Scanning electron microscope

SEM was used to investigate the surface morphology of prepared sample. The polychloromethylstyrene was synthesized with clay Cloisite 10A and casted. The image of SEM (Figure 2) showed that the film has no hole after casting and functionalization which can anchor any catalyst into it for further use. Polymer-clay film was treated with a long chain tertiary amine which reacts with chloromethyl groups converting them into quaternary ammonium functions and followed by crosslinking the polymer chain; end capping the remaining unreacted chloromethyl group. Therefore, Fe-TAML can be anchor to the film.

Viscosity of polymer-clay

Viscosity is the internal friction of a fluid to resist flow. Meanwhile, shear stress is stress component applied tangentially and commonly recognized by sigma (σ) symbol. It is equal to the force vector (a vector has magnitude and direction) divided by the area of application and is expressed in the unit of force per unit area (Pa). Shear rate is the velocity gradient established in a fluid as a result of an applied shear stress and expressed in unit of reciprocal seconds, s^{-1}. Viscosity inconsistent and gives different results. Therefore, to ensure that we can produce the same viscosity of the film, this result can be referred.

$$\text{Viscosity} = \frac{\text{Shear Stress}}{\text{Shear Rate}} \quad (1)$$

Toxicity studies using MTT assay

MTT assay was conducted for studying the toxicity of the oxidize solution that was treated by the catalytic polymer-clay film. After the cells were treated with oxidized solution at varying concentrations for 24 hours at 30 ^{o}C in a 96-well plate, the medium in the plate was discarded. The plate was washed with PBS solution 3 times before loading the MTT solution. This was to ensure that the glucose content in the medium has been completely removed. The presence of glucose can be calculated as shear stress over shear rate. Based on the Figure 3, the shear stress increased as the shear rate increase. The viscosity of different polymers is in the medium will reduce the efficiency of MTT reaction. MTT specific activity can be reduced by cells that extensively metabolized D-glucose. Cytotoxicity of oxidized solution on *Acanthamoeba* sp. was determined based on the percentage of viable *Acanthamoeba* sp. after exposure to oxidized solution for 24 hours at 30 ^{o}C. In general, the viability of *Acanthamoeba* sp. decreased with increasing concentrations of oxidized solution. This shows that the growth of the cells was affected once exposed to oxidized solution. However, the percentage of viability of *Acanthamoeba* sp. as illustrated in Figure 4 showed that more than 70%. This means that the oxidize solution was not highly toxic to the cell and environment.

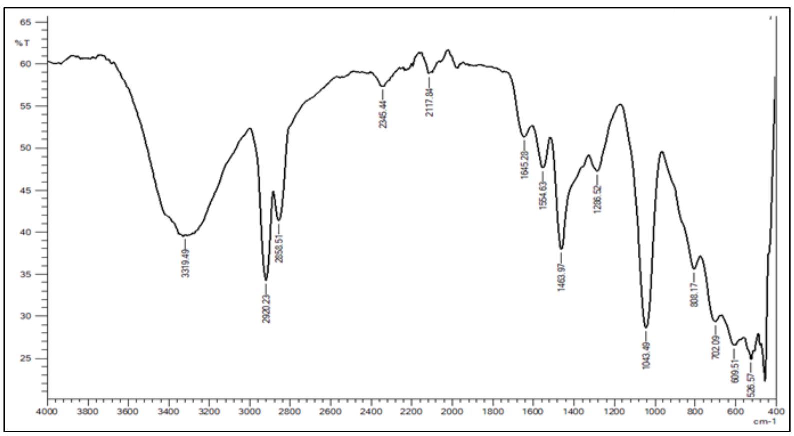

Figure 1. FTIR Spectrum of Polymer-Clay Films after functionalization

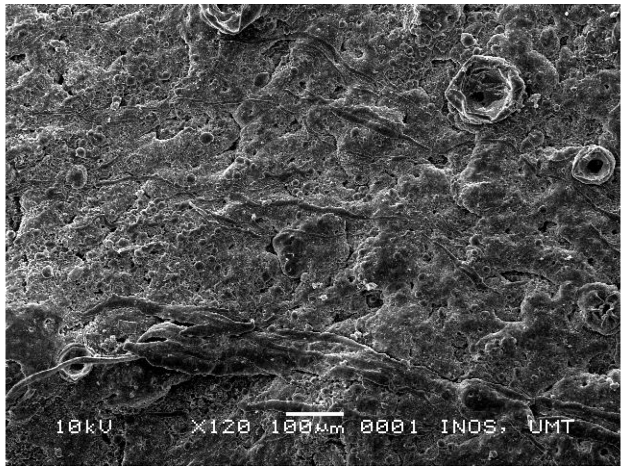

Figure 2. SEM image of functionalized polymer-clay film

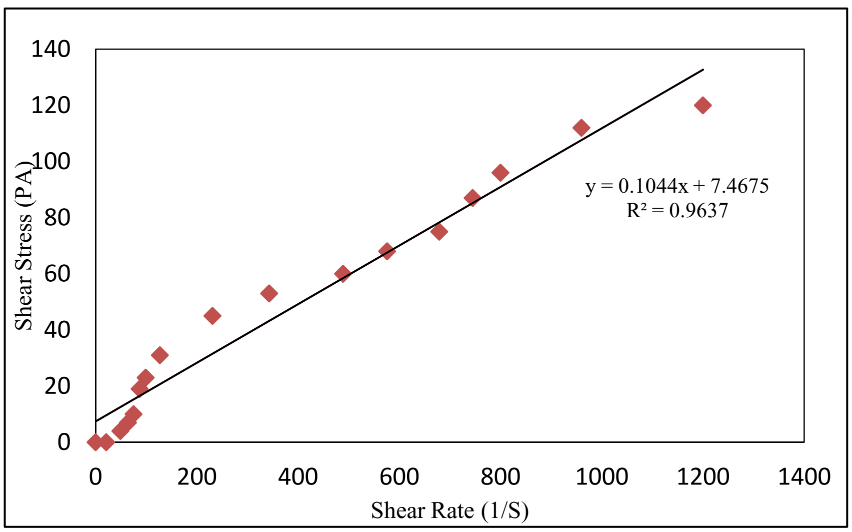

Figure 3. Shear rate against shear stress graph

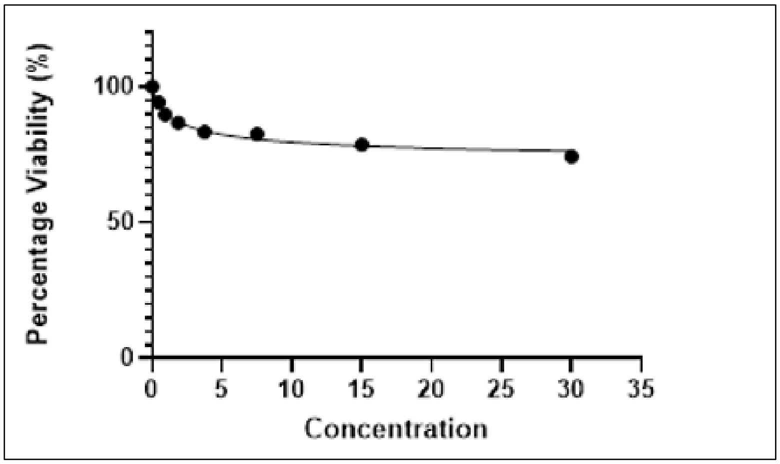

Figure 4. Graph of percentage of viability of *Acanthamoeba* sp. from MTT assay

Conclusion

The catalytic polymer-clay employed in this work in treating 17α-Ethinylestradiol has been successful. Characterization of the catalytic polymer-clay shows that the film contains functional group that helps the catalyst to oxidize EE_2. The results have showed that the production of film does not give harmful effects towards the environment based on the result of toxicity study obtained. Therefore, EE_2 can be treated using polymer and clay in the presence of catalyst.

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