嘉南藥理科技大學專題研究計畫成果報告
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主持人蕭明達　　嘉南藥理科技大學醫藥化學系

中文摘要

為了提升陽離子型聚酯之轉染效率，合成了聚(胺基甲酸酯-酯)(PUE)含有胺基甲酸酯及酯的官能基於主鏈，導入三級胺之官能基於側鏈的之共聚合物，而利用新合成之聚酯類(PEE)此聚酯含有酯及三級胺分別於主鏈及側鏈來當作對照。利用動力光學散射儀測定此兩種陽離子型高分子;皆可以與 DNA 形成奈米級的複合體，從實驗結果中得到 PUE 與 PEE 形成複合體，此時粒徑大小約為 80-115 nm 及 170-180 nm 且帶正電，和 DNA 之重量比分別為 2/1 與 20/1 (polymer/DNA)。水解實驗中可得到 PUE 水解半生期為 20 小時。且從緩衝實驗可得知此高分子具有不錯的緩衝效率。最後利用 COS-7 細胞株做毒性測試與 PEI 做比較可得到一個較低毒性的結果。從上述結果中可得知此高分子可應用於基因治療且可供日後研究之材料。

關鍵詞：轉染, PUE, PEE, 陽離子型高分子, 細胞毒性, 緩衝能力

1、Abstract

To improve the transfection efficiency of cationic polyester, we synthesized poly (urethane-co-ester) (PUE) bearing urethane and ester linkages in the backbone and tertiary amines in the side chain, and polyester (PEE) bearing ester linkages in the backbone and tertiary amines in the side chain. Both poly (urethane-co-ester) (PUE) and polyester (PEE), readily self-assembled with the plasmid DNA (ρ CMV- β gal) in HEPES buffer, were characterized by dynamic light scattering, zeta-potential. The results revealed that PUE and PEE were able to combine with DNA and yield complexes with positive charge of size around 80-115 nm and 170-180 nm at a mass ratio (W/W) of 2/1 and 20/1, respectively. The degradation studies indicated that the half-life of PUE in HEPES buffer is 20 hours. Titration studies were performed to determine the buffering capacities of the polymers. In addition, the COS-7 cell viabilities in the presences of PUE, PEE, and PEI were studies. In this, article, PUE seemed to be a novel cationic poly (urethane-co-ester) for gene delivery and an interesting candidate for further study.

Keywords: transfection, PUE, PEE, polycation, cytotoxicity, buffer capacity

2、Introduction

Non-viral gene vectors have attracted much attention because viral vectors have many drawbacks including the indication of immunological responses, random insertion of viral sequences into the host chromosomes, and recombination events, which can cause virulent viral particals, or limitations associated with DNA size. From there variety of materials, polycations[1-3] have been investigated widely in nonviral gene delivery. Cationic polymers not only condense DNA into nano-particle small enough to enter cell, but also protect negatively charged strands of DNA from nuclease degradation. Several amine-containing polymers such as poly (urethane), [4] poly (β-amino ester), [5] poly (amido-amine), [6, 7] and poly (ethylene imine) (PEI)[8] are positively charged donors at physiological pH. Polyethylenimine (PEI) with high transfection efficiency is generally considered as the standard polycation-based gene delivery, is branched or linear cationic polymer, which mediates gene transfer to a broad variety of cells. PEI has high pH buffering capacity, that is due to the so-called "proton-sponge" effect in which unprotonated amino moieties on the polymer buffer the pH inside the endocytic vesicle. [9] Under physiological conditions PEI, has only 20% of its amines functions protonated, and a further 25% gets protonated in the endosomes where the pH drops to about 5.5, resulting in the some type of endosome buffering effect that helps in the release of the complexes into the cytoplasm.[10] Release of the delivery vehicle from these sections into the cytoplasm is believed to be the limiting step in transfection mediated by many cationic polymers. [11] Molecular weights of polymers and polymer/DNA ratio have been shown too important for transfection efficiency. Molecular weight of polymer affects the affinity between the cation-chain of polymer, and the anion-strand of DNA.[12-14] At is found that polymer chain are too short to effectively and stably condense DNA, but then it has too long retard the "unpacking" of DNA required for transcription and translation. Polymer/DNA ratio controls the ratio of charges, to affect the complexation of polymer with DNA. In our previous researches, we synthesized cationic biodegradable polyurethane containing one tertiary amine in the side chain and backbone as non-viral gane vector. In the study, a new cationic biodegradable poly (urethane-co-ester), bearing urethane and ester linkages in the backbone and tertiary amines in the side chain, was synthesized.

3、Materials and methods

3.1 Materials

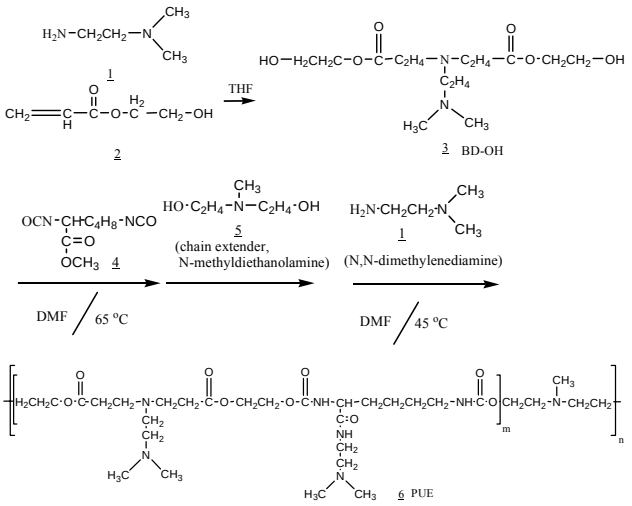
1, 4-butanediol diacrylate was purchased from Lancaster. L-lysine methyl ester diisocyanate (LDI, Kyowa Hakko Kogyo, Japan). 2-hydroxyethyl acrylate (HEA), N-hexane, 2-dimethyiaminoethylamine (DMAE), and glutaraldehyde were obtained from Fluka co. (Switzerland).The solvent of N,N-dimethylformamide (DMF, Tedia co., USA) was dried over calcium hydride and distilled just before use. N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) was obtained from Sigma co. (USA). N-methyl dibenzopyrazine methyl sulfate (electron-coupling reagent) and sodium (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carb oxanilide) (XTT) were purchased from Roche co. (USA). The plasmid pCMV-LacZ (pCMV-βgal),[2, 18] which contained a CMV promoter to drive the β-galactosidase (LacZ) gene expression.[2, 8, 22] The plasmid DNA was amplified in *Escherichia coli* (DH5α strain) and purified by the column chromatography (Qiagen® Plasmid Mega kit, Germany). Monkey SV40 transformed kidney fibroblast COS-7 cells were obtained from American Type Culture Collection (ATCC, CRL-1651). The cells were cultured in the Dulbecco's modified Eagle's medium (DMEM, GibcoBRL Co., Ltd.) supplemented with 10% FBS, 4.5g/L glucose, 1.5 g/L sodium bicarbonate, and 4mM L-glutamine and maintained at 37 °C in a humidified 5% CO_2-containing atmosphere.

3.2 Polymer characterizations

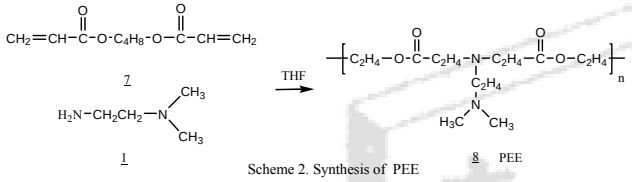
The structures of the polymers were characterized by nuclear magnetic resonance (NMR, Bruker AMX-400 spectrometer) and Fourier transform infrared (FT-IR, Mattson Galerxy Series 5000 spectroscopy). The molecular weight and distribution of polymer was determined by gel permeation chromatography analysis (GPC, Waters Model LC-2410) based on polystyrene standards in THF.

3.3 Synthesis of the polymers

The BD-OH (3), PUE (6) and PEE (8) were synthesized via standard steps process and the structures of PUE (6) and PEE (8) are revealed below scheme 1, 2 :



Scheme 1. Synthesis of PUE



Scheme 2. Synthesis of PEE

3.4 Acid-base titration assay

The extent of pH between 12 and 2 was determined by acid-base titration. 10 mg of PUE was dissolved in 10 mL of 150 mM NaCl, the polymeric solution was added to 100 uL of NaOH, and the pH was recorded. The solution was titrated with increasing volumes of 0.1 N HCl solutions and the data was measured by pH meter. The pH range of the PEE was determined by the same procedure.

3.5 Hydrolytic degradation of polymers

The PEE and PUE were dissolved in buffer solution (pH 7.4 and pH 5.1) with a concentration of 10mg/mL, and then incubated in a water bath at 37°C for various times. After hydrolysis for various times, the solution was dried in vacuum for several hours to remove the water and the molecular weight of polymer was determined by high performance liquid chromatography/gel permeation chromatography (HPLC/GPC).

3.6 XTT assay

The influence of the polymer concentration on the cell viability was evaluated in cell culture for the different polymers. Cytotoxicity of the PEE and PUE in comparison with the PEI was evaluated using the XTT assay.[2, 18] In a 96-well plate, the COS-7 cells were cultured in complete DMEM then seeded at a density of 1.0×10^4 cells/well. The cells were incubated at 37°C and 5% CO_2 in humidified atmosphere for 24 hours. Subsequently, the cells were incubated for one hour in 200 μL FBS-free DMEM containing polymer with various concentrations. The cells were incubated in DMEM only for a negative control. After 1 hour, the cells were washed with 200 μL PBS solution and replaced by complete DMEM for further 48 hours incubation. Then, 50 μl XTT labeling mixture was added to each well and incubated at 37°C for 1 hour. Results were expressed as the relative cell viability (%) with respect to control wells containing culture medium.

3.7 Formation of polymer/DNA complexes

10.0 mg/mL of the polymer was dissolved in the 20mM HEPES buffer (pH7.4) and its serial dilutions were made in which the mass ratio of PUE/DNA (w/w) was from 1/2 to 50/1. And then, the complexes were allowed to self-assemble in HEPES buffer and incubated at room temperature for 30 minutes before measurements.

3.7.1 Characterizations of polymer/DNA complexes

The particle sizes and surface charges of the polymer/DNA complexes were determined by dynamic light scattering (Nicomp 380 system, USA) and the electrophoretic mobility at 25°C with a Zeta-potential system (Nicomp Instrument, USA).

3.7.2 Hydrolytic degradation of Polymer/plasmid DNA complexes

The PEE/DNA and PUE/DNA complexes were prepared at mass ratio of 50/1. The complexes were incubated in a 37 °C water bath and 20 *u*L aliquot samples were removed at various time points and stored at -70 °C for analysis on a 0.7 % agarose gel that was stained with ethidium bromide (0.3 *u*g/mL).

3.7.3 DNA gel retardation assay of polymer/DNA complexes

The PEE/DNA complexes and PUE/DNA complexes were loaded into a 0.7 % agarose gel containing ethidium bromide (0.3 μg/mL) in a tris-acetate-EDTA (TAE) buffer and performed at 100 V for 45 min. After electrophoresis, the DNA bands were visualized by UV-irradiation. The PEE/DNA complexes and PUE/DNA complexes at ratios of 0.5/1 、 1/1 、 2/1 、 3/1 、 4/1、 5/1 、 6/1 、 10/1 、 20/1 、 50/1 (w/w) were prepared.

4、Results and Discussion

4.1 Structural characterizations of PUE (6) and PEE (8)

PUE and PEE were synthesized according to schemes 1 and 2, respectively. In curve (A) of figure 1, the peaks at 1720 cm^{-1} (C＝O, stretching, urethane), 1668 cm^{-1} (C＝O, stretching, amide), 1537 cm^{-1} (N-H, bending, amide), and 3340 cm^{-1} (N-H, stretching, urethane) represent the absorptions of urethane in the PUE. In curve (B) of figure 1, the peak at 1740 cm^{-1} (C＝O, stretching, ester) represents the absorption of ester group of PEE. The chemical shifts of characterized protons of PUE and PEE are listed in Tables 1 and 2, respectively. The chemical shifts of characterized carbons in PUE and PEE are listed in tables 3 and 4. In addition, the GPC date of PUE and PEE showed that the weight-averaged molecular weight were 17600 and 16800 with a polydispersity of 1.8 and 1.7, respectively relative to polystyrene standards in THF.

4.2 Size and Zeta-potential analysis of polymer / DNA complexes

Figures 3 and 4 showed the size and Zeta-potential of the PUE / DNA and PEE / DNA complexes at various mass ratios, determined by the dynamic light scattering (DLS) and electrophoretic mobility at 25 ℃ with the Zeta-potential analysis. The size of complex decreased with the increase of mass content of the polymers until the mass ratio of PUE / DNA reached 2 / 1 and PEE / DNA reached 20 / 1. The results showed that the average diameters (80-115 nm) of PUE / DNA and the average diameters (170-180 nm) of PEE / DNA all fall within the size required for cellular endocytosis. The result demonstrated that urethane was introduced into PUE backbone to increase the condensation ability with DNA. Zeta-potential of the resulting complex changed from negative charge to positive charge with increasing the amounts of PUE and PEE. Mass ratio of PUE / DNA complexes being higher than 2 / 1, the surface of pCMV-β-gal was fully occupied with the PUE molecules to form positive charge complexes, and then PEE / DNA complexes must be higher than 20 / 1. The complexes with extra positive charges on their surfaces allowed better interaction with the target call membrane, resulting in an enhanced uptake.

4.3 DNA gel retardation assay

The electrophoretic mobility behaviors of free DNA, PUE / DNA and PEE / DNA were illustrated by gel retardation in Figure 5. The DNA mobility on agarose gel was influenced by the presence of PUE and PEE. Plasmid DNA was totally retained at mass ratio of (2/1) (PUE/DNA) as indicated in lane 4. Plasmid DNA was partially retained by the presence of PEE at mass ratio of 6 / 1 (lane 8) and totally retained at a mass ratio of 10 / 1 (lane 9). The results suggest DNA could fully completed with PUE and PEE bearing

amino groups to form complexes.

4.4 Buffering capacity of polymers

Titration studies were performed to determine the buffering capacities of the various polymers regarding a proton buffering effect within the endosomal / lysosomal compartments of the cell (Figure 6). All of the polycation solutions had pH 11.5 to 11.8 after added with 1.0 N NaOH. The best nonviral vector is PEI showed buffering capacity over a wide pH range that is probably due to the high amount of amine functions present in the polymeric chain. The initial high pH protonation of the ε–amine groups of PLL can be seen as a horizontal trend above pH 8. The titration curve trend turns nearly vertical below pH 8 suggesting little buffering capacity of PLL, as all the amine groups have already been protonated. The incorporation of tertiary amine content changed the buffering region of the polycation. The data Figure 6 showed pH range of PUE between 11.45 and 5.4 and PEE with pH range from 11.5 to 7.5 because PUE has more tertiary amines than PEE. It was found that the polymer with a high tertiary amine content have a high buffering capacity.

4.5 Cytotoxicity of polymers

The important critical element for the overall transfection efficacy of gene delivery system is cyctotoxicity. Cell damage resulting from a cytotoxic delivery system is deleterious because cell must be capable of supporting translation and transcription. To determine the cytotoxicity of PUE and PEE in comparison with PEI that we performed a XTT assay using the COS-7 cell line. Cells were incubated with increasing amounts of PUE and PEE (ranging from 5 μg/mL to 800 μg/mL) and PEI (ranging from 5 μg/mL to 20 μg/mL). The results showed that PUE and PEE exhibited substantially lower toxicity on COS-7 cells than PEI in Figure 7. The IC_{50} [defined as the concentration resulting in 50 % inhibitory activity (cell death)] for the PEI was around 15 μg/mL, whereas PUE and PEE showed more than 75% viable cells even at a higher dose (800 μg/mL) with no significant change in cell morphology and proliferation relative to controls. We infer that the poly(urethane-co-ester) can provide better cytotoxicity profiles than presently used gene carrier (PEI) due to the introduction of amino groups and urethane units in the polycation backbone alternately to reduce the high charge density of the polycation.

4.6 In vitro hydrolysis of polymer/DNA complexes

Figure 8 showed the degradation profiles of polymers were monitored at 37℃ at buffer pH values of 5.1 and 7.4 in order to approximate the environments within endosmal vesicles and the lysosome, respectively. In general, the polymer degradation was faster at pH 7.4 than at pH 5.1. PUE had a half-life of approximately 32-33 h at pH 5.1. In contrast, PUE was completely degraded in less than 16 h at pH 7.4. There results show higher degradation rate at higher pH and in consistent with the pH-degradation profiles of other amine-containing polyester displayed similar results. From the result, it is found that PUE had a lower degradation rate and longer half-life.

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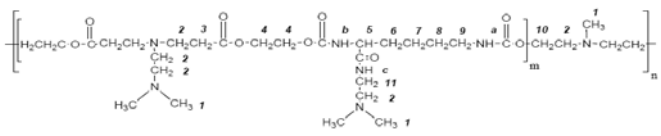
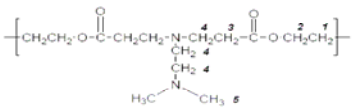
Table 1. ^{1}H NMR Data of PUE

chemical structure of PUE



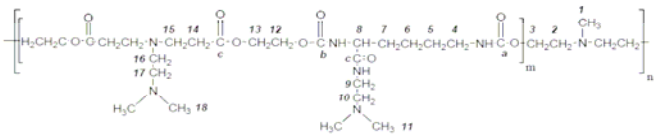
Types of proton	1	2	3	4	5	6,7,8
(ppm)	2.0~2.2	2.3~2.5	3.1	3.7~4.2	3.0	1.3~1.8
Types of proton	9	10	11	a	b	c
(ppm)	3.6	3.7~4.2	3.6	5.3	5.7	7.0



Types of proton	1	2	3	4	5
(ppm)	3.7	4.1	3.1	2.3~2.5	2.2

Table 3. ^{13}C NMR Data of PUE

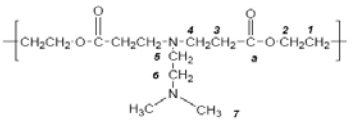
Chemical structure of PUE



Aliphatic carbons									
1	2	3	4	5	6	7	8	9
43.7	56.1	58.8	40.3	22.3	29.2	31.8	53.6	36.5
Aliphatic carbons									
10	11	12	13	14	15	16	17	18
58.6	44.6	57.5	60.2	53.9	59.3	58.9	59.1	44.2
Carbonyl carbons									
a	b	c	d					
156.1	156.7	162.5	174.5					

Table 4. ^{13}C NMR Data of PEE

Chemical structure of PEE



Aliphatic carbons							carbonyl carbon
1	2	3	4	5	6	7	a
57.5	60.2	53.9	59.3	58.9	59.1	44.2	174.5

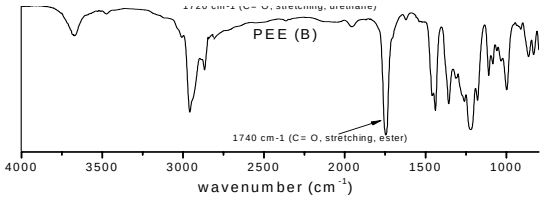


Figure 1. FT-IR spectra of (A) PUE and (B) PEE

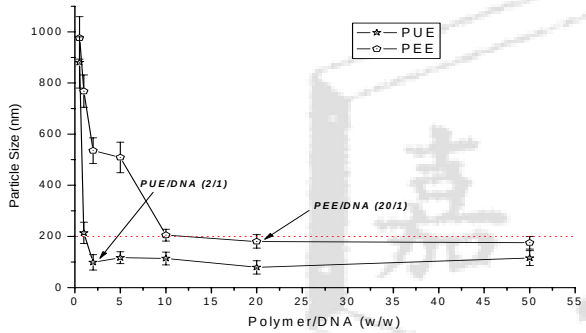


Figure 2. Sizes of PUE and PEE / pCMV-βgal complexes prepared at different mass ratios. Results are presented as mean ± SD (n=3).

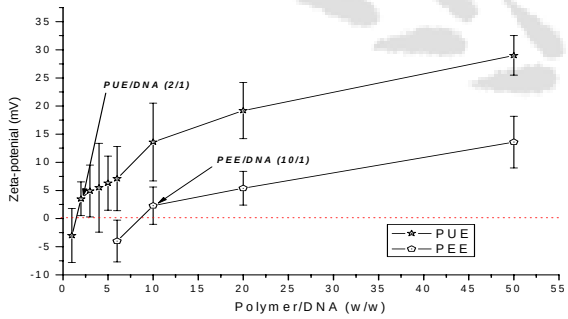


Figure 3. Zeta-potential of AUE and PEE / pCMV-βgal complexes prepared at different mass ratios. Results are presented as mean ± SD (n=3).

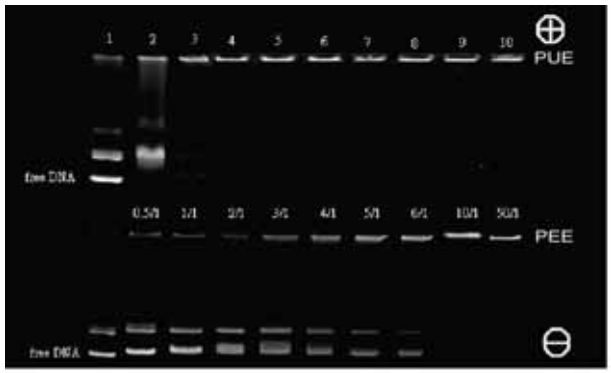


Figure 4. DNA gel retardation assay of PUE and PEE. Lanes:
(1) 400 ng pCMV-βgal;
(2) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):0.5/1;
(3) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):1/1;
(4) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):2/1;
(5) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):3/1;
(6) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):4/1;
(7) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):5/1;
(8) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):6/1;
(9) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):10/1;
(10) PUE/pCMV-βgal and PEE/pCMV-βgal (w/w):20/1.

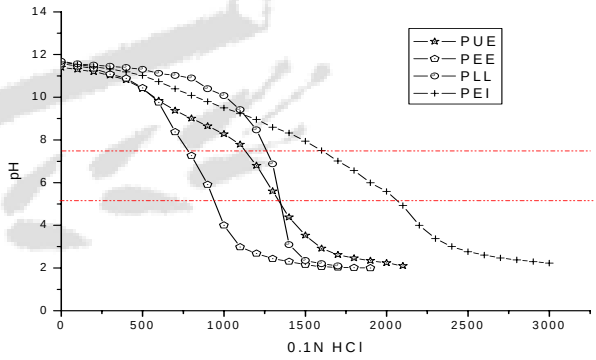


Figure 5. Acid-base titration profile of various polymers with 0.1 N HCl solution.

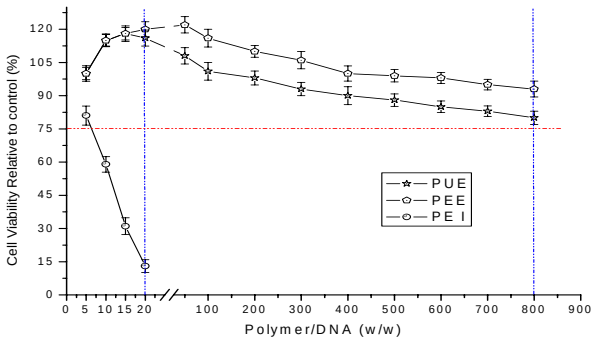


Figure 6. Cytotoxicity of polymer/pCMV-βgal complexes in COS-7 cells. Results are presented as mean ± SD (n=3).

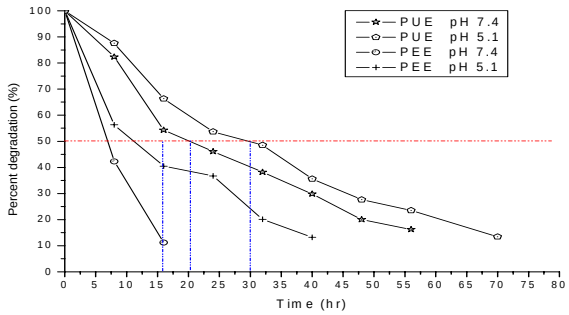


Figure 7. Hydrolytic degradation of PUE and PEE at 37°C at pH 7.4 and 5.1. Degration is expressed as percent degration over