A Study of Biological Significance and Toxicity of Short-Chain Nonylphenol Ethoxylates

(ノニルフェノールエトキシレートの生物的意義及び毒性に関する研究)

Liu Chuang

リュウ　チン

Division of Environmental Science Development

Graduate School of Environmental Science

Hokkaido University, Japan

August, 2015

Contents

List of Tables

List of Figures

A Study of Biological Significance and Toxicity of Short-Chain Nonylphenol Ethoxylates

Abstract

Nonylphenol ethoxylates belong to one of the largest groups of non-ionic surfactants known as alkylphenol ethoxylates. Nonylphenol ethoxylates(NPEOs) represent majority of all alkylphenols produced, and are part of the broader category of surfactants known as alkylphenol ethoxylates (APEOs) of which they represent approximately 80-85% of the volume . Since nonylphenol discovery in 1940, nonylphenol production has increased exponentially, and 100 to 500 million tons of nonylphenol are produced globally every year. Main use of nonylphenol is in the manufacture of nonylphenol ethoxylate surfactants. Nonylphenol ethoxylates have been used in a wide variety of applications including industry (especially in pulp and paper, and textile) as detergents, emulsifiers, wetting agents, dispersing agents, daily goods as surface cleaners, lubricants and shampoos, agriculture (additives in pesticide formulations).

After use and disposal, more than 60% of the products have been discarded into the aquatic environment by discharges of industrial and municipal wastewater. The majority of nonylphenol ethoxylates may degrade in wastewater treatment systems to protect environments. Nonylphenol ethoxylates can be biodegraded as shortening of the ethoxy chain and subsequent degradation to nonylphenol under anaerobic conditions, to short-chain NPEOs (NP_1EO, NP_2EO and NP_3EO) under aerobic conditions.

Although primary biodegradation of nonylphenol ethoxylates is quick, it leads to more toxic and persistent metabolites: nonylphenol (NP), nonylphenol monoethoxylate (NP_1EO), nonylphenol diethoxylate (NP_2EO). They are estimated to be accumulated in the environment. Recently, these biodegradation metabolites have been found to have endocrine disrupting activity and cause harmful effects, including feminization and carcinogenesis on various organisms. Therefore, the use of nonylphenol and its

ethoxylates delivatives had been prohibited in the European Union. However, they are still as the main non-ionic surfactants on a large scale production and use in some developing country, because of NPEOs efficient decontamination ability and its low price.

Biodegradation is the main process for treatment of nonylphenol ethoxylates. Majority of nonylphenol ethoxylates is adsorbed to solids in primary sewage treatment plants. Abiotic processes like hydrolysis or photolysis are negligible in degradation of nonylphenol and short chain nonylphenol ethoxylates in water. Primary nonylphenol ethoxylates are transformed to nonylphenol mono- or di- ethoxylates under anaerobic condition, and nonylphenl carboxylates under aerobic condition, before they are degraded finally into nonylphenol under anaerobic conditions.

In this study, it was revealed that in active sludge process, absorption rates of nonylphenol mono-ethoxylates to nonylphenol tri-ethoxylates were more than 60%. These compounds could be accumulated quickly in sludge within 10 min. It meant that biodegradation process had some roles in treatment of short-chain nonylphenol ethoxylates. After 24 hr, although nonylphenol di-ethoxylates and nonylphenol tri-ethoxylates contents were markedly low, nonylphenol mono-ethoxylates content was relative higher than those of others. It was indicated that nonylphenol di-ethoxylates and nonylphenol tri-ethoxylates were converted to nonylphenol mono-ethoxylates by microorganisms. Furthermore, it could be estimated that absorption process was more than 60% by inactivated sludge system. As short-chain nonylphenol ethoxyaltes could be accumulated rapidly in sludge within 10 min, they gave certain effects on COD reduction.

Nonylphenol and short-chain nonylphenol ethoxylates such as nonylphenol di-ethoxylates are present in aquatic environment as wastewater contaminants, and their toxic effects on aquatic species have been reported. Apoptosis has been shown to be induced by serum deprivation or copper treatment. To understand the toxicity of nonylphenol di-ethoxylates, we investigated the effects of NP_2EO on apoptosis induced by serum deprivation and copper by using PC12 cell system, because it is

well known that apoptosis is induced by serum deprivation or copper treatment in the cells. Nonylphenol diethoxylate itself showed no toxicity, and recovered decreased cell viability caused apoptosis. In addition, nonylphenol diethoxylate decreased DNA fragmentation due to apoptosis induced by serum deprivation and copper treatment in PC12 cells. These phenomena were confirmed after treating apoptotic PC12 cells. In addition, the cytochrome c release into the cytosol was decreased when nonylphenol diethoxylates was added in apoptotic cells. Furthermore, Bax contents in apoptotic cells were reduced after treatment with nonylphenol diethoxylate. From these results, nonylphenol diethoxylate inhibits apoptosis induced in the cells. It has an opposite effect of nonylphenol on apoptosis in PC12 cells, because nonylphenol enhances apoptosis induced by serum deprivation. It was hypothesized that the difference between nonylphenol and nonylphenol diethoxylates might be depending on the differences for the structure of the two compounds. These results suggested that nonylphenol diethoxylate has capability to affect cell differentiation and development and has potentially harmful effect on organisms because of its unexpected impact on apoptosis.

Chapter 1 General introduction

1.1 Introduction

1.1.1 Nonylphenol Ethoxylates

Activities of economic and social benefits for human being lead to various environmental pollutions. Environmental problem has been one of the most serious issues for human being in 21st century. Developing industry, agriculture and other economical activities bring air, water and soil pollutions. Among them, the water pollution is most strict. Majority of wastewater derive from municipal sewage including domestic sewage, industrial effluent and rainfall. They contain large amounts of pollutants such as organic pollutants, surfactants and volatile organic chemicals (VOCs). In addition, endocrine disrupters have to do a lot of attentions because they are toxic, persistent and easy to accumulate in organisms.

Surfactants are a unique kind of chemical compounds which radically alter surface and interfacial properties and represent one of the more ubiquitous contaminants in aquatic system (Schram et al., 2003). Alkylphenols are synthetic organic chemicals, which are widely used in industrial and domestic application. Nonylphenol ethoxylates (NPEOs) belong to one of the largest groups of non-ionic surfactants known as alkylphenol ethoxylates (APEOs) of which they represent approximately 80–85% of the volume (EPA, 2010). Since nonylphenol (NP) was discovered in 1940, NP production has increased exponentially (EPA, 2010, Fiege et al., 2002). Using NP is in the manufacture of NPEOs as surfactants.

NPEOs are one kind of nonionic surfactants produced in large volumes. For about 40 years, due to their surface active properties, NPEOs have been used in a wide variety of applications including industry (especially in pulp and paper, and textile) as detergents, emulsifiers, wetting agents, dispersing agents, commercial and daily goods as surface cleaners, lubricants, and shampoos, and agriculture (additives in pesticide formulations) (CEPA, 2001; US EPA, 2005). A variety of cleaning products, degreasers and

detergents are also available for institutional and domestic use. A typical NPEO surfactant formulation is comprised of NP with an average of 10 ethoxy units, usually within the range of 1 to 20 ethoxy units. Commercial NPEOs are complex mixtures of isomers and oligomers, with hydrophilic chain that mainly consists of 3-20 ethoxy units (Di Corcia et al., 2000).

After use and disposal, more than 60% of the products have been discarded into the aquatic environment by discharges of industrial and municipal wastewater. The majority of NPEOs may degrade in wastewater treatment systems to protect environments. NPEOs can be biodegraded as shortening of the ethoxy chain and subsequent degradation to NP under anaerobic conditions. In this process, short-chain NPEOs (NP_1EO, NP_2EO and NP_3EO) under aerobic conditions during wastewater treatment processes were detected (Ahel and Giger, 1993a; Ahel et al., 1994a, b; Giger and Brunner, 1984). Although primary biodegradation of NPEOs is immediately going, it leads to more toxic and persistent metabolites: NP, NP_1EO and NP_2EO, which are accumulated in the environment (Mann and Boddy, 2000; Planas et al., 2002; TenEyck and Markee, 2007).

Recently, these biodegradation metabolites have been found to be recognized as endocrine disrupters, and cause harmful effects including feminization and carcinogenesis on various organisms (White et al., 1994; Sonnenschein and Soto, 1998; Sumpter, 1998). Therefore, the European Directive No. 2003/53/EC (2003) prohibited the use of NP and its ethoxylates. However, because of NPEOs efficient decontamination ability of NPEOs and its low price, they are still as the main non-ionic surfactants on a large scale production and use in some developing countries. Their accumulation in environment has raised public concern.

1.1.2 The structure and properties of NPEOs

Commercial APEOs are manufactured by the base-catalyzed ethoxylation reaction of ethylene oxide and alkylphenol (AP). The reaction with ethylene oxide is rapid and consumes all of the AP. The structure of NPEOs is shown in Fig. 1.1(Carter et al., 1995).

The structure of NPEOs contains a hydrophilic ethoxylate chain attached to phenolic oxygen and a hydrophobic nonyl part.

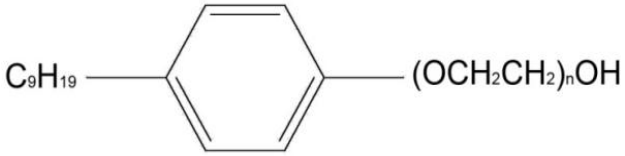
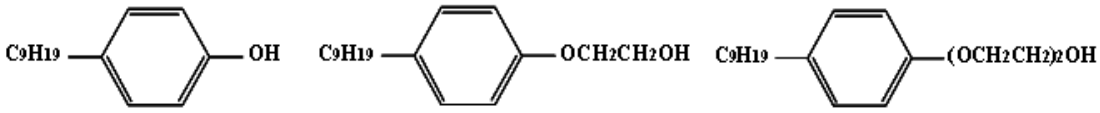


Fig 1.1 The structure of NPEOs

Where n is the average number of moles of ethylene oxide per mole of NP and ranges from 1 to 100. By far the most commercially important AP is NP. NP is used primarily to produce NPEOs surfactants; other uses include phenolic resins, rubber curatives and antioxidants. NPEOs account for about 80% of the total APEOs market. Also significant commercially are octylphenol (about. 15-20%), dodecylphenol and dinonylphenol ethoxylates (about 1-2% each). NPEOs' main degraded products are NP、NP_1EO and NP_2EO. The structures are as follow,



NP **NP_1EO** **NP_2EO**

Fig 1.2 Structure of NP、NP_1EO and NP_2EO

Water solubility is directly proportional to n. NPEOs is water soluble for values of n > 7. The NPEOs most commonly used in cleaning products has n = 9-10 (NP_9E0, $NP_{10}EO$). Solubility of NP and the lower NPEO at 25℃ is shown in follows; 5 mg/L (NP), 3 mg/L (NP_1EO), 6 mg/L (NP_3EO), 10 mg/L (NP_5EO) (Ahel and Giger, 1993b).

The physicochemical properties of NPEO metabolites are listed in Table 1.1.

Table 1.1 The physicochemical characteristics of NPEO metabolites

	NP	NP_1EO	NP_2EO
Molecular formula	$C_{15}H_{24}O$	$C_{17}H_{28}O_2$	$C_{19}H_{32}O_3$
Molecular weight	220.35	264.41	308.46
Water solubility (mg/L, 25 ℃)	5.42	3.02	3.38
Log Kow water partition coefficient	4.48	4.17	4.21
Organic carbon adsorption coefficient (Koc)	240.470	288.403	151.356

The main characteristics of NPEOs are shown as follows (Witorsch et al., 2002):

(1) Surface tension is altered with the change of the number of ethylene oxide (EO). With the increase of the value of the chain of EO, the surface tension of the aqueous solution is increased.

(2) NPEOs have the properties of chemical stability, acid-resisting, alkali-resisting and high temperature resistance.

(3) They can be used in metal pickling and strong alkaline detergent.

(4) They can be used as penetrating agent, emulsifier, detergent and peeling agent of dyeing and so on.

(5) They show stability for the antioxidant. They are not easy to be oxidized by some high oxidants such as sodium hypochlorite, borate and peroxide.

(6) These compounds are not easy to be biodegraded as compared with other nonionic surfactant.

1.13 NPEOs in daily life

Due to the characteristic of the structure of NPEOs, they have the resistance to washing, wetting, and dispersion. They are widely used in industry, agriculture and household as detergents, emulsifiers, wetting agents, dispersing agents, surface

cleaners, lubricants, shampoos and other products (Fig. 1.3). These products have numerous applications, including controlling deposits on machinery, cleaning equipment and scouring fibers, as wetting and de-wetting agents, in dyeing, in machine felt cleaning and conditioning and in product finishing. Because of low price of them, NPEOs were once commonly used in household laundry detergents. EPA and the detergent manufacturers have cooperated to eliminate this use. However, NPEOs are still widely used in large quantities in industrial laundry detergents and have some additional uses which lead to releases to water.

NPEOs are an effective and economical emulsifier, wetting agent, dispersant and solubilizer. When NPEOs are used in formulated cleaning products, the contents of NPEOs can range up to 20% in liquid laundry detergents, and down to less than 1% in hard surface cleaners and personal care products. NPEOs contribute to the performance of cleaning products by penetrating through oily soils, dislodging them and carrying them away, leaving the surfaces clean. Different applications of NPEOs are shown in table 1.2 in European Union market.

Table 1.2 Different applications of NPEOs in European Union market, based on the survey results of the European Chemical Industry Council (Cefic) (Groshart, 2001b).

Functional use in EU	%
Surface active agents	46.1
Cleaning/washing agents	44.7
Foaming agents	2.8
Flotation agents	1.7
Construction materials and additives	1.4
Dust binding agents	1.5
Intermediates	0.2
Plant protection products, agricultural	0.1
Others	0.1

Fig. 1.3 The application of NPEOs

As shown in Fig. 1.3, many studies have reported about the wide uses of APEO metabolites in the environment (Ahel and Giger, 1985; Naylor et al., 1992; Bennie et

al., 1997; Ferguson et al., 2001; Tabata et al., 2001).

1.1.4 The fate of NPEOs and its degradation products in the environments

An annual growth of 1.5% of NPEOs has been expected to continue to drive the NP market (Kirschner, 2007), even though developed countries like as Canada, EU member countries, Korea, Taiwan and USA have enacted regulations or guidelines to limit or restrict the use of APEOs for cleaning and washing detergents. NPEOs and their degradation products (NP, NP_1EO and NP_2EO) are not natural sources; their existences in the environments are mainly caused by human activities. A total of 190-950 tons of NPEOs per year and 5-16 tons of NP per year are still used in Finland (Mehtonen et al., 2012).

The variations of physical/chemical properties of NP/NPEOs and their rapid degradation to other metabolites make their environmental fate extremely complex. They are widely existed in environments such as air, water, soil, sludge and sediment.

1.1.4.1 Air

NP and NPEOs are semi-volatile organic compounds，which are not expected to readily volatilize into air and degrade rapidly in the atmosphere. Dachs et al. (1999) detected for the first time NP in all atmospheric samples collected from the urban and coastal regions of the Lower Hudson Estuary for the first time. NP was also detected in the air of urban area, industrial region and coastal region affected by discharged pollutants of sewage. It was predicted that NP may volatilize from water to the air in areas where NP concentrations are elevated in surface waters. The U.K. Environment Agency (1998) has estimated 0.3 days as a half-life for the reaction of hydroxyl radicals with NP in the atmosphere, indicating that it would be unlikely for any NP in air to be transported to remote regions. NPEOs are far less volatile than NP, and thus it is expected that they would not partition to the atmosphere. Because of the presence of NPEOs in aerially applied pesticide formulations, however, it is needed to determine their atmospheric chemistry, photochemistry and fate. Rudel et al. (2001)

determined NP, NP_1EO and NP_2EO concentrations in the dust of residence and office among 2.3-7.82 μg/g, 2.5-14.5μg/g and 0.82-1.45μg/g, respectively.

1.1.4.2 Water and sediment

In general, NPEOs and NP cannot readily biodegraded by using laboratory standard test methods. Substantial biodegradation will occur after a period of acclimation. NPEOs are, therefore, inherently biodegradable, and their mechanisms involve in stepwise loss of ethoxy groups to lower NPEO congeners, followed by the production of NPEC and NP, depending upon experimental conditions (Rudling and Solyom, 1974; Maki et al., 1994). During the degradation process, some metabolites and final products of metabolism are more toxic and persistent than the primary NPEOs; however, some researchers indicated that these compounds can also be ultimately biodegraded by the adequate methods. It was also noted that the use of high concentrations of the chemicals in biodegradability tests may result in artificially high risk if the chemicals poison the test organisms. This possibility has been suggested to account for the differences between results for the biodegradability of NPEOs (e.g., U.K. Environment Agency, 1997).

It has been reported that full-scale municipal wastewater treatment plants (WWTPs) can provide greater efficiencies for the removal of NPEOs than bench-scale systems, which may due to a greater variety of microbial populations and nutrients in the WWTPs (Holt et al., 1992).

Wastewater from industries or agricultures containing NPEOs is usually entered to conventional activated sludge treatment plants, where they are partially degraded to short chain NPEOs and other metabolites such as NP (Hayashi et al., 2005).

In general, primary biodegradation of NPEOs in WWTPs is readily completed; however, ultimate biodegradation is difficult. Substantial differences in treatment efficiencies for NPEOs and their degradation products exist in WWTPs. These differences have been attributed to the degradation process of NPEOs in influent streams and WWTP design and operating conditions, including temperature of

treatment. The persistent products such as NP and lower-chain NPEOs have been observed in WWTP final effluents and receiving waters. In addition, substantial concentrations of NP and lower-chain NPEOs are found in sludge from WWTPs. The application of NP-containing sludge to agricultural land may result in potential exposure in terrestrial environments. In general, primary biological degradation of NPEOs is the major pathway and occurs more rapidly in WWTPs than in natural environments, because the concentration of microorganisms in WWTPs is higher than in natural environments. Most municipalities in Canada have some type of wastewater treatment. WWTPs play a significant role in the transformation and degradation of NP and NPEOs before their entry into the environment. More than 60% of the higher-chain APEOs in WWTPs exit as stable metabolites (e.g., AP and short-chain APEOs) in either their effluents or their sludge (Ahel et al., 1994a). Short-chain NPEOs (n = 1 to 2) and NP have been detected at relatively high concentrations in sewage influents, ranging from 0.69 to 280 μg/L (Maguire, 1999; Loyo-Rosales et al., 2007).Furthermore, they could be accumulated in sediment and sludge depending on because their physiochemical properties.

1.1.4.3 Soil

Providing sludge to agricultural land is an easy way of sludge treatment for sewage treatment plant. At present, it is good way to restore soil fertility in all over the world. However, this disposal ways cause eventually the organic pollutants in the sludge, including NPEOs and NP are introduced into the soil environments. Studies have shown that NPEOs and NP in the sludge concentration were 1 ~ 680 mg/kg and 1.8 ~ 2530 mg/kg, respectively (Thiele et al., 2001). About NP and NPEOs behavior in the soil, Marcomini et al. (1989) reported that initial concentrations of NP, NP_1EO and NP_2EO in sludge soil for a year were 4.7 mg/kg, 1.1 mg/kg and 0.1 mg/kg (dry weight), respectively. According to above concentrations, it was indicated that NP and short chain NPEOs are widely existed in various environment.

1.2 Degradation study of NPEOs

Contaminants in the water environments are usually removed by following ways; absorption, volatilization and degradation. Possibility of NPEOs volatilities is not so high. The main removal pathways of NPEOs are absorption and degradation. In general, they are divided into physical method, chemical method, biological method, and combined methods with above mentioned methods.

1.2.1 Physical and chemical methods

It was indicated that activated carbon could adsorb $NP_{0\sim2}EO$ (Nevskaia et al., 2001); however, absorption efficiency for removal of NPEOs was restricted by contaminated organic compounds, pH and temperature. In addition, the cost using activated carbon is high.

Other convincing methods for removing organic compounds include use of oxidant reaction. At present, advanced oxidation was widely applied by using reactions of free radical production which include ultrasonic oxidation method, electrochemistry method, photochemical method and catalytic oxidation method. However, these methods consume large amounts of energy (Yim et al., 2003; Kuramitz et al., 2002; Deborde et al., 2005).

1.2.2 Biological method

Biodegradation mainly utilizes dominant bacterial community or engineering bacterial communities to degrade targeted compounds. Some studies have revealed to isolate dominant bacterial communities to be shortening EO chain of NPEOs (Liu et al., 2007). It is confirmed that to degrade NPEOs is better way than others because of its high efficiency and cost saving. Until now, it has been widely developed methods for biodegradation of NPEOs.

1.3 Toxicity study of NP and NPEOs

There are many chemicals which disrupt the endocrine systems of fish, wildlife, and

humans, thereby adversely affecting their growth, development, reproduction, and health. Although the evidence about precise endocrine disrupting mechanism is not yet unequivocally conclusive, the occurrence of environmental endocrine-disrupting chemicals (EDCs) has been linked to the feminization of male fish, and sex transposition in wildlife (Tyler et al. 1998), to decline in sperm counts in men (Sharpe 1998), and to increase of hormone-related cancers in humans (Soto et al. 1994).

1.3.1 Endocrine disrupting properties

Similarities in estrogenic structure of NP and short chain NPEOs as well as other synthetic compounds were being studied, and were recognized as early as 1938 (Ying, 2006). Later findings showed that some chemicals and its degraded products are weakly estrogenic activity in vitro and in vivo to mammals and aquatic organisms (in particular fishes). It has been concerned about human health and species preservation in environments. The degraded product, NP and short chain NPEOs found in sewage effluent and sludge are even more toxic than the parent chemical long chain NPEOs (Jobling and Sumpter, 1993).

Due to structural similarities between NP and short chain NPEOs, they are able to mimic estrogen hormone through a direct binding with the estrogen receptor, blocking the action of the original hormone. It may cause reproductive diseases. For this reason NP and short chain NPEOs are listed as endocrine disrupting chemicals.

The parent NPEOs surfactants, short chained intermediates and NP exhibit various degree of their estrogenic potency and toxicity. Health Canada has published a priority substance list about NP and its ethoxylates. Based on a number of findings from acute and chronic studies, the relative estrogenic effects and toxicities are shown when the relative estrogenicity factor (17β-estradiol-equivalent) of NP is defined as 1.0 as listed in Table 1.3 (Health Canada, 2007)

Table 1.3 Relative estrogenicity based on 17β-estradiol-equivalence of NP and relative toxicity of NP and NPEO degraded products (Health Canada, 2007).

Chemical	Relative estrogenicity to NP (based on 17β-estradiol)	Relative toxicity based on acute and chronic data
NP	1	1
NP_1EO	0.67	0.5
NP_2EO	0.67	0.5
NP_nEO ($n \geq 9$)	0	0.005
NP_1EC	0.63	0.005
NP_2EC	0.63	0.005
OP	4.1	1
OP_1EC	0.63	0.005
OP_2EC	0.63	0.005

1.3.2 Toxicities of NP and NPEOs

It is well known that NP and short chain NPEOs can interact with some proteins. They may change the structure and activity of enzymes. Toxicities of NPEOs are increased in proportion of shortness of EO chain length. LC_{50} values for NP and NP_9EO are reported to be 190 μg/L and 14,000 μg/L, respectively. Toxicities of NPEOs to aquatic organisms tend to decrease with increasing degree of ethoxylation.

1.4 Aims and objectives

The one of the purposes in the current study is to develop an effective biological method technique for the removal of short chain NPEOs from the aquatic environments. Main purpose of the study is to clarify toxic mechanism of short chain NPEOs using apoptotic reactions in PC12 cells. To attain this purpose, individual objectives of the research are shown as follows;

1. To clarify aerobic degradation of short-chain NPEOs by activated sludge process
2. To examine absorption rates of these compounds in activated sludge

3. To detect cytotoxicity of short chain NPEOs in PC12 Cells.
4. To clarify toxic mechanism of short chain NPEOs in apoptotic PC12 Cells.
5. To investigate effect of NPEOs including the different EO chain length on apoptotic status caused by copper exposure.
6. To clarify the mechanisms of the effects of short chain NPEOs and long chain NPEOs on apoptotic conditions.

1.5 Outline of thesis

The total research work presented in this dissertation is organized into five chapters. Chapter I explained comprehensively introduction of the overall background, concept and purpose of this study along with the review of relevant literatures to understand short chain NPEOs application, characteristics and contamination in the environments, and toxicity study of short chain NPEOs. Chapter II presented degradation techniques of NPEOs, using activated sludge process and inactivated sludge. It would be clarified biodegradation role and absorption rate in sludge. Chapter III showed the cytotoxicity of NP_2EO and its mechanism using apoptotic PC12 cells. In addition, meanings of the obtained results were discussed. Chapter VI presented effects of NP_1EO, NP_5EO and $NP_{10}EO$ on apoptotic conditions of PC12 cells to prove the hypothesis presented in chapter III. Finally chapter V was section total conclusions and recommendation, summarized all chapters.

References

Ahel M, Giger W, Koch M, 1994a. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment–I. Occurrence and transformation in sewage treatment.Water Res, 28: 1131-1142.

Ahel M, Giger W, Schaffner C, 1994b. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment–II. Occurrence and transformation in sewage treatment.Water Res, 28: 1143–1152.

Ahel M, Giger W, 1993a. Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. Chemosphere, 26: 1471-1478.

Ahel M. and Giger W, 1993b. Aqueous solubility of alkylphenols and alkylphenol polyethoxylates, Chemosphere, 26: 146 1-1470.

Ahel M. and Giger W, 1985. Determination of alkylphenols and alkylphenol mono and diethoxylates in environmental samples by high-performance liquid chromatography. Anal Chem, 57: 1577-1583.

Bennie DT, Sullivan CA, Lee HB, Peart TE, Maguire RJ, 1997. Occurrence of alkylphenols and alkylphenol mono- and diethoxylates in natural waters of the Laurentian Great Lakes basin and the upper St. Lawrence river. Sci Total Environ, 193: 263-275.

Carter GN, Huntsman C, Austin, Texas, 1995. Environmental fate and safety of nonylphenol ethoxylates. Surfanctants and Wastewater, 27: 29-33

CEPA (Canadian Environmental Protection Act, 1999), 2001. Priority Substances List Assessment Report. Nonylphenol and its Ethoxylates. Environment Canada, Health Canada.

Dachs J, Van Ry DA, Eisenreich SJ, 1999. Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. Environ. Sci. Technol. 39: 2676-2679.

Deborde M, Rabouan S, Duguet JP, Legube B, 2005. Kinetics of aqueous

ozone-induced oxidation of some endocrine disruptors., Environ Sci Technol, 39: 6086-6092.

Di Corcia A, Cavallo R, Crescenzi C, Nazzari M, 2000. Occurrence and abundance of dicar-boxylated metabolites of nonylphenol polyethoxylate surfactants in treatedsewages. Environ Sci Technol, 34: 3914-3919.

EPA, 2010. Nonylphenol (NP) and Nonylphenal Ethoxylates (NPEs) Action Plan U.S. Environmental Protection Agency; Available from: http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/RIN2070-ZA09 NP-NPES%20Action%20Plan Final 2010-08-09.pdf.

Ferguson PL, Iden CR, Brownwell BJ, 2001. Distribution and fate of neutral alkylphenol ethoxylate metabolites in a sewage-impacted urban estuary. Environ Sci Technol, 35: 2428-2435.

Fiege H, Voges HW, Hamamoto T, Umemura S, Iwata T, Miki H, Fujita Y, Buysch HJ, Garbe D, Wilfried Paulus "Phenol Derivatives" in Ullmann's Encyclopedia of Industrial Chemistry 2002, Wiley-VCH, Weinheim.doi:10.1002/14356007.a19_313.

Giger W, Brunner P H, 1984. 4-Nonylphenol in sewage-sludge accumulation of toxic metabolites from nonionic surfactants [J]. Science, 225: 623-625.

Groshart CP, 2001. Chemical Study on Alkylphenols. Table 3.5 Use Of nonylphenol ehtoxylates. Den Haag: Rijksinstituut voor kust en zee.

Hayashi S, Saito S, Kim JH, Nishimura O, Sudo R, 2005. Aerobic biodegradation behaviour of nonylphenol polyethoxylates and their metabolites in the presence of organic matter. Environ Sci Technol 39: 5626-5633.

Holt MS, GC Mitchell, RJ Watkinson, 1992. The environmental chemistry, fate and effects of nonionic surfactants. In: O. Hutzinger (ed.), Handbook of environmental chemistry. Vol. 3. Anthropogenic compounds. Part F. Detergents. Springer-Verlag, Berlin. pp. 89-144 (ISBN 3-540-53797-X).

Jobling S, Sumpter JP, 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (Oncorhynchus mykiss)

hepatocytes. Aquatic Toxicology, 27: 361-372.

Kirschner M, 2007. Nonylphenol. ICIS Chemical Business Americas, 271: 42-42.

Kuramitz H, Saitoh J, Hattori T, Tanaka S, 2002. Electrochemical removal of p-nonylphenol from dilute solutions using a carbon fiber anode. Water Res, 36: 3323-3329.

Liu X, Tani A, Kimbara K, Kawai F, 2007. Xenoestrogenic short ethoxy chainnonyphenol is oxidized by a flavoprotein alcohol dethydrogenase from Ensifer sp strain AS08. Appl Microbiol Biotechnol, 73: 1414-1422.

Loyo-Rosales JE, Rice CP, Torrents A, 2007. Fate of octyl- and nonyl-phenol ethoxylates and some carboxylated derivatives in three American wastewater treatment plants. Environ Sci Technol, 41: 6815-6821.

Maguire RJ, 1999. Review of the persistence of nonylphenol and nonylphenol ethoxylates in aquatic environments. Water Qual Res J Canada, 34: 37-78.

Maki H, N Masuda, MY Fujiwara, M Ike, M Fujita, 1994. Degradation of alkylphenol ethoxylates by Pseudomonas sp. strain TR01. Appl Environ Microbiol, 60: 2265-2271.

Mann RM, Boddy MR, 2000. Biodegradation of a nonylphenol ethoxylate by the autochthonous microflora in lake water with observations on the influence of light. Chemosphere, 41: 1361-1369.

Marcimini A, Capal P D, Liechtensteiger T *et al.*, 1989 Behavior of aromatic surfactants and PCBs in sludge-treated soil and landfills, Journal of Environmental Quality, 18: 523-528.

Mehtonen J, Munne P, Verta M, 2012. Work package 4: identification of sources and estimation of inputs/impacts on the Baltic Sea, Summary Report Finland.

Miles-Richardson, SR, Pierens SL, Nichols KM, Kramer VJ, Snyder EM, Snyder SA, Render JA, Fitzgerald SD, Giesy JP, 1999. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (Pimephalespromelas). Environ Res Sect A, 80: S122-137.

Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR, 1992. Alkylphenol ethoxylates in the environment. J Am Oil Chem Soc, 69: 695-703.

Nevskaia, DM, Guerrero-Ruiz A, 2001. Comparative study of the adsorption from aqueous solutions and the desorption of phenol and nonylphenol substrates on activated carbons. J Colloid and Interface Sci, 234: 316-321.

Planas C, Guadayol JM, Droguet M, Escalas A, Rivera J, Caixach J, 2002. Degradation of polyethoxylated nonylphenols in sewage treatment plant. Quantitative analysis by isotopic dilution-HRGC/MS. Water Res, 36: 982-988.

Rudel RA Brody JG, Spengler JD, Vallarino J, Geno PW, Sun G, Yau A, 2001. Identification of selected hormonally active agents and animal mammary carcinogens in commercial and residential air and dust samples. J Air Waste Manage Assoc, 51: 499-513

Rudling, L, Solyom P, 1974. The investigation of biodegradability of branched nonylphenol ethoxylates. Water Res, 8: 115-119.

Schramm LL, Stasiuk EN, Marangoni G, 2003. Surfactants and their applications. Annu Rep Prog Chem C, 99:3-48.

Sharpe RM, 1998. Environmental estrogens and male infertility. Pure Appl Chem, 70: 1685-1702.

Sonnenschein C, Soto AM, 1998. An updated review of environmental estrogen and androgen mimic and antagonists. J. Steroid Biochem, 65: 143-150.

Soto AM, KL Chung, C Sonnenschein, 1994. The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environment Health Perspect, 102: 380-383.

Sumpter JP, 1998. Xenoendocrine disrupters - environmental impacts. Toxicol Lett, 102-103: 337-342.

Tabata A, Kashiwa S, Ohnishi Y, Ishikawa H, Miyamoto N, Itoh M, Magara Y, 2001. Estrogenic influence of estradiol-17h, p-nonylphenol and bisphenol A on Japanese Medaka (Oryzias iatipes) at detected environmental concentrations.

Water Sci Technol, 43: 109-116.

TenEyck MC, Markee TP, 2007. Toxity of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate and mixtures of these compounds to Pimephales promelas (Fathead Minnow) and Ceriodaphnia dubia. Arch Environ Contam Toxicol, 53: 599-606.

Thiele B, Günther K, Schwuger MJ, 1997. Alkylphenol ethxylates:trace analysis andenvironmental behavior. Chem Rev, 7: 3247-3272.

Tyler CR, Jobling S, Sumpter JP, 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol, 28: 319-361.

U.K. Environment Agency, 1997. Draft comprehensive risk assessment report for nonylphenol. National Centre for Ecotoxicity and Hazardous Substances, London, U.K.

U.K. Environment Agency, 1998. Risk assessment of 4-nonylphenol (branched) and nonylphenol. Draft, September 1998. London, U.K.

US EPA (United States Environmental Protection Agency), 2005. Aquatic Life Ambient Water Quality Criteria-Nonylphenol. Environmental Protection Agency, Office of Water, Office of Science and Technology, Washington, DC, EPA-822-R-05-005, US.

Warhurst AM, 1995. An environmental assessment of alkylphenol ethoxylates and alkylphenols, Published jointly by Friends of the Earth, Scotland and Friends of the Earth, England, Wales and Northern Ireland.

White R, Jobling S, Hoare SS, Sumpter JP, Parker MG, 1994. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology, 135: 175-182.

Witorsch RJ, 2002. Endocrine disruptors: Can biological effects and environmental risks be predicted. Regul Toxicol Pharm, 36: 118-130.

Yim BB, Yoo YG, Maeda Y, 2003. Sonolysis of alkylphenols in aqueous solution with Fe(II) and Fe(III). Chemosphere, 50: 1015-1023.

Ying GG, 2006. Fate, behavior and effects of surfactants and their degradation products in the environment. Environment International, 32: 417O431.

Chapter 2 Biodegradation study of NP_2EO

Abstract

Biodegradation is a natural process carried out by microorganisms to break down organic matter into smaller compounds. The biodegradation is depending on the organisms can utilize target organic compounds as nutrient and energy. It is an important recycling process in nature to allow the breakdown of complex organic compounds into small and simple molecules or inorganic ones. They are expected to be able to be reused and rebuilt again into useful forms by the organisms.

In activated sludge process, absorption rates of NP_1EO to NP_3EO were more than 60%. These compounds could be accumulated quickly in the sludge within 10 min. It was clear that these biodegradation reactions play some roles in degradation process of short-chain NPEOs. After 24 hr, although NP_2EO and NP_3EO contents were extremely reduced, NP_1EO content was relative higher than others. It was suggested that NP_2EO and NP_3EO were converted to NP_1EO by microorganisms.

Furthermore, it was confirmed that absorption process was more than 60% by inactivated sludge system. Short-chain NPEOs affected on COD removal.

2.1 Biological treatment process of organic matters

Biological treatment for organic matters using microorganism can be divided into aerobic and anaerobic biological processes. Aerobic biological process for wastewater was shown in Fig. 2.1. Aerobic biological process is defined that organic chemical substances are degraded by aerobic microorganisms under the presence of free oxygen (molecular oxygen) conditions. This process is well known as stable and harmless methods. Microbes utilize organic pollutants in wastewater as nutrient for aerobic metabolism. These high-energy organic matters are converted to low energy inorganic material step by step through a series of biochemical reactions.

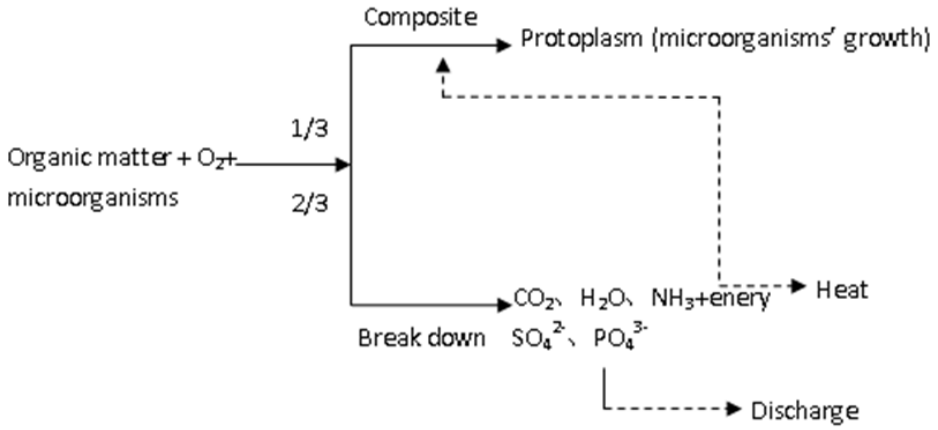


Fig. 2.1 Metabolic mechanism of organic chemical substances in aerobic biological process.

Anaerobic treatment of wastewater is that facultative bacteria and anaerobic bacteria degrade and stabilize pollutants without the presence of free oxygen. In anaerobic biological processes, complex organic matters can be degraded and converted into simple compounds. The transformation of organic matter is divided into three parts: first one is conversion into CH_4; second one is changing into CH_4, CO_2, NH_3, H_2S, and last one is that small amount of organic matter is converted to new protoplasm. In these processes, different microbial metabolic processes influence, restrict each other, and form complex ecosystems. Anaerobic biological process for wastewater is shown in Fig. 2.2.

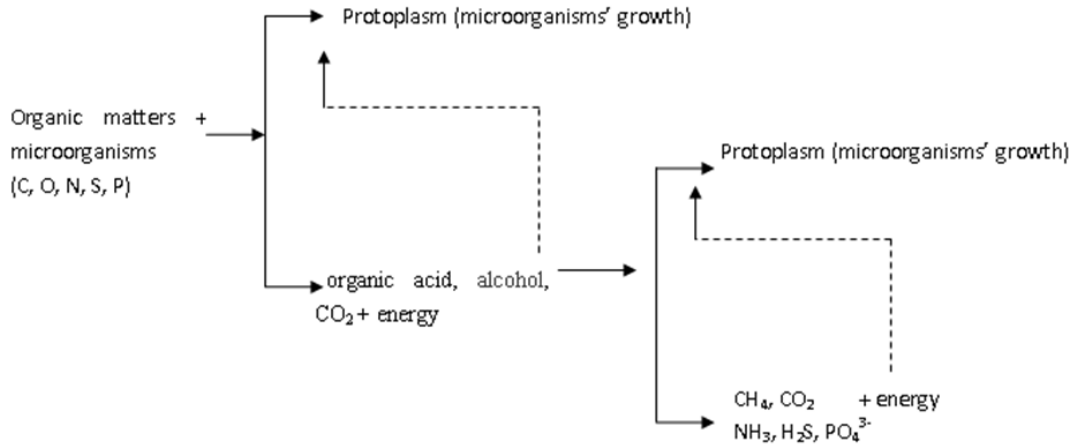


Fig. 2.2 Metabolic mechanism of organic chemical substances in anaerobic biological process.

2.2 Biodegradation process of NPEOs

Biodegradation is a natural process carried out by microorganisms to break down organic matter into smaller compounds. The biodegradation is depending on the organisms can utilize target organic compounds as nutrients or energy. It is an important recycling process in nature to allow the breakdown of complex organic compounds into small and simple molecules or inorganic ones. They are expected to be able to be reused and rebuilt again into useful forms by the organisms. The rate of biodegradation depends on 1) the presence of the involved microorganisms, 2) physical environment for growth, e.g. temperature, pH, salinity and contaminants in wastewater, 3) structure of the chemicals (Corvini et al., 2006), and 4) bioavailability of the chemicals (Merrettig-Bruns and Jelen, 2009). Biodegradation is the main process for the removal of NPEOs, and its important factor is intermediate metabolites as compared with the other degradation processes (Ahe et al., 1994).

Although majority of NPEOs is absorbed into the sludge in primary sewage Treatment plants (STW). Biological process is difficult to remove NPEOs from water body. However, abiotic processes like as hydrolysis or photolysis are negligible in degradation step of NP and short chain NPEOs (ECHA, 2012). The two simplified pathways under aerobic and anaerobic conditions are illustrated in Fig. 2.3. Primary NPEOs are transformed to NP_1EO or NP_2EO under the anaerobic condition, and nonylphenl carboxylates (NP_1EC and NP_2EC) under the aerobic condition, before they finally degraded into NP under the anaerobic conditions (Giger et al., 1984).

On basis of the experiments reported (Giger et al., 1984), significantly higher concentrations of NP in digested sludge were as compared with those in active sludge. Therefore, it was suggested that anaerobic biodegradation is likely predominantly degradation process to degrade from short-chained NPECs and/or NPEOs to NP.

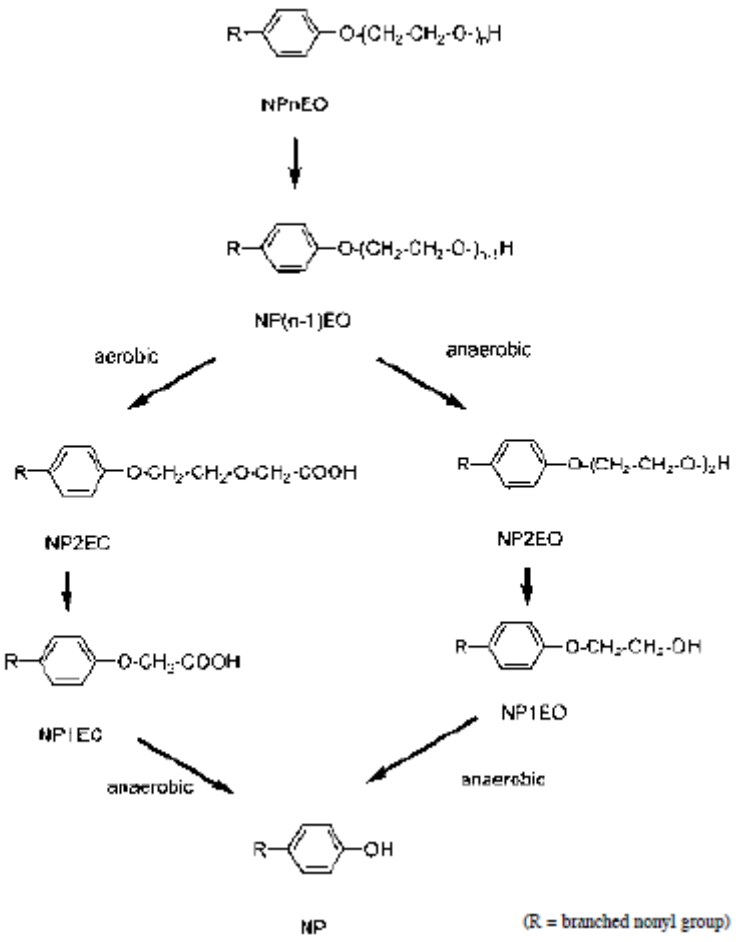


Fig. 2.3 Biodegradation pathways for NPEOs

As shown in Fig. 2.4, the processes of two kinds of aerobic biodegradation pathway for NPEOs were indicated (John and White, 1998; Sato et al., 2001).

From the fact presented in Fig. 2.4, it was indicated that short chain NPEOs is difficult to process further degradation. In this study, degradation and adsorption of short chain NPEOs in activated sludge might provide some evidences how to further degrade for short chain NPEOs.

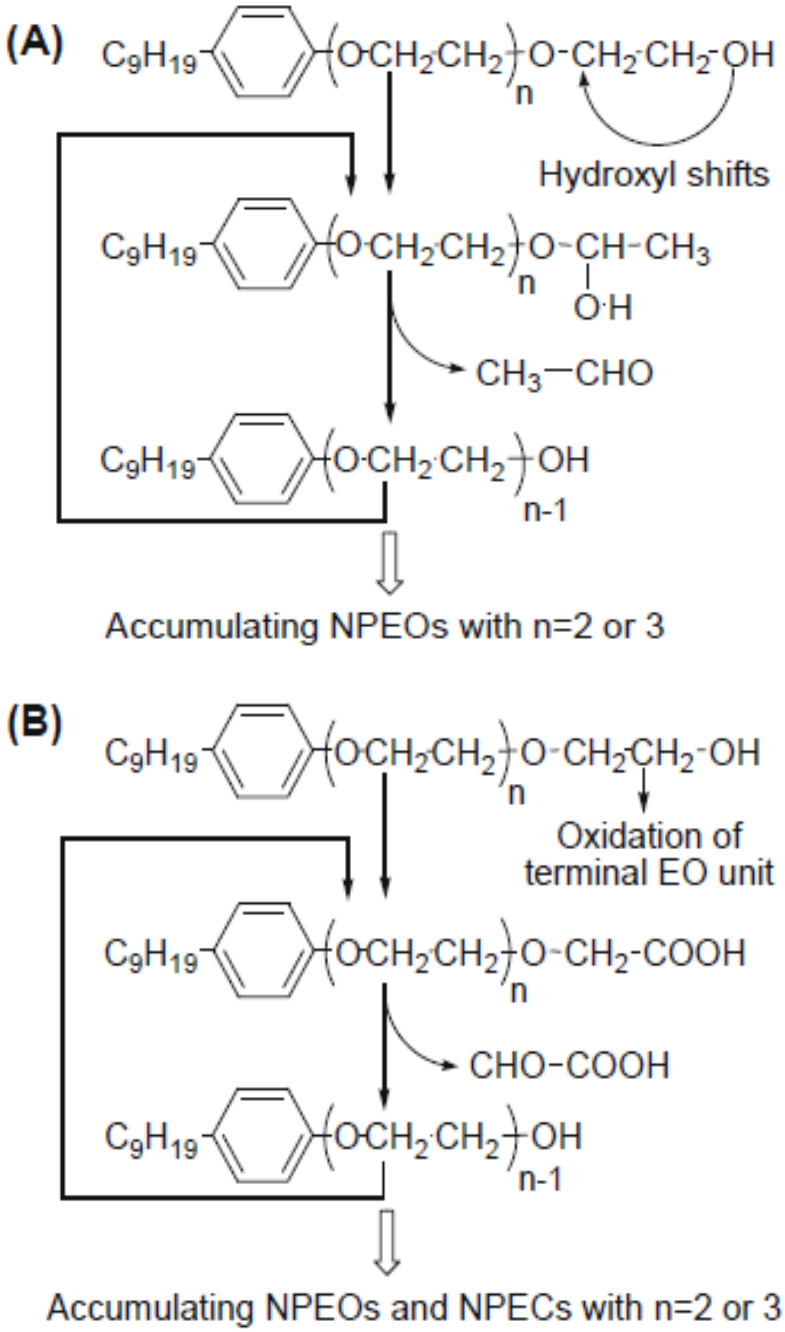


Fig. 2.4. Generally proposed aerobic biodegradation pathway of NPEOs (A) Hydroxyl shift cleavage model, and (B) terminal oxidation cleavage model.

2.3 Materials and Methods

2.3.1 Materials and Reactors

The activated sludge is kindly donated from aerobic tank of Soseigawa Wastewater Treatment Center. Initially, activated sludge was incubated with artificial domestic

wastewater. The main composition of it was shown in Table 2.1. In this way, increase of sludge biomass can well degrade NP_2EO. Three small reactors were prepared as follows; control reactor (without NP_2EO), target reactor (treatment of NP_2EO), and inactivated sludge reactor to confirm biodegradation and absorption rate. NP_2EO is defined as a mixture consisting of $NP_{1\sim5}EO$.

Table 2.1 Synthetic wastewater composition (Smolders et al., 1994 a and b)

Category	Reagents	Initial concentration (mg/L)	Remarks
C source	Glucose	400	COD≈400 mg/L
	NP_2EO	60	
N source	NH_4Cl	202	TN≈50 mg/L
P source	K_2HPO_4	45	TP≈8 mg/L
Buffer	$NaHCO_3$	120	
Inorganic salt	$CaCl_2$	6	
	$FeSO_4 \cdot 7H_2O$	0.55	
	$MgSO_4 \cdot 7H_2O$	6	
	$MnSO_4 \cdot H_2O$	6	

After that, a small aerobic reactor was set up. Adjusting factors as listed in Table 2.2 reactor condition will be better for microorganisms to degrade NP_2EO. When biomass reaches certain contents, NP_2EO dissolved in 1 mL methanol was added into reactor. The fresh wastewater was added into the reactor once 24 hr after addition of NP_2EO. At the same time, to confirm sludge activity, reactor containing inactive sludge inactivated by heat treatment at 110 ℃ was also prepared. The same concentration of NP_2EO was also added into the reactor containing inactive sludge.

Table 2.2 Parameters defined in the reactors

SRT (sludge retention time)	10 days
DO (dissolved oxygen) conc.	more than 5 mg/L
pH	about 7.5
Temperature	about 20 ℃
Operation time	24 hr
Wastewater amount:	500 mL/day
MLSS (biomass) conc.	4000 mg/L

2.3.2 Sample collection and preparation

Sludge samples and aquatic samples were collected at 10 min and 24 hr after reaction is started from every reactors. Sample volumes of water and sludge are 500 mL and50 mL, respectively. Water sample collected was and separated by liquid-liquid extraction. The procedures are as follows.

1. Five hundred mL wastewater sample is filtered by 0.45 μm organic filter membrane.

2. Five mL 1 mol/L HCl, 25 g NaCl, 50 mL OF CH_2Cl_2 are added to 500 mL bottle. Then the mixture is shaken for 2 min, and is kept on sand at room temperature afterward for 20 min.

3. Organic phase is transferred into 50 mL tube, and is centrifuged at 5,000 rpm/min for 10 min by centrifuge. Water phase is transferred back into 500mL bottle.

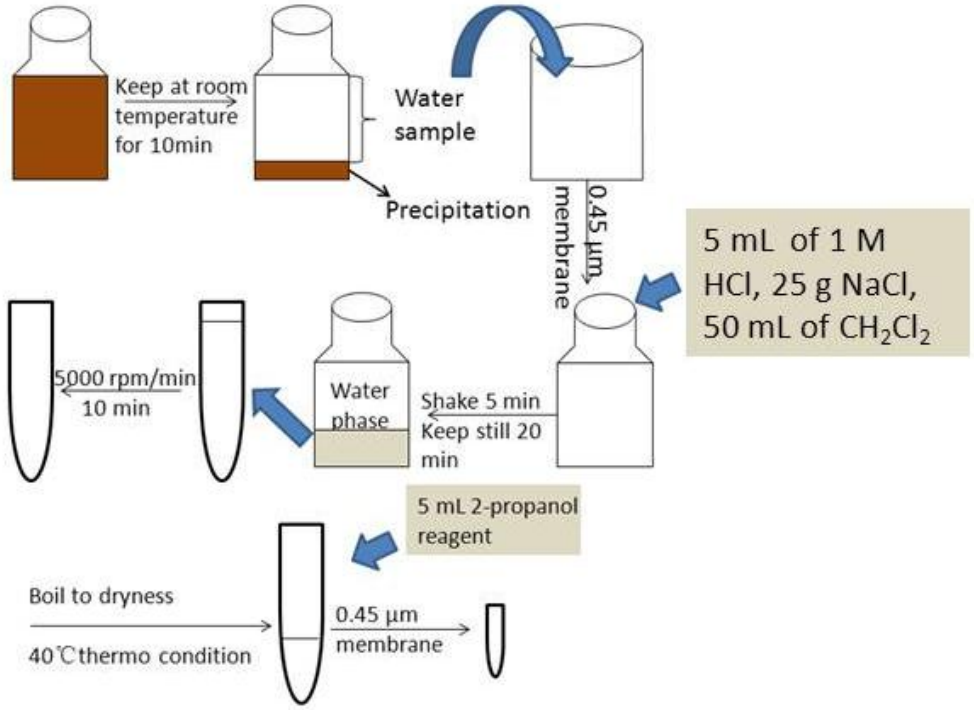
4. Above mentioned procedures are repeated again. Then the both extracts are mixed.

5. The extract is dried on 40 ℃.

6. Then the extracts are resolved in 5 mL of 2-propanol reagent. Then the resolved

sample is filter with 0.45 μm organic filter membrane.

The method procedure is shown as follows.



Sludge sample was collected and extracted by sonication-assisted extraction.

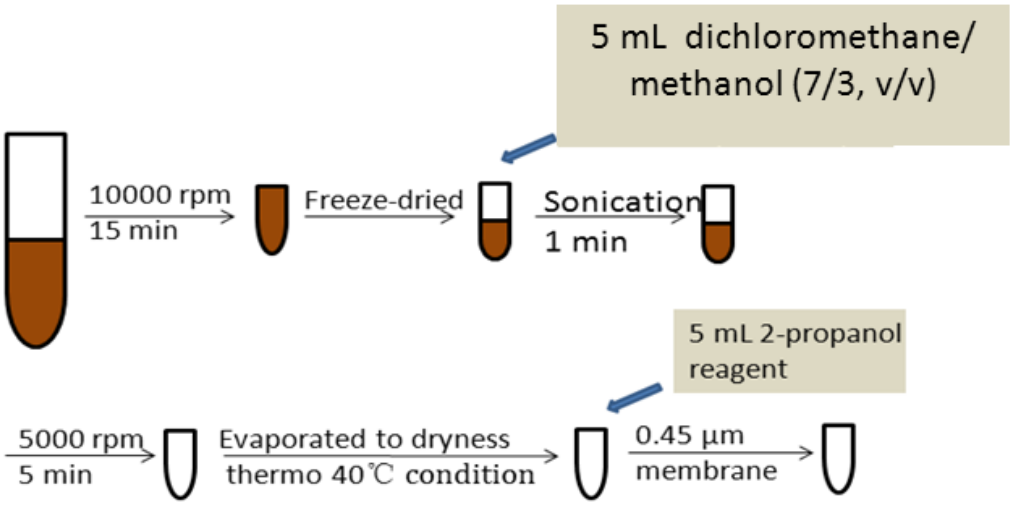
1. Fifty mL of sample is separated by centrifugation at 10,000 rpm for 15 min. Sludge phase is lyophilized (freeze-dried). Then dried sludge is stored in -20 ℃ before extraction.

2. A freeze-dried sludge (0.18 g) is sonicated for 1 min with 5 mL of dichloromethane / methanol (7/3, v/v). The extract is separated by centrifugation at 5,000 rpm for 5 min. The extraction is repeated three times with a fresh solvent mixture.

3. All of extracts are evaporated to dry at 40 ℃.

4 Then the extracts are resolved in 5 mL of 2-propanol reagent. Then the resolved sample is filtered by using 0.45 μm organic filter membrane.

The method procedure is shown as follows.



2.3.3 Analysis

Chemical oxygen demand (COD) is measured by pack test (Kyoritsu Chemical – Check Lab. Corp., Tokyo, Japan).The amounts of NP_2EO extracted from water and sludge samples were measured by high-performance liquid chromatography (HPLC) equipped with an ultraviolet detector and APS-2 NH_2 column (250 mm, 4.6 µm, 5 µm). Detector was adjusted at a wavelength as 277 nm. N-hexane/2-propanol (98:2 v/v) was used as mobile phase at a flow rate of 1 mL/min. The column temperature was fixed at 25 ℃ during the measurements. Injection volume was selected as 20 µL.

2.3 Results and discussion

The biomass acclimation process to removal of organic compounds is a critical step in order to induce microbial selection and physiological transformations of the metabolic pathways required for biodegradation. In order to ensure effective biomass acclimation, it is important to realize a biomass exposure to the compound (NPEOs) and each step increase has been given only when stable operating conditions in terms of COD removals were reached (Tomei et al., 2003). In this study, three reactors (control reactor (without NP_2EO), target reactor (treatment with NP_2EO) and inactivated sludge reactor (to estimate absorption rate in the sludge)) were used. For target reactor, when the reactor was stable, it means that the activated sludge reached the best

activity and condition, NP_2EO were added into the reactor. To investigate biodegradation rate and absorption rate, the short chain NPEOs contents were detected by HLPC.

2.3.1 COD removal

In this study, at the beginning of setting up the reactors, synthetic domestic wastewater without NP_2EO was added to reactors to keep certain biomass (MLSS: 4,000mg/L). Original COD concentration is about 500 mg/L. When NP_2EO (60 mg/L) was added into the target reactor. COD concentration was 460 mg/L. As shown in Fig. 2.5, COD was decreased to about 300 mg/L in control reactor at 10 min. After 24 hr, final COD concentration was about 50 mg/L. However, COD concentration in target reactor was relative higher at 10 min than that in control reactor. After 24 hr, the finial COD concentration in target reactor was around 100 mg/L. This result indicated that NP_2EO has some impacts on COD removal in the reactor at the beginning of operational time.

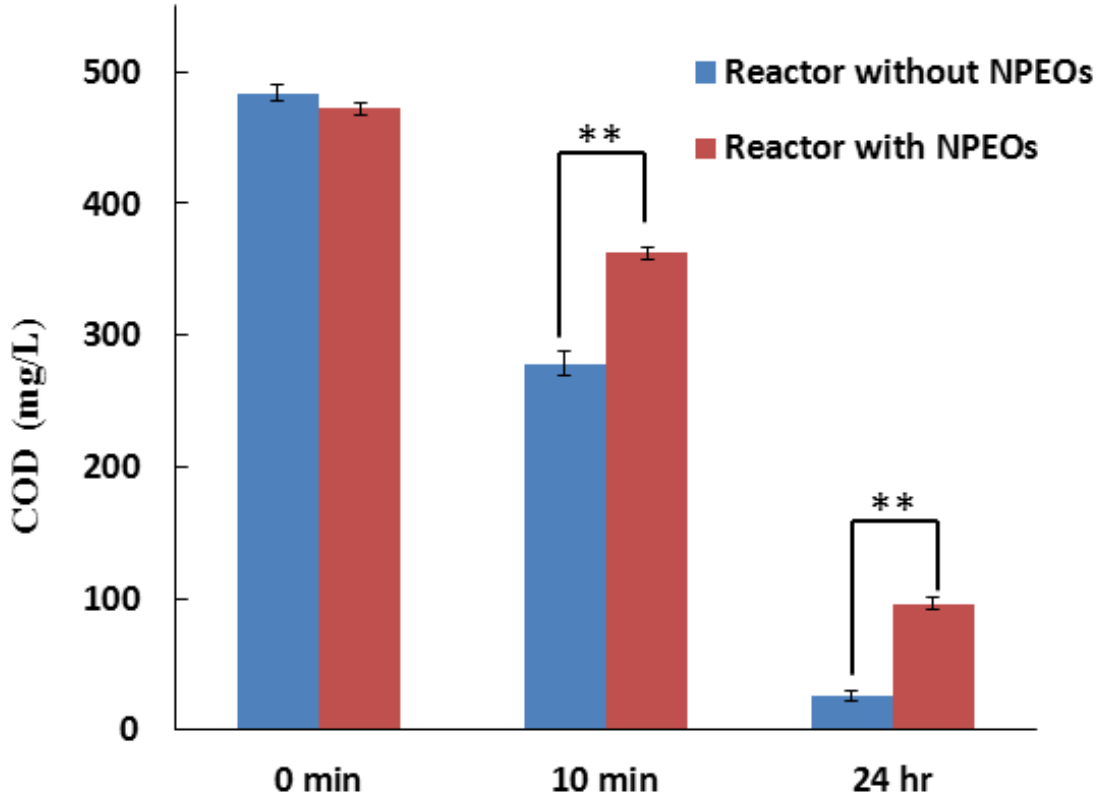


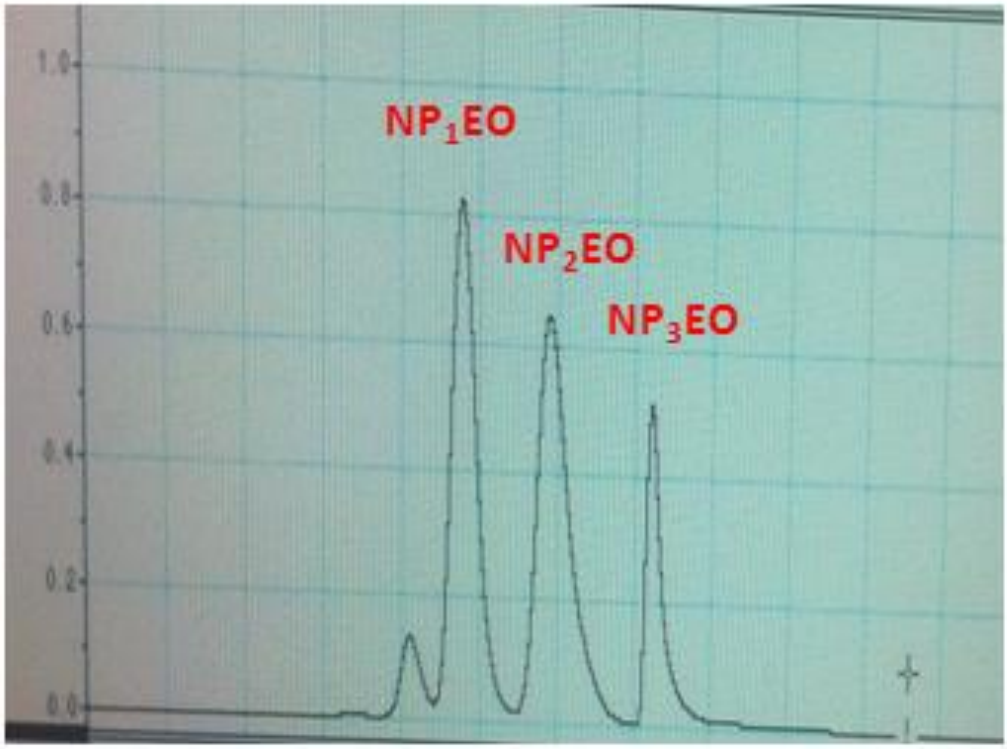
Fig. 2.5. COD concentrations in activated sludge at 24 hr, Error bar indicates mean ± S.E.M (n=6). **: significant differences (P<0.01)

2.3.2 Short chain NPEO concentration in reactors

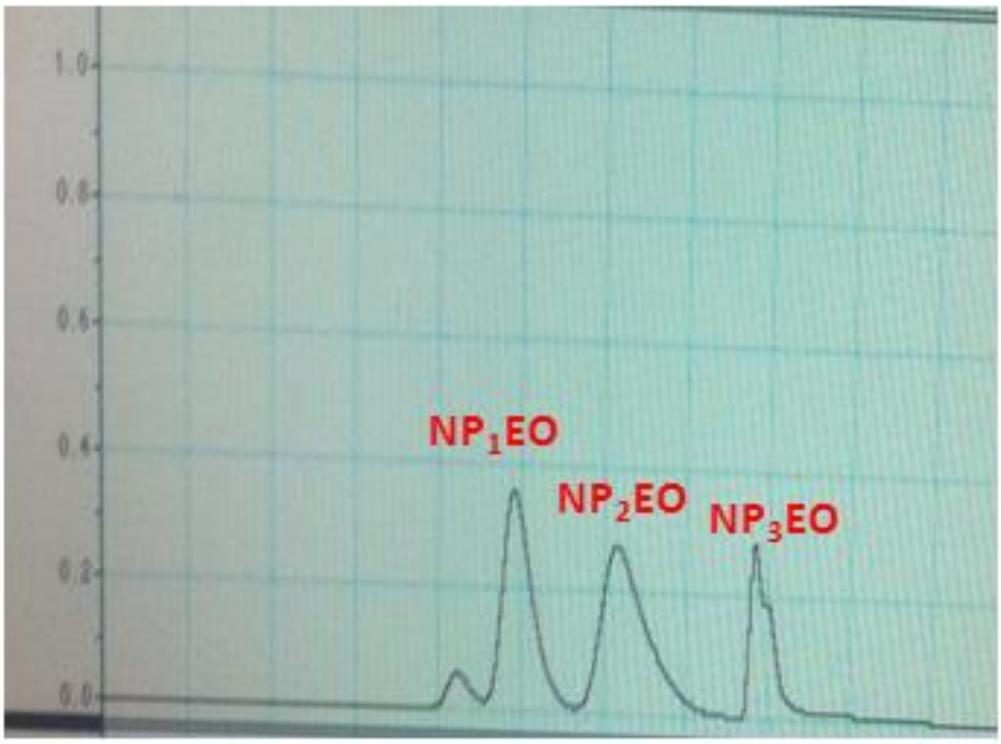
When NP_2EO was added into reactors, water sample and sludge sample at 10 min and 24 hr were collected and extracted by the extraction methods. The NP_nEO extracted in the samples were measured by HPLC. The peaks of NP_1EO to NP_3EO were identified under the HLPC conditions. The HPLC profiles were shown in Fig. 2.6. Amount of short chain NPEOs in water samples detected were calculated according to area for the each peak values.

NP_1EO could be rapidly adsorbed at 10 min in target reactor. About 60% of $NP_{1\sim3}EO$ can be adsorbed in the activated sludge after 24 hr (Fig. 2.8). After 24 hr, NP_2EO and NP_3EO contents in water were absolutely low; however, NP_1EO content in water was relative higher than others (Fig. 2.7). It was indicated that NP_2EO and NP_3EO were converted to NP_1EO by microorganisms.

A



B



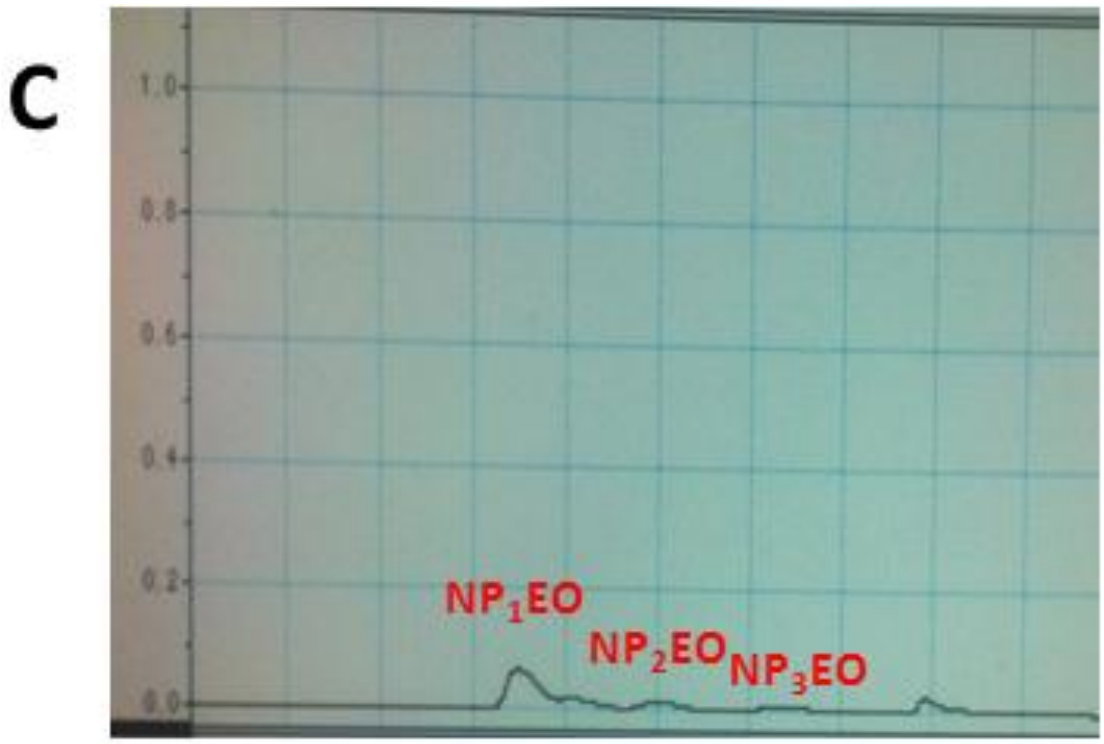


Fig. 2.6. HPLC Chromatograms of NP_1EO to NP_3EO of water samples, A; original peak conditions of NP_1EO, NP_2EO and NP_3EO, B; peak conditions of NP_1EO, NP_2EO and NP_3EO at 10 min after operation of reactors, C; peak conditions of NP_1EO, NP_2EO and NP_3EO at 24 hr after operation of reactors.

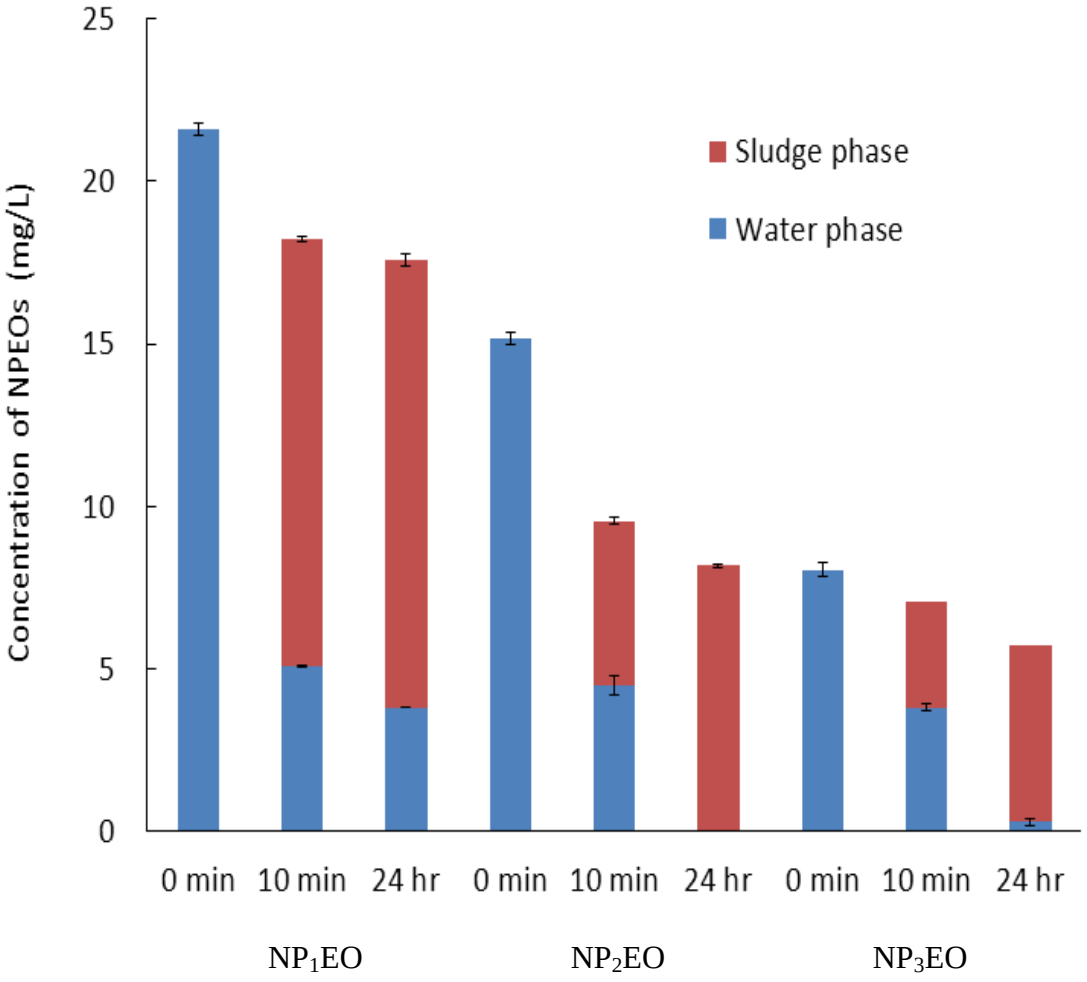


Fig. 2.7. Degradation of NPEOs in activated sludge at 24 hr (n=6)

To further confirm whether majority of short chain NPEOs were adsorbed in sludge, NPEOs contents in reactor containing an inactivated sludge were measured. It was indicated that absorption process was progressed more than 60% by inactivated sludge system. Short-chain NPEOs could be accumulated rapidly in sludge within 10 min.

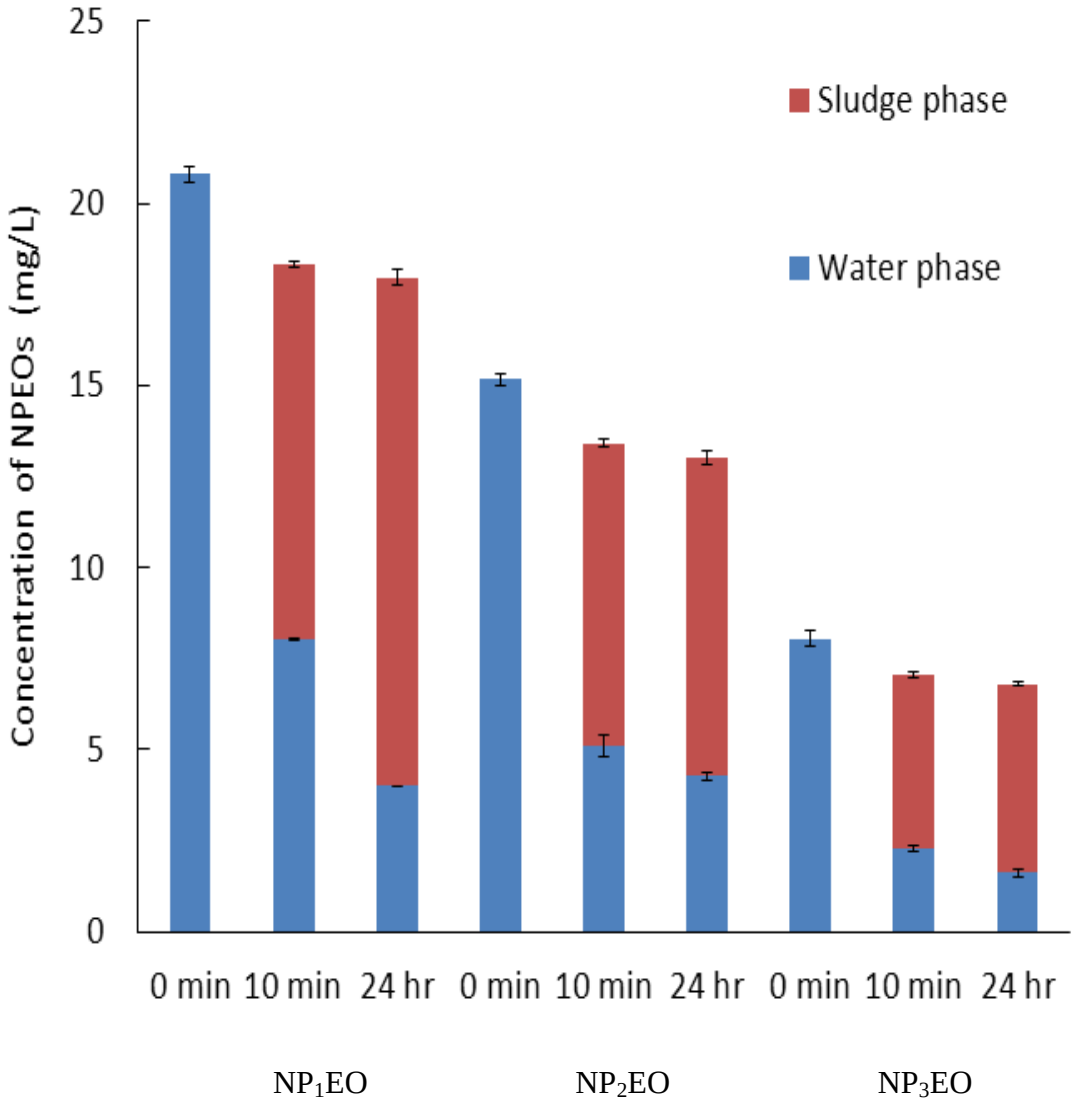


Fig. 2.8. Amounts of NPEOs in inactivated sludge at 24 hr (n=6)

2.4 Conclusions

In activated sludge process, absorption rates from NP_1EO to NP_3EO were more than 60%. It meant that these compounds could be accumulated quickly in sludge within 10 min. However, biodegradation process had some roles in treatment of short-chain NPEOs. After 24 hr, NP_2EO and NP_3EO contents were absolutely low, NP_1EO content was relative higher than the others. It was indicated that NP_2EO and NP_3EO were converted to NP_1EO by microorganisms.

Furthermore, it was shown that absorption process was more than 60% by inactivated

sludge system. Short-chain NPEOs could be accumulated rapidly in sludge within 10 min. It was suggested that behavior of short- chain NPEOs affected on COD removal.

References

Ahel M, Giger W, Koch M, 1994. Behaviour of alkylphenol polyethoxylate Surfactants in the aquatic environment-I. Occurrence and transformation in sewage treatment. Water Res, 28: 1131-1142.

Corvini PFX, Schäffer A, Schlosser D, 2006. Microbial degradation of nonylphenol and other alkylphenols-our evolving view. Applied Microbiol Biotechnol, 72: 223-243.

ECHA (European Chemicals Agency), 2012. Community Rolling Action Plan. 29 February 2012.

Giger W, Brunner PH, Schaffner C, 1984. 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from non-ionic surfactants. Science, 225: 623-625.

John DM, White GF, 1998. Mechanism for biotransformation of nonylphenol polyethoxylates to xenoestrogens in Pseudomonas putida. J Bacteriol, 180: 4332-4338.

Merrettig-Bruns U, Jelen E, 2009. Anaerobic biodegradation of detergent surfactants. Materials, 2: 181-206.

Sato H, Shibata A, Wang Y, Yoshikawa H, Tamura H, 2001. Characterization of biodegradation intermediates of non-ionic surfactants by matrix-assisted laser desorption/ionization-mass spectrometry 1. Bacterial biodegradation of octylphenol polyethoxylate under aerobic conditions. Polym Degrad Stab, 74: 69-75.

Smolders GJF, Vandermeij J, Vanloosdrecht MCM, Heijnen JJ, 1994a. Stoichiometric model of the aerobic metabolism of the biological phosphorus removal process. Biotechnol Bioeng, 44: 837-848.

Smolders GJF, Vandermeij J, Vanloosdrecht MCM, Heijnen JJ, 1994b. Model of the anaerobic metabolism of the biological phosphorus removal process-stoichiometry and pH influence. Biotechnol Bioeng, 43: 461-470.

Tomei MC, Annesini MC, Luberti R, Cento G, Senia G, 2003. Kinetics of 4-nitrophenol biodegradation in a sequencing batch reactor. Water Res, 37: 3803-3814.

Chapter 3 Effect of NP_2EO on apoptosis

Abstract

NP and short-chain NPEOs such as NP_2EO are presented in aquatic environment as wastewater contaminants, and their toxic effects on aquatic species have been reported. Apoptosis has been shown to be induced by serum deprivation or copper treatment. To understand the toxicity of NP_2EO, we investigated the effects of NP_2EO on apoptosis induced by serum deprivation and copper by using PC12 cell system. NP_2EO itself showed no toxicity and recovered cell viability from apoptosis. In addition, NP_2EO decreased DNA fragmentation caused by apoptosis in PC12 cells. This phenomenon was confirmed after treating apoptotic PC12 cells with NP_2EO, whereas the cytochrome c release into the cytosol decreased compared to that in apoptotic cells not treated with NP_2EO. Furthermore, BAX contents in apoptotic cells were reduced after exposure to NP_2EO. Thus, NP_2EO has the opposite effect on apoptosis in PC12 cells compared to NP, which enhances apoptosis induced by serum deprivation. The difference in structure of the two compounds is hypothesized to be responsible for this phenomenon. These results indicated that NP_2EO has capability to affect cell differentiation and development and has potentially harmful effect on organisms because of its unexpected impact on apoptosis.

3.1 Introduction

APEOs are nonionic surfactants found in industrial, institutional, and household products. NPEOs are the most commercially abundant APEOs, representing about 80% of the market (APERC, 2006). NPEOs are often used in non-agricultural pesticides, cosmetic and cleaning products, and correction fluids and inks (Stasinakis et al., 2008). Because of the widespread application of these products, NPEOs enter the environment via industrial effluents and wastewater. NPEOs released into the environment are biodegraded to shorter-chain metabolic intermediates including NP, NP_1EO, and NP_2EO by the loss of EO (Ahel et al., 1994), which are more toxic, lipophilic, and

stable compared to long chain NPEOs (Ying, 2006; Sharma et al., 2009). In recent years, the toxicity of NPEOs and NP has been studied particularly in the aquatic environment (Vazquez et al., 2005; Soares et al., 2008). The toxicity of NPEOs to aquatic organisms increases as the length of EO chain decreases (Teneyck and Markee, 2007). NP and short chain NPEOs are frequently identified as major contaminants in waste and even drinking water (Ying, 2006; Soares et al., 2008; Sharma et al., 2009). Recently, these sub-products have been detected in vegetables (Cai et al., 2012).

NP and NPEOs are toxic to both aquatic and terrestrial organisms probably as a result of their interaction with proteins (Scott and Krogh, 2004). NPEOs are produced by a base catalyzed reaction of ethylene oxide with NP (Naylor, 1995). NP and NPEOs have different structures; hence, they disrupt differently the biochemical processes in organisms. It has been reported that enzymes such as P-glycoprotein interact with various NPEO compounds, but not with NP (Loo et al., 1998). NP exhibits stable hydrophobicity and has limited biodegradation potential (Tollefsen et al., 2008). It accumulates in organisms, and causes acute toxicity to algae, clams, shrimp, crustaceans, and fish (Tollefsen et al., 1998). NP and short chain NPEOs have also been reported to interact with intracellular and extracellular estrogen binding proteins (Knudsen and Pottinger, 1999), and to interfere with reproductive functions and normal development of fish (Jobling et al., 1996; Seki et al., 2003).

It was found that more than 80% of the male Medaka exposed to concentrations of 50 μg/L to 100 μg/L NP exhibited gonadal intersex (i.e., intermediate characteristics between males and females). Treatment with 300 μg/L NP_1EO produced a similar outcome, indicating that when NP was mixed in NP_1EO, the toxicity of NP_1EO was enhanced. Because of the differences in the structure of the two compounds, NP had a much stronger effect on fish and mammals compared to the short chain NPEOs (Balch et al., 2006).

Some studies have demonstrated that NPEOs with relatively small number of EO units (s = 0-30) showed greater cytotoxicity (inhibition of cell proliferation) in human skin fibroblast cells compared to long chain NPEOs (Goto et al., 2004). The genotoxic

effects of NPEOs on mammalian cells have also been studied. NPEOs were found to cause direct DNA damage (DSBs, double strand breaks), but did not contribute to reactive oxygen species (ROS) (Toyooka et al., 2012). NPEOs also act as tumor initiators and promoters. In contrast, NP decreases the viability of Raji cells via ROS (Qi et al., 2013). These differences of the effect of NPEOs and NP may be dependent on their structures, although the reason for the observed discrepancy between NP and short chain NPEOs remains unclear.

Most of the research on NPEOs degradation from NP has focused on the growth of reproductive organs in rats (Laws et al., 2000; Lee et al., 1996; Odum et al., 1999). Beside reproductive organs, NP also affects nonreproductive organs, such as kidney and liver (Chapin et al., 1999; Nagao et al., 2001). It has been found that hydroxyl radical formation in the striatum was induced in rats treated with NP, which might contribute to certain stages in the advancement of brain damage (Obata and Kubota, 2000). Furthermore, behavioral alterations were observed in the open-field activity of rat offspring exposed to NP (Ferguson et al., 2000). These findings indicate that NP may affect the central nervous system. Thus, NP deleteriously affects several organs, in addition to adipose tissue and adipocytes (Ferguson et al., 2000; Obata and Kubota, 2000; Masuno et al., 2003). However, the effects of short chain NPEOs on cells or organisms have received comparatively little attention because of its smaller toxicity compared to NP.

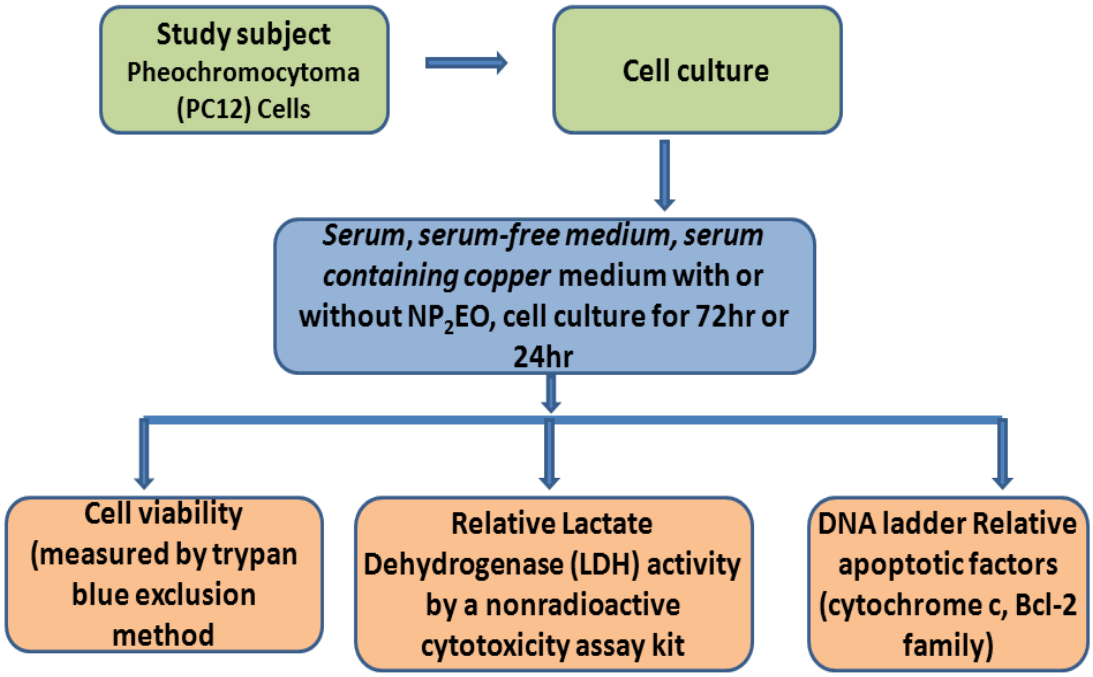
Few studies have demonstrated the influence of NPEOs on three cell lines; namely, ASF4-1 cells, Jurkat cells, and NIH3T3 cells, which represent normal human skin fibroblasts, human leukemia cells, and mouse fibroblasts, respectively. Certain $NP_{70}EO$ showed no cytotoxicity in any of the cell lines, whereas other $NP_{10}EO$ exhibited significant toxicity. $NP_{10}EO$ toxicity was caused by detachment of cells due to the structural change of the cell membrane. In addition, it was also shown that cytotoxicity was dependent on the number of EO units in all three cell lines. With decreasing the number of EO units, cell proliferation was increasingly inhibited (Goto et al., 2004). Moreover, LC_{50} of NPEOs (s = 1.5, 9, 15, 40, and 50) in Mysidopsis bahia was 0.11,

1.41, 2.57, over 100 and over 4110 mg/L, respectively (Goto et al., 2004). These findings indicate that the toxicity of NPEOs increases with decreasing the number of EO units. Further detailed cytotoxicity studies of short chain NPEOs are needed.

PC12 is a rat pheochromocytoma clonal cell line that responds to nerve growth factor by extending neuritis, thus acquiring the appearance of neurons (Greene and Tischler, 1976). PC12 cells have become a very suitable model to study neuronal function and differentiation (Vaudry et al., 2002; Ravni et al., 2006). On the other hand, it is well known that apoptosis is induced by serum deprivation in PC12 cells (Maroto and Perez-Polo, 1997). Recently, we have reported that some endocrine disrupters inhibited apoptosis induced by serum deprivation (Yamanoshita et al., 2000; 2001). In addition, we have examined whether NP affects apoptosis in PC12 cells. Our results of DNA fragmentation and relative expression of apoptotic factors indicated that NP enhances apoptosis induced by serum deprivation in PC12 cells (Aoki et al., 2004). The effects of short chain NPEOs on apoptosis have not been clarified, although it is expected that the toxicity of NPEOs will decrease with increasing the number of EO units. Apoptosis is a physiological cell-death mechanism commonly associated with programmed events, including morphological and biochemical changes, which are necessary for the differentiation and development of organs and organisms (Maroto and Perez-Polo, 1997). Investigation of effects on apoptosis is expected to contribute new insight into the mechanisms of the effect of NPEOs on the differentiation and development of an organism. In addition, PC12 cells are a good model for study of apoptotic changes because apoptotic condition can be regulated by bovine fetal albumin (Maroto and Perez-Polo, 1997) and copper (Kawakami et al., 2008). The objective of our study is to investigate the role of NPEOs regulation of the changes of apoptotic status caused by serum deprivation or copper, and to clarify the mechanisms of the effects of short chain NPEOs on apoptotic conditions. The significance of obtained results was discussed in detail.

3.2 Materials and Methods

The procedures of present study are shown as follows.



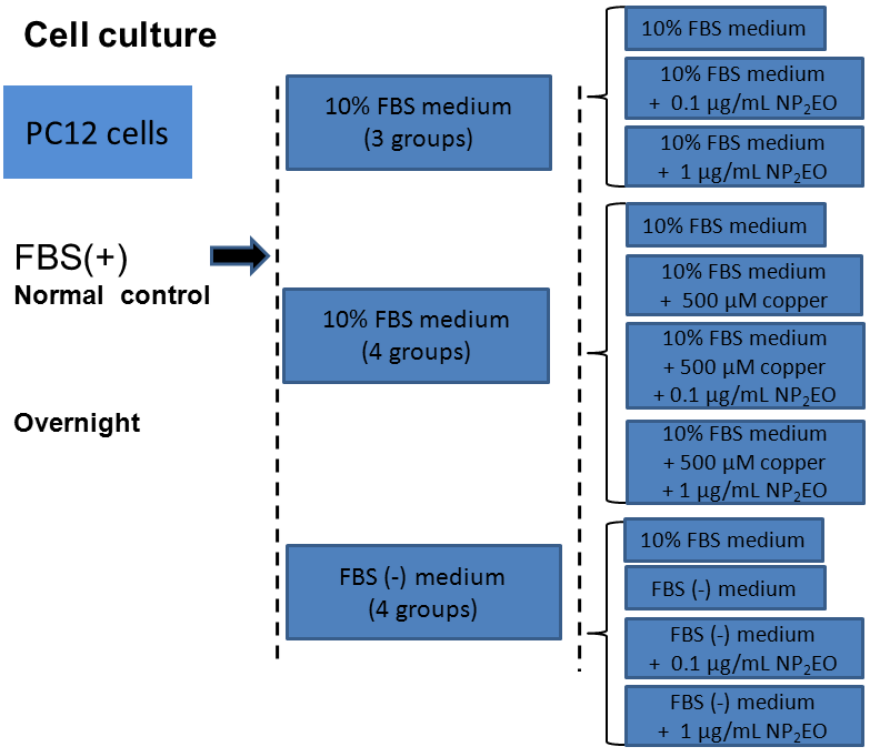
3.2.1 Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle medium (DMEM), ribonuclease A and o-phenylenediamine dihydro chloride (OPD) were obtained from Sigma-Aldrich (St. Louis, MO, USA). NP_2EO was purchased from Tokyo Chemical Industry Corporation (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from HyClone (Rockville, MD USA). Monoclonal antibody against cytochrome c, and polyclonal antibody against Bcl-2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nonradioactive cytotoxicity assay kit and purified horseradish peroxidase conjugate of goat anti-mouse IgG (H+L) were obtained from Promega (Madison, WI, USA). Polyclonal antibody against Bax was provided by Calbiochem (San Diego, CA, USA). Anti-rabbit immunoglobulin peroxidase conjugate was obtained from Amersham Bioscience UK Limited (UK). AmershamTM ECLTM western blotting detection reagents were

obtained from GE Healthcare UK Limited (UK). The High Pure PCR template Preparation Kit for genomic DNA and Proteinase K was obtained from Roche Diagnostics (Basel, Switzerland). Trypan blue stain solution (0.5%) was obtained from Bio-Rad (Hercules, CA, USA). The Cytochrome c Releasing Apoptosis Assay kit was from Medical and Biological Laboratories Co., Ltd (Japan). All other chemicals were analytical grade.

3.2.2 Cell Culture

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37 ℃ and 5% CO_2. The cells were pre-incubated overnight in Nunclon TM Delta treated 25-cm^2 flasks (ThermoFisher Scientific; Waltham, MA, USA), and the medium was then replaced with serum/serum-free DMEM with and without NP_2EO, and serum DMEM containing $CuSO_4$ with and without NP_2EO. Before the transfer to the serum-free medium that was replaced with medium without serum, the cells in the flask were washed twice with serum-free DMEM. The procedures of cell culture are shown as follows.



3.2.3 Cell Viability

Cell viability was measured by trypan blue exclusion assay. PC12 cells were incubated in the serum and serum-free medium with 0-1 μg/mL of NP_2EO or serum medium containing 0-500 μM copper with and without NP_2EO for 72 hr. After incubation, the cells were stained in 0.25% trypan blue solution in phosphate-buffered saline (PBS). Total cells and trypan blue-stained cells were counted using a hemocytometer (TC10TM Automated Cell Counter, Bio-Rad). Cell viability was expressed as a percentage against the total cell number in each experiment. Each experiment was repeated 3-6 times.

3.2.4 Lactate dehydrogenase (LDH) leakage method

The cytotoxicity of NP_2EO was assessed under different experimental settings by measuring the amount of leaked LDH with a nonradioactive cytotoxicity assay kit. PC12 cells were cultured on serum/serum-free DMEM with and without NP_2EO, and serum DMEM containing $CuSO_4$ with and without NP_2EO. The number of cells was counted using a hemocytometer (TC10TM Automated Cell Counter, Bio-Rad), and aliquot of each medium was kept at -20 ℃ until examination. The storage medium (50 μL) was transferred to multititer plates. Same volume of substrate mixture containing tetrazolium salts was added, and the plates were incubated for 30 min. The reaction was terminated by adding stop solution, and the developed formazan dye was quantified by measuring absorbance at 495 nm in a Micro Plate Reader model 450 (Bio-Rad). The cytotoxicity of NP_2EO was expressed as follows (relative LDH activity for sample- relative LDH activity for medium)/1×106 cells.

3.2.5 DNA Extraction from PC12 Cells

The PC12 cells were incubated in serum/serum-free DMEM containing 0-1 μg/mL NP_2EO or serum DMEM containing 0-500 μM $CuSO_4$ with and without NP_2EO for 72 hr. After the treatment, the cells were harvested with a scraper and transferred into 15-mL tubes. The cells were washed with 1×PBS. Genomic DNA was isolated with

High Pure PCR Template Preparation Kit following the manufacturer's instructions. Finally, the DNA was recovered using ethanol precipitation method.

The DNA ladder pattern was quantified using agarose gel electrophoresis. From 3 μg to 5 μg of DNA was subjected to electrophoresis on 1.5% agarose gel. After electrophoresis, the gel was soaked in ethidium bromide solution for 15 min in the dark, and DNA was visualized and photographed under UV illumination with ChemiDoc XRS (Bio-Rad) to determine the extent of cell death. Density of the DNA ladder was estimated by Image J software.

3.2.6 Western Blotting Analysis of Cytochrome c Release in Cytosol

Cytochrome c release in the cytosol was assessed using Cytochrome c Release Apoptosis Assay Kit (Calbiochem, Darmstadt, Germany). The cells prepared following kit instructions were disrupted by sonication for 30 s in a Sonifier 250 (Branson Ultrasonic). Lysed cells were separated from the intact cells by centrifugation at 650 ×g for 5 min. Each supernatant was transferred into a separate 1.5-mL tube and centrifuged at 10,000 ×g at 4 ℃ for 30 min. The treated lysate including released cytochrome c (20-30 μg proteins) was separated by polyacrylamide gel electrophoresis (12.5% polyacrylamide for the separation gel and 3% for the stacking gel), and the electrophoresed proteins were transferred to nitrocellulose membranes with a wetting blotting system, type-AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 ℃ in 0.15% Tween-20, 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 2.5 g dry non-fat milk. The membranes were incubated for 45 min at 37 ℃ with anti-cytochrome c antibody (dilution 1: 200) in 0.15% Tween-20, 40 mM Tris-HCl buffer (pH 7.4), and 150 mM NaCl. The membranes were washed with the same buffer for 3 min 3 times, and then incubated at 37 ℃ for 30 min using anti-mouse IgG-conjugated peroxidase as a secondary antibody (dilution; 1:2500) in the same buffer. The membranes were washed for 3 min 5 times. Finally, the protein bands that responded to antibodies were detected with an enhanced chemiluminescence imaging system (ChemiDoc XRS, Bio-Rad). The cytochrome c content was measured using

Image J software.

3.2.7 Enzyme Linked Immune-Sorbent Assay (ELISA) of BAX and BCL-2 in PC12 Cells

The ELISA procedure was carried out according to Yamanoshita et al. (2000). The cells under different experimental settings were washed with 1×PBS and harvested by centrifugation at 650×g for 5 min. The cells were resuspended in the lysis buffer consisting of 2 mM HEPES, 100 mM NaCl, 10 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 5 mM b-glycerophosphoric acid disodium salt, 50 mM NaF, 1 mM $MgCl_2$, 2 mM DTT, and 1% TritonX-100, and disrupted by sonication (Branson Sonifier 250) for 30 s. The cell debris and intact cells were removed by centrifugation. The contents of BAX and BCL-2 in the cell extracts of the sample solution were measured by ELISA. In brief, 100 μL of each sample was placed in titerplate wells. The plate was incubated for 1 hr at 37℃. After the incubation, the titerplate was washed twice with 40 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20 and blocked by adding 200 μL of the same buffer containing 2% blocking reagent. The plates were then incubated for 1 hr at 37℃. The first antibody for BAX or BCL-2 (1:750 or 1:100 diluted with the same buffer) was immobilized with antigens in the samples for 1 hr at 37℃. Then, the plate was washed with the same buffer 3 times. The second antibody, an anti-rabbit immunoglobulin peroxidase (1:500 diluted with the same buffer), was added and the plate was incubated for 1 hr at 37℃. Then the plate was washed with the same buffer 5 times. After washing, 0.1% OPD in 50 mM phosphate-citrate buffer, pH 5.0, containing 0.03% sodium perborate was added into the wells. After 10 min, HCl was added to stop the enzyme reaction. Absorbance at 495 nm was measured with Microplate Reader model 450 (Bio-Rad).

3.2.8 Statistical Analysis

Each value is expressed as the mean ± S.E. Statistical analyses were performed by the

student's t-test.

3.3 Results

3.3.1 Cell Viability

To evaluate the cytotoxicity of NP_2EO, cell viability was measured by trypan blue staining after the PC12 cells were exposed to 0, 0.1 and 1 μg/mL NP_2EO for 72 hr. As shown in Fig. 3.1 A, no significant difference in viability was observed in cells exposed to 0.1 or 1 μg/mL NP_2EO compared to the viability of cells cultured in serum medium without NP_2EO. A significant decrease in viability was observed in cells cultured in the serum-free medium free of NP_2EO. Furthermore, after the addition of NP_2EO to the serum-free medium, a significant increase of cell viability was observed between 0.1 and 1 μg/mL NP_2EO (Fig. 3.1 B).

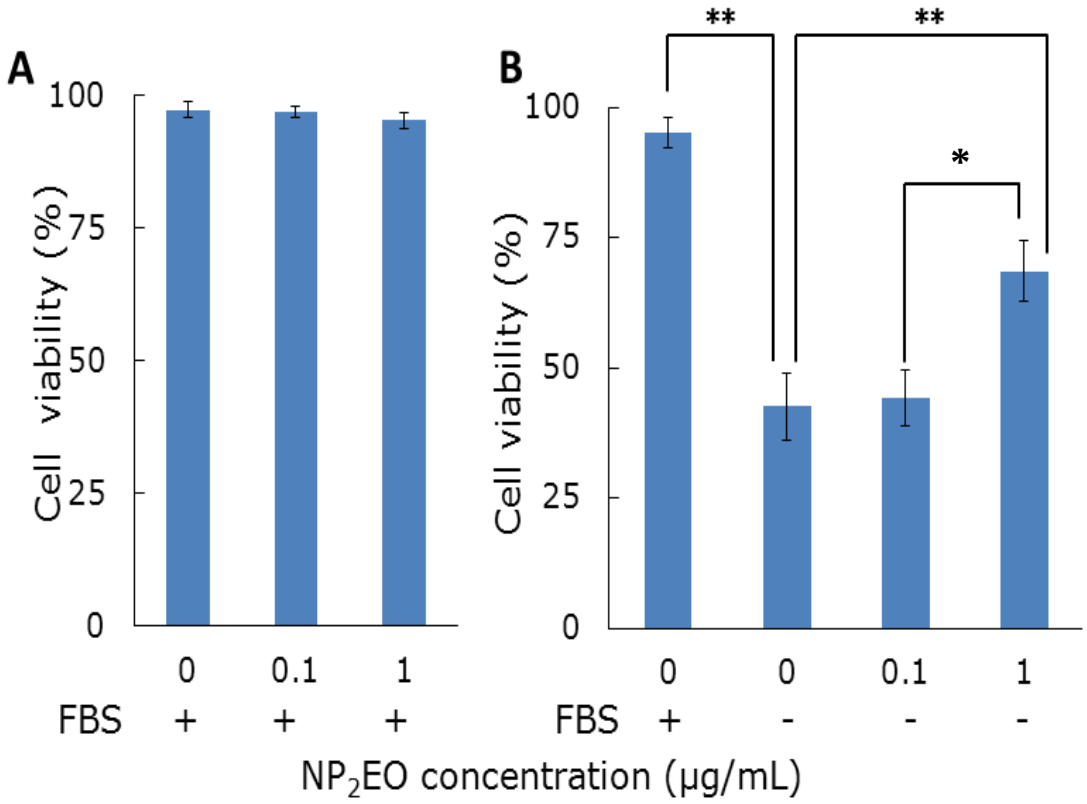


Fig. 3.1 Cell viability of PC12 cells in serum medium (A) and serum-free medium(B) containing 0-1 μg/mL NP_2EO for 72 hr. Error bars indicate SEM (n = 6). **: significant difference ($p < 0.01$). *: significant difference ($p < 0.05$).

Copper has been reported to induce apoptosis in PC12 cells (Kawakami et al., 2008). Cell viability in the serum medium containing $CuSO_4$ (500 μM) decreased significantly (Fig. 3.2). When copper and NP_2EO were co-present in the medium for PC12 cells, cell viability significantly increased compared to the cells exposed to copper only. Therefore, NP_2EO recovers viability of copper treated cells.

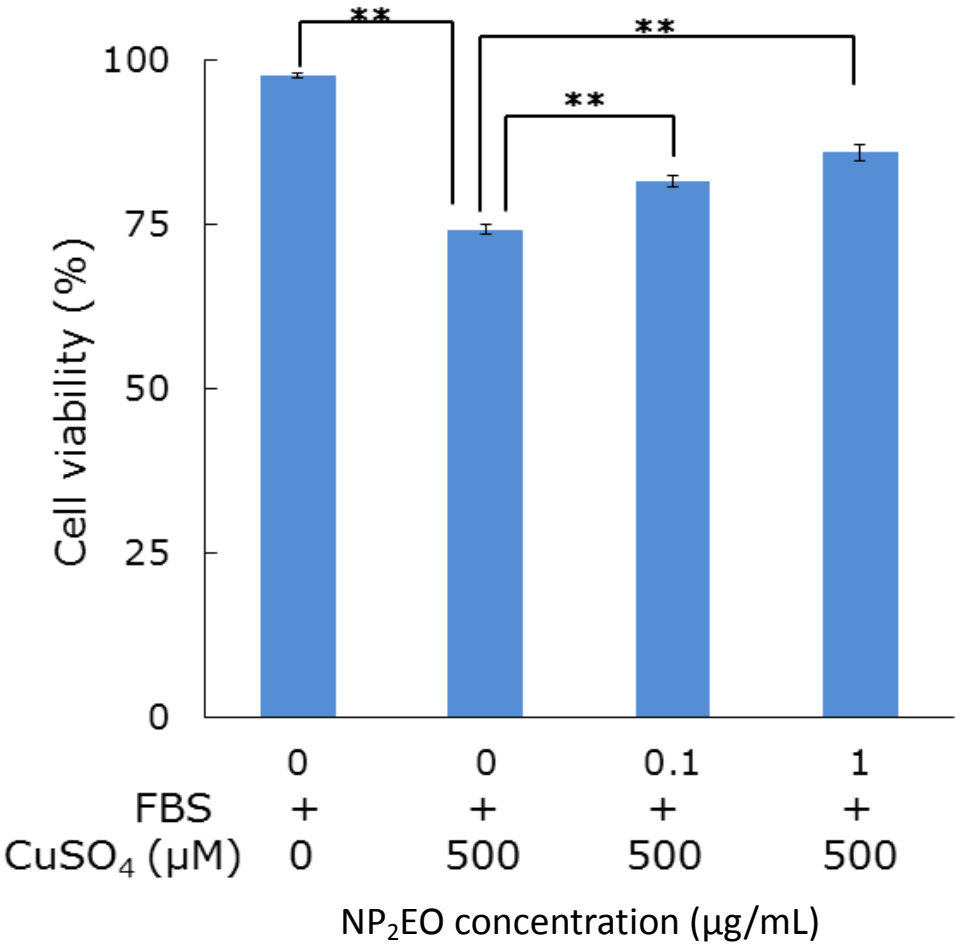


Fig. 3.2 Cell viability of PC12 cells exposed to 500 μM $CuSO_4$ containing 0-1 μg/mL NP_2EO for 72 hr. Error bars indicate SEM (n = 3). **: $p < 0.01$ *: $p < 0.05$.

3.3.2. LDH leakage in cell medium

To confirm the results of the cell viability assay, relative LDH activity/1×106 cells was measured by the PC12 cells were exposed to 0-1 μg/mL NP_2EO for 72 hr. As

shown in Fig. 3.3 A, no significant difference in activity was observed in the medium with cells exposed to 0.1 or 1 μg/mL NP_2EO compared to the activity in the medium with cells cultured in serum medium without NP_2EO. A significant increase in activity was observed in the medium with cells cultured in the serum-free medium free of NP_2EO. This increase was considered to be caused by apoptosis induced by serum deprivation. Similarly to the cell viability profile (Fig. 3.3 B), the addition of NP_2EO to the serum-free medium slightly decreased, the activity in the medium with cells treated with NP_2EO (Fig. 3.3 B). These results indicate that it was indicated that serum deprivation breaks membrane integrity, and NP_2EO protects the membrane permeability.

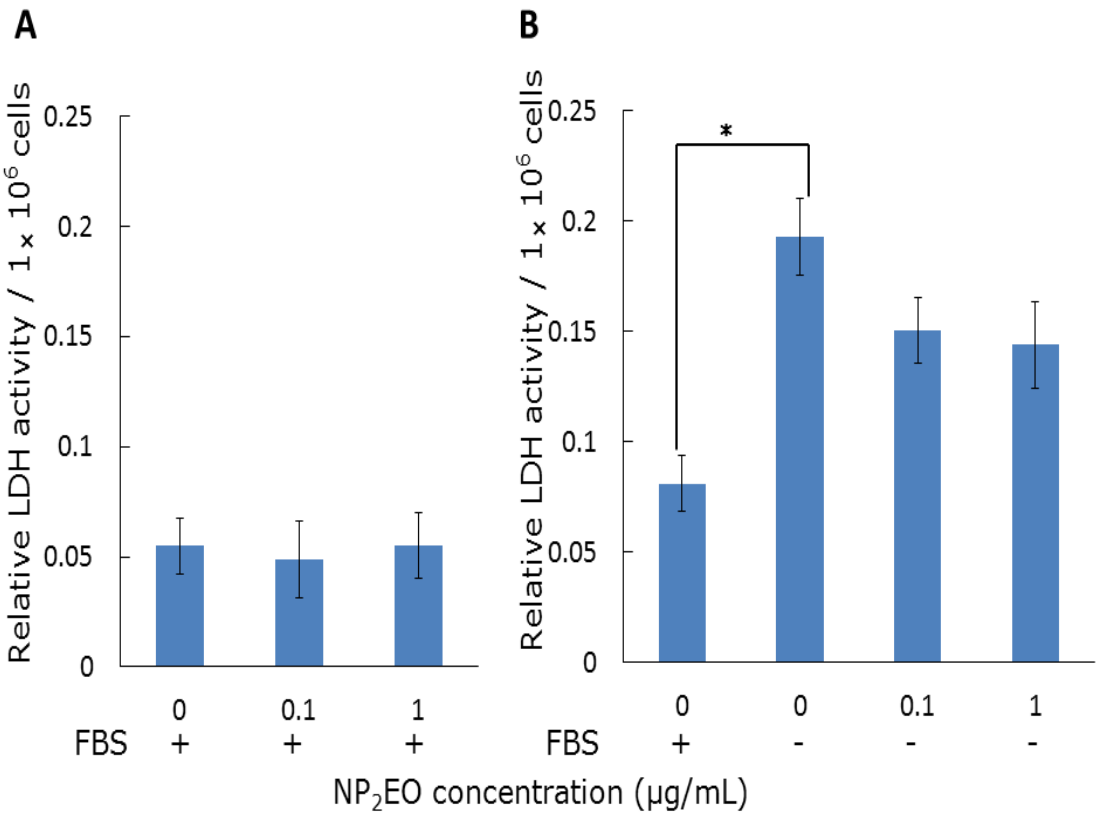


Fig. 3.3 Relative LDH activity of PC12 cells in serum medium (A) and serum-free medium(B) containing 0-1 μg/mL NP_2EO for 72 hr. Error bars indicate SEM (n = 4). *: significant difference ($p < 0.05$).

The activity profile of the medium containing $CuSO_4$ (Fig. 3.4) was very similar to one given in Fig.3.2.

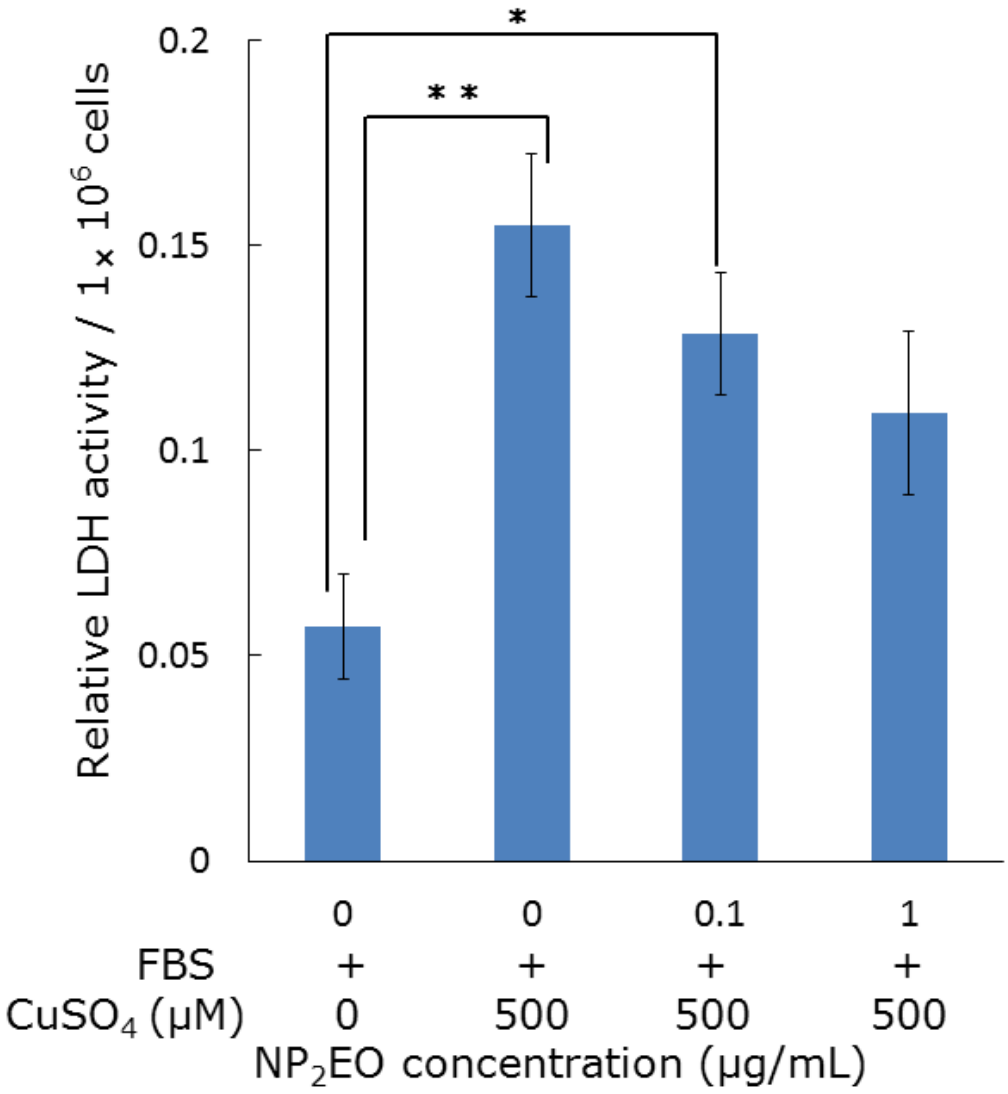


Fig. 3.4 LDH activity of PC12 cells exposed to 500 μM $CuSO_4$ containing 0-1 μg/mL NP_2EO for 72 hr. Error bars indicate SEM (n = 4). **: $p < 0.01$ *: $p < 0.05$.

3.3.3 DNA Fragmentation by Agarose Gel Electrophoresis

Mechanism of apoptosis is shown as follow (Fig. 3.5).

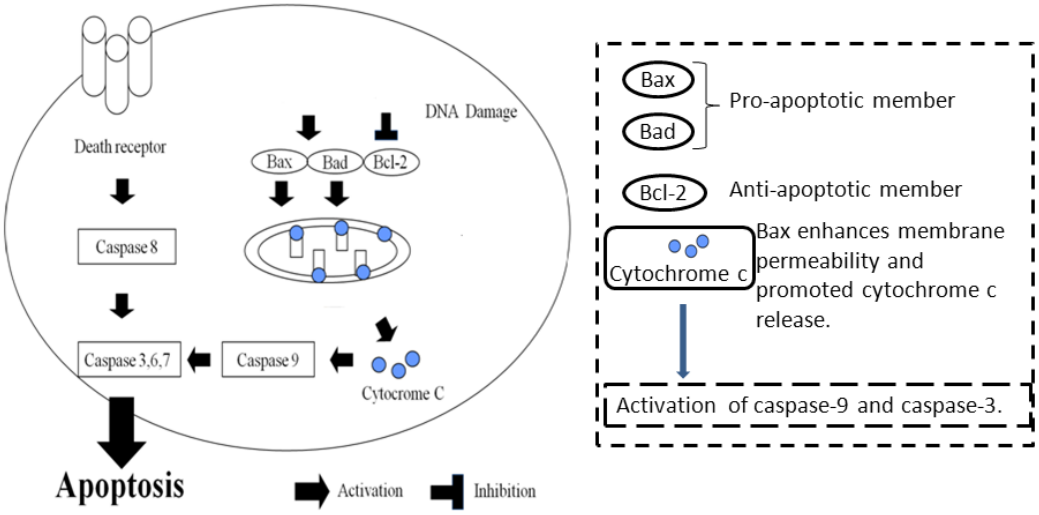


Fig. 3.5 Mechanism of apoptosis

Previous studies reported that apoptosis is induced by serum deprivation in PC12 cells (Maroto and Perez-Polo, 1997). To investigate the effect of NP_2EO on apoptosis, DNA fragmentation of PC12 cells cultured in serum/serum-free medium with and without NP_2EO was studied (Fig. 3.6).

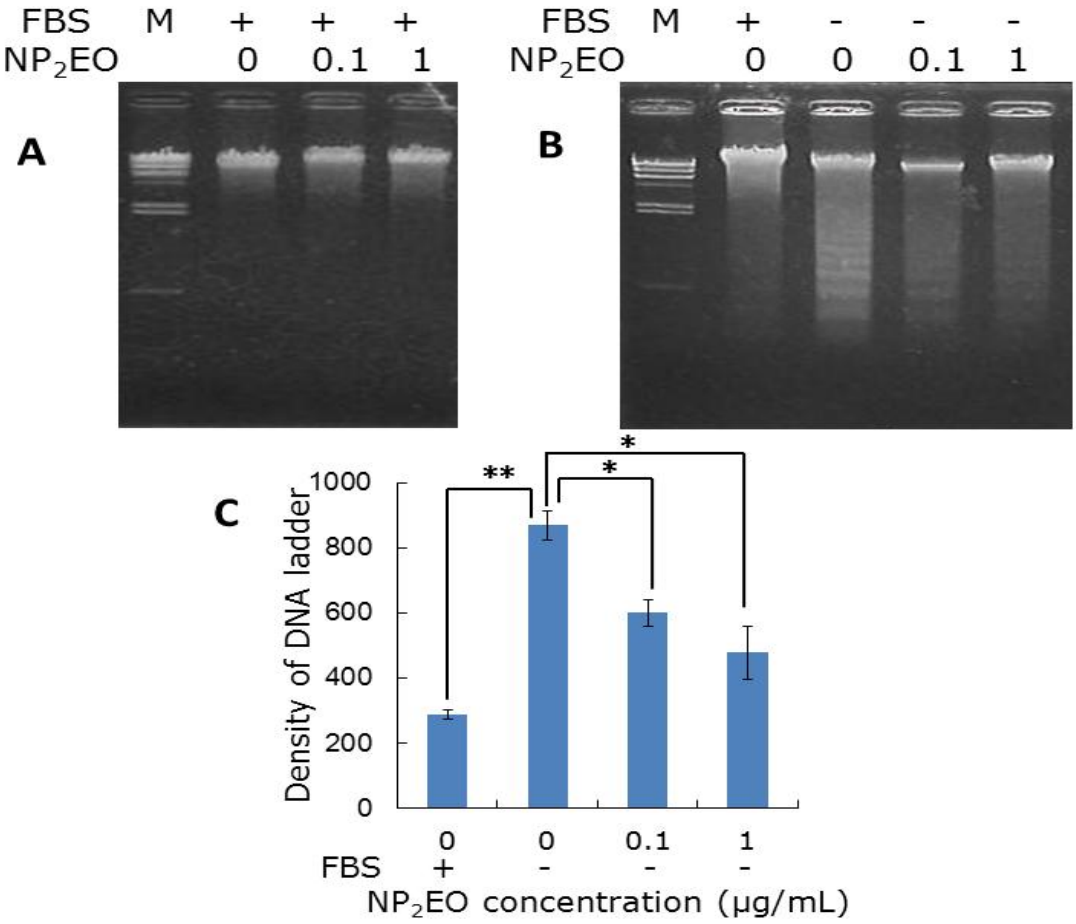


Fig. 3.6 DNA fragmentation of PC12 cells cultured in serum-containing medium (A) and serum-free medium (B) for 72 hr after exposure to 0–1 μg/mL NP_2EO. Fragmentation was evaluated by measurement of density (C). * and ** mean significant differences of $p < 0.05$ and $p < 0.01$ from the cells incubated in serum-free medium without NP_2EO, respectively.

The morphological characteristics of apoptosis are frequently accompanied by multiple cleavage of DNA into fragments 180–200-bp long (Woodgate et al., 1999). DNA fragmentation was observed when apoptosis was induced by serum-deprivation in the cells (Fig. 3.6 B), and it was absent in PC12 cells cultured in the serum containing medium with 0-1 μg/mL NP_2EO (Fig. 3.6 A). These results indicate that NP_2EO does not induce apoptosis in PC12 cells. On the other hand, the DNA ladder pattern gradually decreased in cells cultured in the serum-free medium in a dose-dependent

manner of NP_2EO (Fig. 3.6 B). In addition, the densities of the DNA ladders also decreased when the cells were exposed to 0.1 or 1 μg/mL NP_2EO (Fig. 3.6 C). These results indicate that NP_2EO reduce the apoptosis induced by serum deprivation in PC12 cells.

As expected, the same DNA fragmentation pattern was observed in the serum medium containing $CuSO_4$ (500 μM) treated with 0.1 or 1 μg/mL NP_2EO (Fig. 3.7 A). NP_2EO reduced the DNA fragmentation in the cells grown in serum medium containing CuSO4, with the densities of DNA ladders also decreasing when the cells were exposed to 0.1 or 1 μg/mL NP_2EO (Fig. 3.7 B).

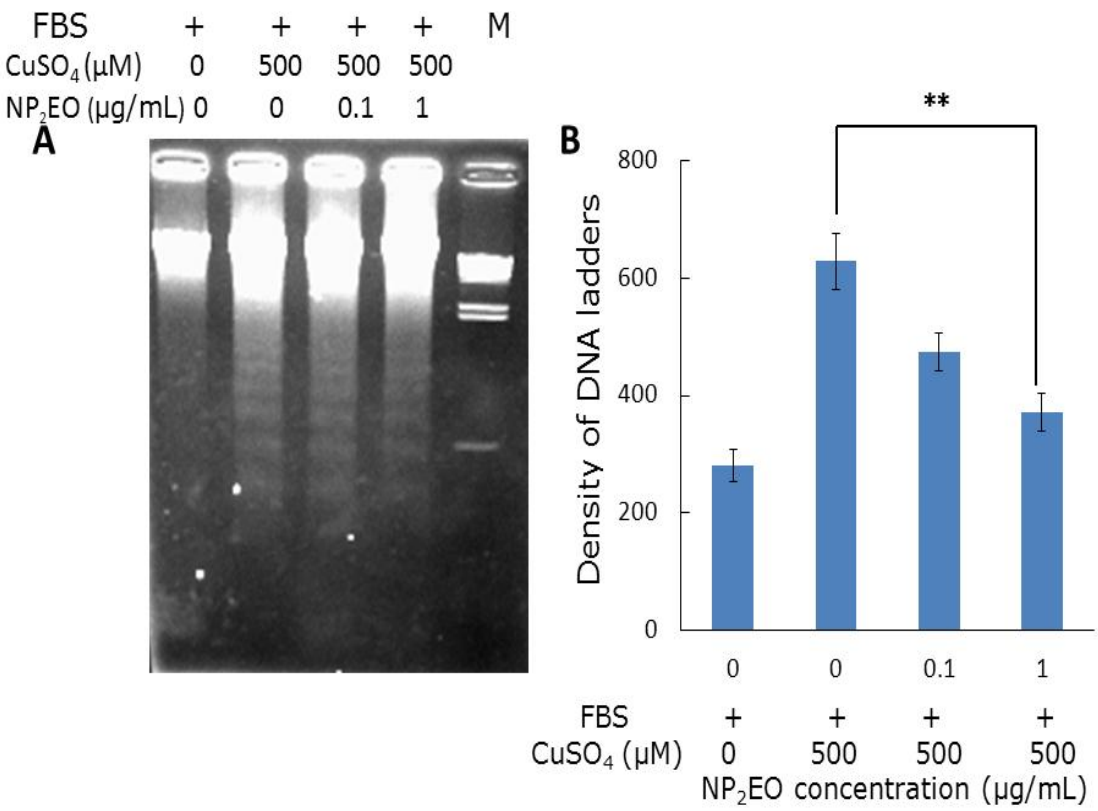


Fig. 3.7 DNA fragmentation of PC12 cells cultured in serum containing medium with 0-500 μM $CuSO_4$ and 0-1 μg/mL NP_2EO for 72 hr (A). Fragmentation was evaluated by the measurement of DNA ladder patterns (B) ** mean significant differences from the cells incubated in serum medium including copper without NP_2EO ($p < 0.01$).

3.3.4 Detection of Cytochrome c Release by Western Blotting

As shown in Fig. 3.8 B, no significant changes in cytochrome c release were observed among the cells cultured in serum medium with and without NP_2EO. These results further confirmed that NP_2EO does not induce apoptosis. Yet, after treating PC12 cells with NP_2EO in the serum-free medium, cytochrome c content declined in comparison to the PC12 cells without NP_2EO (Fig. 3.8 A). Hence, relatively low concentrations of NP_2EO might inhibit the apoptosis induced by serum deprivation in PC12 cells.

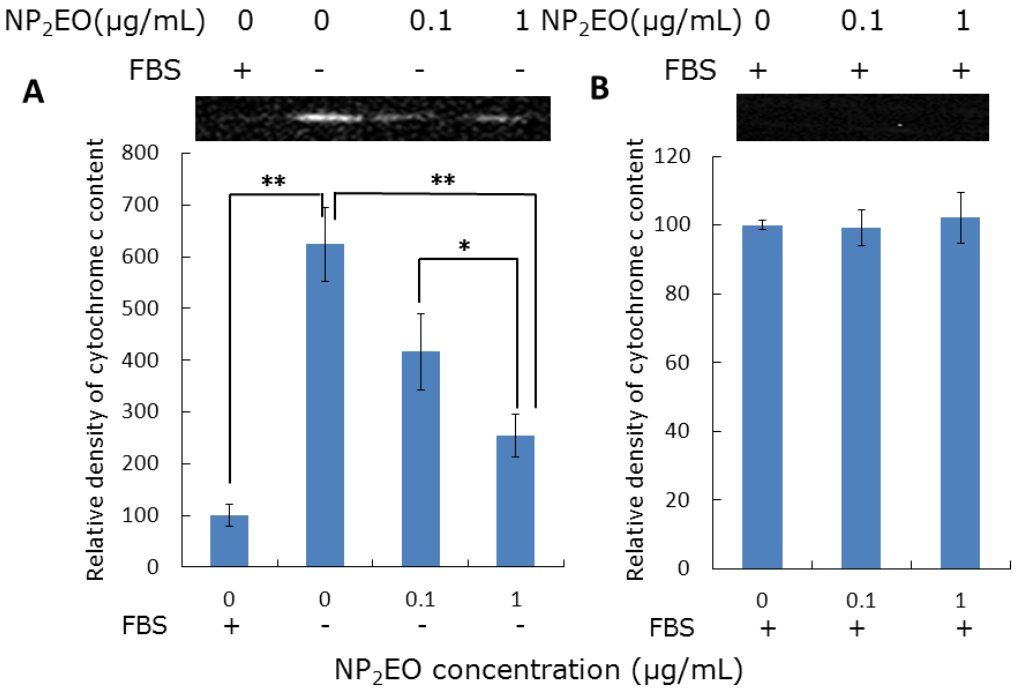


Fig. 3.8 Representative western blot analyses for cytochrome c in PC12 cells cultured in serum-containing medium (A) and serum-free medium (B) for 72 hr after exposure to 0-1 μg/mL NP_2EO. **: significant differences from the cells incubated in serum medium including copper without NP_2EO ($p < 0.01$). *: significant differences ($p < 0.05$).

Furthermore, the cytochrome c release decreased significantly in PC12 cells cultured in serum medium containing copper with 0.1 or 1 μg/mL NP_2EO (Fig. 3.9). Thus, NP_2EO reduced the cytochrome c release under the apoptotic situation caused by copper.

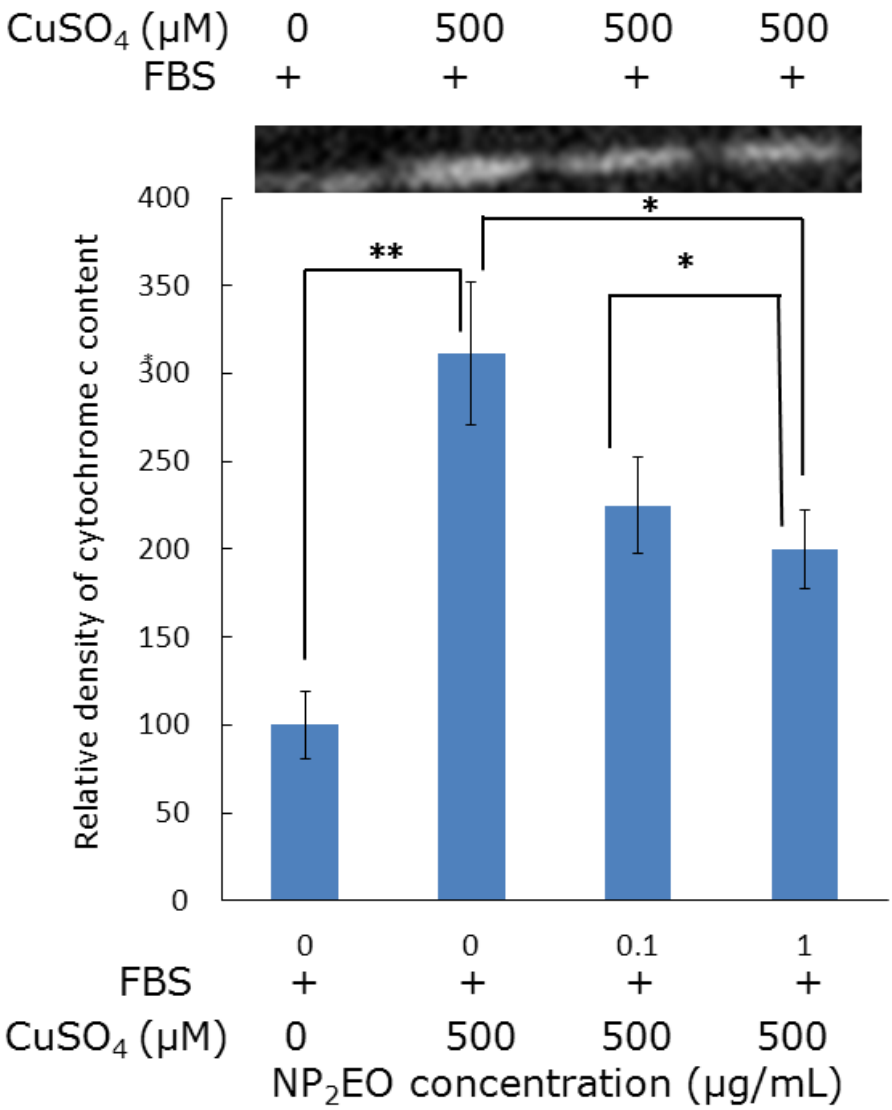


Fig. 3.9 Representative western blot analyses for cytochrome c in PC12 cells cultured in serum medium containing with 0-500 μM $CuSO_4$ for 72 hr after exposure to 0-1 ppm NP_2EO. Error bars indicate SEM (n = 5). * and ** mean significant differences of $p < 0.05$ and $p < 0.01$, respectively.

3.3.5 Contents of BAX, BCL-2 in PC12 Cells by ELISA

To examine whether bcl-2 family is related to reduction of apoptotic situation caused by NP_2EO, the contents of BAX and BCL-2 were measured by ELISA after PC12 cells were exposed to 0-1 μg/mL NP_2EO for 24 hr (Fig. 3.10). NP_2EO significantly decreased BAX contents in PC12 cells cultured in serum-free medium (Fig. 3.10 A).

However, as shown in Fig. 3.10B, there was no significant difference in the contents of BCL-2 between the cells cultured in serum-free medium with and those without NP_2EO.

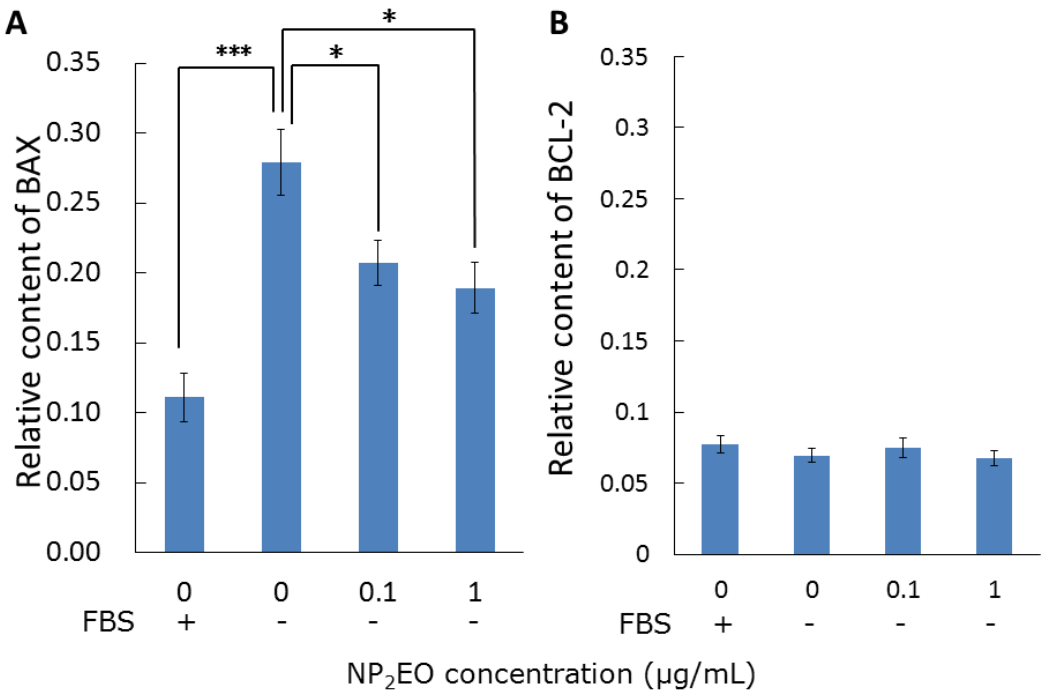


Fig. 3.10 The contents of BAX (A) and BCL-2 (B) in PC12 cells cultured in serum-containing medium or serum-free medium for 24 hr after exposure to 0-1 μg/mL NP_2EO. Error bars indicate SEM (n = 6).* and *** mean significant differences of $p < 0.05$ and $p < 0.001$, respectively.

Similarly, the content of BAX decreased significantly in PC12 cells cultured in serum medium containing copper with 0.1 or 1 μg/mL NP_2EO (Fig. 3.11 A), although the content of BCL-2 did not change significantly in the cells cultured on copper containing medium with and without NP_2EO (Fig. 3.11 B).

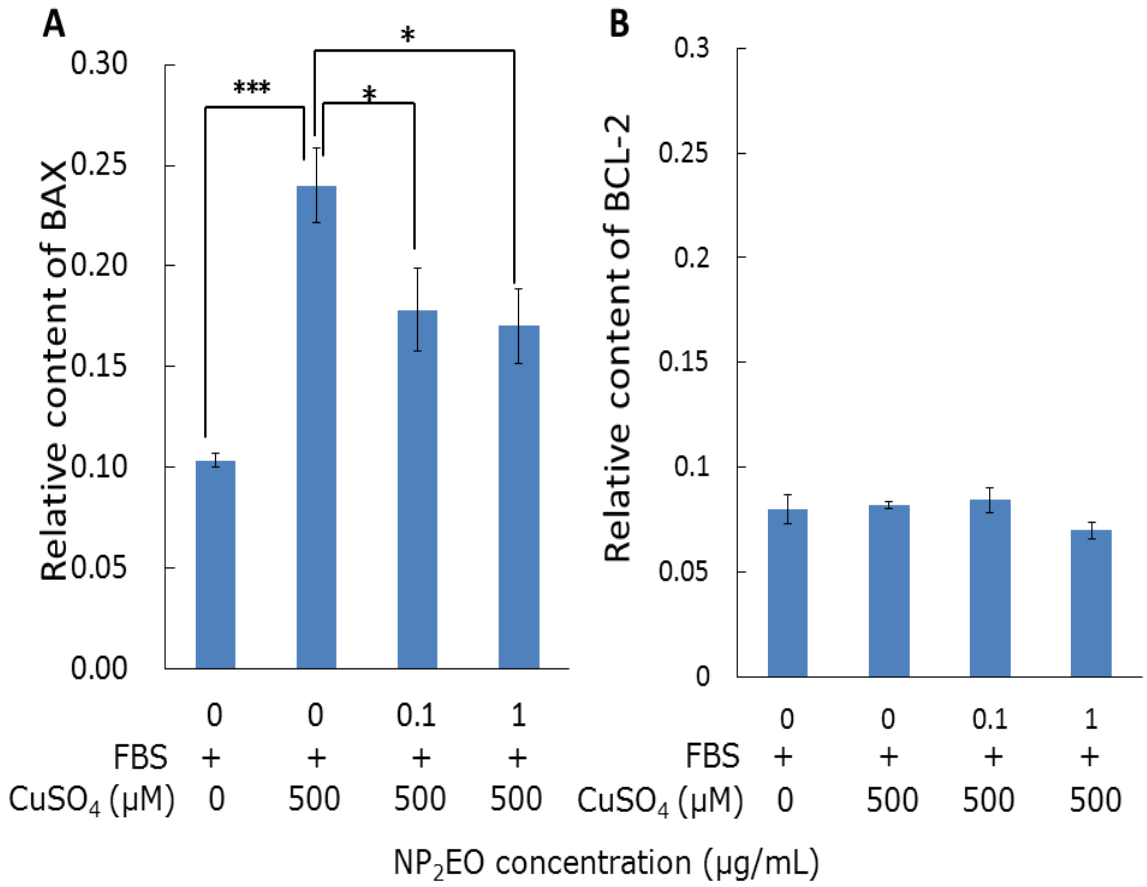


Fig. 3.11 The contents of BAX (A) and BCL-2 (B) in PC12 cells cultured in serum medium containing with 0-500 μM $CuSO_4$ for 72 hr after exposure to 0-1 μg/mL NP_2EO. Error bars indicate SEM (n = 6).* and *** mean significant differences of $p < 0.05$ and $p < 0.001$, respectively.

3.4 Discussion

This study confirmed that NP_2EO inhibits apoptosis induced by serum deprivation through examining cell viability (Fig. 3.1), DNA fragmentation (Fig. 3.6), and by copper exposure through cell viability (Fig.3.2), DNA fragmentation (Fig. 3.7). In addition, there was no cytotoxicity in PC12 cells cultured in serum containing medium with 0.1 or 1 μg/mL NP_2EO, and DNA fragmentation was not observed in these cells (Fig. 3.6 A). The results indicated that the short chain NPEOs do not induce apoptosis. However, compared to PC12 cells without NP_2EO, the cytotoxicity of cells increased

when exposed to 0.1 or 1 μg/mL NP_2EO under apoptotic conditions caused by serum deprivation (Fig. 3.6 B), whereas NP_2EO caused a significant decrease in DNA fragmentation (Fig. 3.6 C). These results indicated that NP_2EO inhibits apoptosis caused by serum deprivation. This finding is additionally supported by the results presented in Fig. 2. These results suggest that NP_2EO could significantly recover the cytotoxicity of cells exposed to $CuSO_4$ because $CuSO_4$ is known to induce apoptosis (Woodgate et al., 1999). In addition, DNA fragmentation decreased with addition of NP_2EO (Fig. 3.7). These results indicated that NP_2EO inhibits apoptosis activity caused by copper exposure.

It has been demonstrated that NP induces apoptosis (Bechi et al., 2006; Kuo et al., 2012). Furthermore, Aoki et al. (2004) reported that NP enhances apoptosis induced by serum deprivation. Yet, the current study showed that NP_2EO itself did not induce apoptosis, and that it inhibited apoptosis induced by serum deprivation and copper exposure. Previous studies have indicated that NP cytotoxicity was higher than the short chain NPEOs (Teneyck and Markee, 2007). Therefore, the strength of toxicity might depend on the length of EO chain, because NP can be defined as NP_0EO.

The current study also aims to determine whether oxidative stress contributes to the observed changes in apoptotic status caused by short chain NPEOs. Cytochrome c release in apoptotic PC12 cells that were and were not exposed to NPEOs was measured by western blotting. Serum deprivation and copper exposure increased the amount of cytochrome c release from mitochondria (Figs. 3.8 and 3.9). This increase was because of the disruption of mitochondrial membrane caused by ROS production (Atlante et al., 2000; Kawakami et al., 2008). The cytochrome c release decreased when PC12 cells were exposed to NP_2EO under the apoptotic condition. These results might be due to NP_2EO causing a reduction in oxidative stress. However, previous research showed that NP exposure induced apoptosis in the liver of malt rats, and enhanced the expression of cytochrome c with increasing oxidative stress (Jubendradass et al., 2012). EO chain length might influence the difference in the toxicity between NP and short chain NPEOs; however, the actual mechanism remains unclear. To elucidate the

different toxicity of these two compounds, examination of NP_2EO under the apoptotic conditions is required.

Serum deprivation has been reported to induce apoptosis in PC12 cells (Maroto and Perez-Polo, 1997). Results obtained in our laboratory suggested that NP enhanced apoptosis by serum deprivation in PC12 cells (Aoki et al., 2004) as indicated by the caspase-3 pathway. In comparison, the contents of BAX and BCL-2 from the BCL-2 family were not altered, with only the content of BAD increasing. These results indicated that enhanced apoptotic cell death might be dependent on the pathway regulating the release of cytochrome c from mitochondria. It has been reported that cytochrome c is released from the mitochondria when apoptosis is induced (Gao et al., 2001). In the current study, it was observed that the relative release of cytochrome c in PC12 cells treated with NP_2EO decreased in serum-free medium and medium containing copper. In addition, the contents of BAX from the BCL-2 family decreased significantly in PC12 cells under apoptotic conditions after exposure to NP_2EO (Figs. 3.10 and 3.11). These results indicated that NP_2EO might inhibit apoptotic pathway because of reduction of Bax contents. It has been reported that tributyl tin and 2,4,5-T completely inhibit apoptosis induced by serum deprivation, decreasing both Bax and proapoptotic protein content (Yamanoshita et al., 2000; 2001). Hence, NP_2EO might have a similar impact on the apoptosis of PC12 cells to these compounds. Therefore, the addition of NP_2EO affects BCL-2 family protein expression, and hence, affects apoptosis induced by serum deprivation or copper exposure.

3.5 Conclusion

NP_2EO inhibited apoptosis induced by serum deprivation and copper. NP_2EO exhibited the opposite effect on apoptosis in PC12 cells compared to NP, which might be due to the difference in the structure of the two compounds, along with different response mechanisms to apoptosis. Ultimately, further investigation is required to clarify the precise toxicity mechanism of short chain NPEOs and NP.

References

Ahel M, Giger W, Koch M. 1994. Behavior of alkylphenol polyethoxylates surfactants in the aquatic environment-I. Occurrence and transformation in sewage treatment. Water Res 28: 1131-1142.

Aoki M, Kurasaki M, Saito T, Seki S, Hosokawa T, Takahashi Y, Fujita F, Iwakuma T. 2004. Nonylphenol enhances apoptosis induced by serum deprivation in PC12 cells. Life Sci 74: 2301-2312.

APERC (Alkylphenols and Ethoxylates Research Council). 2006. Alkylphenols and Alkylphenol Ethoxylates-Product Uses. Alkylphenols and Ethoxylates Research Council, Washington, DC, USA.

Atlante A, Calissano P, Bobba A, Azzariti A, Marra E, Passarella S. 2000. Cytochrome c Is Released from Mitochondria in a Reactive OxygenSpecies (ROS)-dependent Fashion and Can Operate as a ROS Scavenger and as a Respiratory Substrate in Cerebellar Neurons Undergoing Excitotoxic Death. J Biol Chem 275: 37159-37166.

Balch G, Metcalfe C. 2006. Developmental effects in Japanese medaka (Oryziaslatipes) exposed to nonylphenolethoxylates and their degradation products. Chemosphere 62: 1214-1223.

Bechi N, Ietta F, Romagnoli R, Focardi S, Corsi I, Buffi C, Paulesu L. 2006. Estrogen-like response to p-nonylphenol in human first trimester placenta and BeWo choriocarcinoma cells. Toxicol Sci 93: 75-81.

Cai Q, Huang H, Lu H, Mo C, Zhang J, Zeng Q, Tian J, Li Y, Wu X. 2012. Occurrence of Nonylphenol and NonylphenolMonoethoxylate in Soil and Vegetables from Vegetable Farms in the Pearl River Delta, South China. Arch Environ ContamToxicol 63: 22-28.

Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Mintz N, Wolfe G. 1999. The effects of 4-nonylphenol in rats: A multigeneration reproduction study. Toxicol Sci 52: 80-91.

Ferguson SA, Flynn KM, Delclos KB, Newbold RR. 2000. Maternal and offspring toxicity but few sexually dimorphic behavioral alterations result from nonylphenol exposure. Neurotoxicol Teratol 22: 583-591.

Gao W, Pu Y, Luo KQ, Chang DC, 2001. Temporal relationship between cytochrome c release and mitochondrial swelling during UV-induced apoptosis in living HeLa cells. J Cell Sci 114: 2855-2862.

Goto R, Kubota T, Ibuki Y, Kaji K, Go A. 2004. Degradation of nonylpheno lpolyethoxylates by ultraviolet B irradiation and effects of their products on mammalian cultured cells. Toxicology 202: 237-247.

Greene LA, Tischler AS. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci 173: 2424-2428.

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. 1996. Inhibition of testicular growth in rainbow trout (Oncorhynchusmykiss) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15: 194-202.

Jubendradass R, Cynthia D'Cruz SC, Amala J, Rani S, Mathur PP. 2012. Nonylphenol induces apoptosis via mitochondria- and Fas-L-mediated pathways in the liver of adult male rat. Regulatory Toxicology and Pharmacology 62: 405-411.

Kawakami M, Inagawa R, Hosokawa T, Saito T, Kurasaki M. 2008. Mechanism of apoptosis induced by copper in PC12 cells. Food Chem Toxicol 46: 2157-2164.

Knudsen FR, Pottinger TG. 1999. Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid- binding sites in rainbow trout (Oncorhynchus mykiss). Aquat Toxicol 44: 159-170.

Kuo PL, Hsu YL, Tsai MJ, Lien CT, Huang MS, Ko YC. 2011. Nonylphenol induces bronchial epithelial apoptosis via Fas-mediated pathway and stimulates bronchial epithelium to secrete IL-6 and IL-8, causing bronchial smooth muscle proliferation and migration. Basic Clin Pharmacol Toxicol 110: 178-186.

Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 54:

156-167.

Lee PC, Lee W. 1996. In vivo estrogenic action of nonylphenol in immature female rats. Bull Environ Contam Toxicol. 57: 341-348.

Loo TW, Clarke DM. 1998. Nonylphenol ethoxylates, but not nonylphenol, are substrates of the human multidrug resistance P-glycoprotein. Biochem Biophys Res Commun.247: 478-480.

Masuno H, Okamoto S, Iwanami J, Honda K, Shiosaka T, Kidani T, Sakayama K, Yamamoto H. 2003. Effect of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicol Sci 75: 314-320.

Maroto R, Perez-Polo JR. 1997. BCL-2-related protein expression in apoptosis: oxidative stress versus serum deprivation in PC12 cells. J Neurochem 69: 514-523.

Nagao T, Wada K, Marumo H, Yoshimura S, Ono H. 2001. Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reprod Toxicol 15: 293-315.

Naylor CG. 1995. The Environmental Fate and Safety of Nonylphenol Ethoxylates. International Conference & Exhibition;AATCC 1133-1152.

Obata T, Kubota S. 2000. Formation of hydroxyl radicals by environmental estrogen-like chemicals in rat striatum. Neurosci Lett 296: 41-44.

Odum J, Pyrah IT, Soames AR, Foster JR, Van Miller JP, Joiner RL, Ashby J. 1999. Effects of p-nonylphenol (NP) diethylstilbestrol (DES) on the alderley Park (Alpk) rat: Comparison of mammary gland and uterus sensitivity following oral gavage or implanted mini-pumps. J Appl Toxicol 19: 367-378.

Qi Y, Zhang Y, Liu Y, Zhang W. 2013. Nonylphenol decreases viability and arrests cell cycle via reactive oxygen species in Raji cells. Exp Toxicol Pathol 65: 69-72.

Ravni A, Bourgault S, Lebon A, Chan P, Galas L, Fournier A, Vaudry H, Gonzalez B, Eiden LE, Vaudry D. 2006. The neurotrophic effects of PACAP in PC12 cells: control by multiple transduction pathways. J Neurochem 98: 321-329.

Scott-Fordsmand JJ, Krogh PH. 2004. The influence of application form on the toxicity

of nonylphenol to Folsomiafimetaria (Collembola: Isotomidae). Ecotoxicol Environ Saf 58: 294-299.

Seki M, Yokota H, Maeda M, Tadokoro H, Kobayashi K. 2003. Effects of 4-nonylphenol and 4-tert-octylphenol on sex differentiation and vitellogenin induction in medaka (Oryziaslatipes). Environ Toxicol Chem 22: 1507-1516.

Sharma VK, Anquandah GA, Yngard RA, Kim H, Fekete J, Bouzek K, Ray AK, Golovko D. 2009. Nonylphenol, octylphenol, and bisphenol-A in the aquatic environment: a review on occurrence, fate, and treatment. J Environ Sci Health A 44: 423-442.

Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN. 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ Int 34: 1033-1049.

Stasinakis AS, Gatidou G, Mamais D, Thomaidis NS, Lekkas TD. 2008. Occurrence and fate of endocrine disrupters in Greek sewage treatment plants. Water Res 42: 1796-1804.

Teneyck MC, Markee TP. 2007. Toxicity of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate and mixtures of these compounds to pimephalespromelas (fathead minnow) and ceriodaphniadubia. Arch Environ Contam Toxicol 53: 599-606.

Tollefsen KE, Blikstad C, Eikvar S, Finne EF, Gregersen IK. 2008. Cytotoxicity of alkyphenols and alkylated non-phenolics in a primary culture of rainbow trout (Onchorhynchusmykiss) hepatocytes. Ecotoxicol Environ Safe 69: 64-73.

Tollefsen KE, Ingebrigtsen K, Olsen AJ, Zachariassen KE, Johnsen S. 1998. Acute toxicity and toxicokinetics of 4-heptylphenol in juvenile Atlantic cod (Gadusmorhua L.). Environ Toxicol Chem 17: 740-746.

Toyooka T, Kubota T, Ibuki Y. 2012. Nonylphenol polyethoxylates induce phosphorylation of histone H2AX. Mutat Res 741: 57-64.

Vaudry D, Chen Y, Hsu CM, Eiden LE. 2002. PC12 cells as a model to study the neurotrophic activities of PACAP. Ann. NY Acad Sci 971: 491-496.

Vazquez-Duhalt R, MarquezRoche F, Ponce E, Licea AF, Viana MT. 2005 Nonylphenol, an integrate dvision of apollutant. Appl Ecol Environ Res 4:1-25.

Woodgate A, MacGibbon G, Walton M, Dragunow M. 1999. The toxicity of 6-hydroxydopamine on PC12 and P19 cells. Brain Res Mol Brain Res 69: 84-92.

Yamanoshita O, Kurasaki M, Saito T, Takahasi K, Sasaki H, Hosokawa T, Okabe M, Mochida J, Iwakuma T. 2000 Diverse effect of tributyltin on apoptosis in PC12 cells. Biochem Biophys Res Commun 272: 557-562.

Yamanoshita O, Saito T, Takahashi K, Hosokawa T, Okabe M, Ito K, Kurasaki M. 2001. 2,4,5-Trichlorophenoxyacetic acid inhibits apoptosis in PC12 cells. Life Sci 69: 403-408.

Ying GG, 2006. Fate, behavior and effects of surfactants and their degradation products in the environment. Environ Int 32: 417-431.

Chapter 4 Comparison study of effects of short chain NPEOs and relative long chain NPEOs on apoptosis induced in PC12 cells

Abstract

Short chain NPEOs have been reported to more toxic than long chain NPEOs on aquatic species and NPEOs are widely existed in various environments, such as water, sludge, sediment and so on. To understand the different toxicity of various NPEOs from NP_2EOs, we investigated the effects of NP_1EO, NP_5EO, and $NP_{10}EO$ on apoptosis induced by copper in PC12 cell system. NP_1EO belongs to short chain NPEOs; however, $NP_{10}EO$ is long chain NPEOs. As results, NP_1EO, NP_5EO, and $NP_{10}EO$ themselves have no toxicity as same as NP_2EO and NP_1EO. In addition, there is no effect of NP_5EO and $NP_{10}EO$ on cell viability caused by apoptotic condition; however, DNA fragmentation caused by apoptosis was decreased when PC12 cells were treated with NP_1EO. It was indicated that the length of EO part in NPEOs has given different impacts on apoptosis. Although NP enhances apoptosis induced by serum deprivation, short chain NPEOs inhibited apoptosis induced in PC12. Furthermore, long chain NPEOs (more than 5 EO parts) have no effect on apoptosis in PC12 cells. These differences might be considered to be depending on structure of these compounds, especially length of EO part.

4.1 Introduction

Commercial NPEOs are consisting of compounds having hydrophilic chain, 3–20 EO units (Di Corcia et al., 2000). NPEOs in environments or wastewater were degraded into NP and other metabolites (NPEOs) which could be more toxic as compared with their parent compound (Ying et al., 2002; Soares et al., 2008), and be more bioaccumulative in aquatic organisms (Shang et al., 1999).

Usually degradation process of NOEOs was not progressed completely. Biological degradation of NPEOs occurs in natural environments and waste water treatment plants. These substances were mainly contaminated into surface waters via effluents from the wastewater treatment plants. However, NPEOs are easily bioconverted into short chain NPnEOs (n=1-3). These compounds appear to be recalcitrant to further microbial attack and, consequently they accumulate in sediments, ground waters and sewage sludge (Giger et al., 1984; Vallini et al., 2001). In current study, it was shown that degradation of relative short chain was mainly carried out in the sludge. These nonylphenolic metabolites are difficult to further degrade. These environmental endocrine disruptors are exogenous chemicals which affect the balance of normal hormonal functions, and produce adverse developmental, reproductive, neurological, and immune effects in animals (Crisp et al., 1998).

It is well known that toxicity of NPEOs against aquatic organisms decreases with increasing degree of ethoxylation (EC, 2002; EPA, 2010). Xuelei et al. (2011) showed that NP was more toxic to *Moina macrocopa* than $NP_{10}EO$. Depending on length of ethoxylate, 24 and 48 hr after treatment of the chemicals LC50 values were 0.154 and 0.065 mg/L for NP, and 3.37 and 2.11 mg/L for $NP_{10}EO$, respectively.

The objective of this part is to investigate effect of NPEOs including the different EO chain length on apoptotic status caused by copper exposure, and to clarify the mechanisms of the effects of short chain NPEOs and long chain NPEOs on apoptotic conditions. The significance of obtained results was discussed in detail.

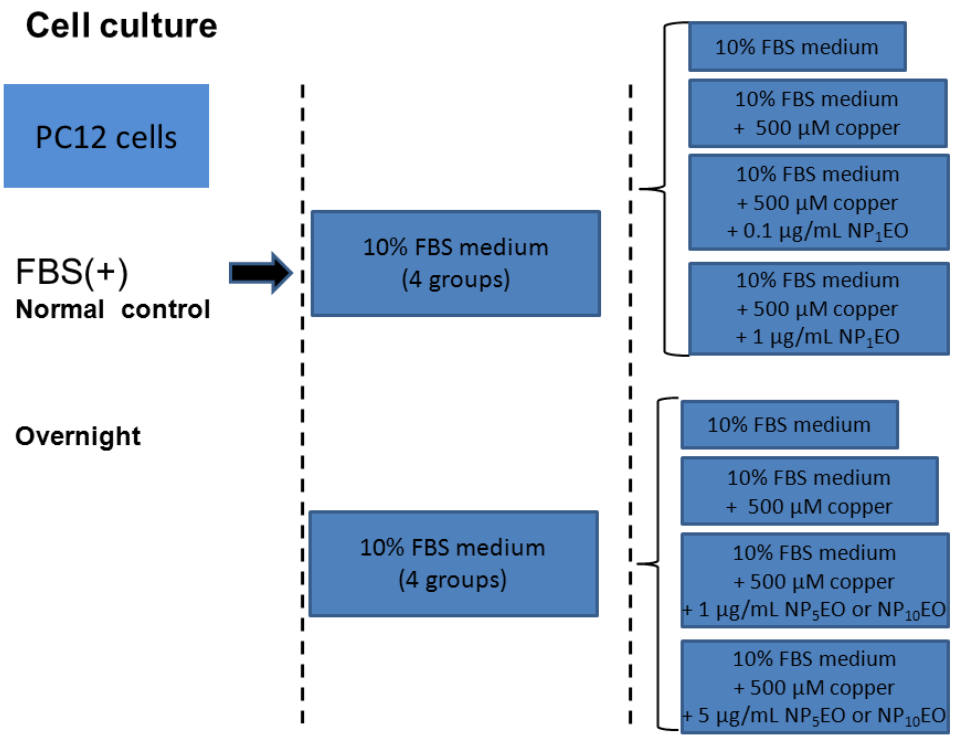
4.2 Material and methods

4.2.1 Materials

In this part, all of materials are the same as chapter 3.

4.2.2 Cell Culture

First of all, PC12 cells were incubated in DMEM supplemented with 10% FBS in a humidified incubator at 37℃ and regulated as 5% CO_2. The cells were pre-incubated for overnight in Nunclon TM Delta treated 25-cm^2 flasks (ThermoFisher Scientific; Waltham, MA, USA), and the medium was then replaced with serum/serum DMEM containing 500 μM $CuSO_4$ with and without NP_1EO, NP_5EO and $NP_{10}EO$. The procedures of cell culture in detail are shown as follow.



4.2.3 Cell Viability

Cell viability was measured by trypan blue exclusion assay. PC12 cells were incubated in the serum medium containing 0-500 μM copper with and without NP_1EO (0-1 μg/mL), NP_5EO (0-5 μg/mL) $NP_{10}EO$ (0-5 μg/mL) for 72 hr, respectively. After incubation, a part of cells was stained in 0.25% trypan blue solution in phosphate-buffered saline (PBS). Total cells and trypan blue-stained cells were counted using a hemocytometer (TC10TM Automated Cell Counter, Bio-Rad). Cell viability was expressed as a percentage against the total cell number in each experiment. Each experiment was repeated 3-6 times.

4.2.4 DNA Extraction from PC12 Cells

The PC12 cells were incubated in serum DMEM containing 0-500 μM $CuSO_4$ with and without NP_1EO, NP_5EO or $NP_{10}EO$ for 72 hr. After the treatments, the cells were harvested with a scraper, and transferred into 15-mL tubes. The cells were washed with 1×PBS. Genomic DNA was isolated with High Pure PCR Template Preparation Kit following the manufacturer's instructions. Finally, the DNA was recovered using ethanol precipitation method.

The DNA ladder pattern was quantified using agarose gel electrophoresis. From 3 to 5 μg of DNA were subjected for electrophoresis on 1.5% agarose gel. After electrophoresis, the gel was soaked in ethidium bromide solution for 15 min in the dark, and DNA was visualized and photographed under UV illumination with ChemiDoc XRS (Bio-Rad) to determine the extent of cell death.

4.3 Results

4.3.1 Cell Viability

To evaluate effects of different EO chain in NPEOs on PC12 cells, these three NPEOs, NP_1EO (0-1 μg/mL), NP_5EO (0-5 μg/mL) and $NP_{10}EO$ (0-5 μg/mL), were selected. No significant difference in cell viabilities in cells exposed to NP_1EO, NP_5EO, and $NP_{10}EO$ was observed as compared with the viabilities of cells cultured in serum medium without NPEOs (data are not shown).

To clarify effects of NP_1EO, NP_5EO, and $NP_{10}EO$ on apoptotic PC12 cells, cell viability was measured by trypan blue staining after the PC12 cells cultured in serum containing medium with copper were exposed to 0-1 μg/mL NP_1EO, 0-5 μg/mL NP_5EO and 1-5 μg/mL $NP_{10}EO$ for 72 hr. It is well known that copper has been reported to induce apoptosis in PC12 cells (Kawakami et al., 2008). Cell viability of the cells cultured in the serum medium containing $CuSO_4$ (500 μM) decreased significantly as compared with that in the medium without copper (Fig. 4.1). When copper and NP_1EO were co-present in the medium for PC12 cells, cell viability significantly increased compared to the cells exposed to copper only (Fig. 4.1 A). Therefore, NP_1EO can recover viability of copper treated cells. However, the same concentration of NP_5EO and $NP_{10}EO$ compared with NP_1EO did not impact on cell viability in the serum medium containing $CuSO_4$ (500 μM).

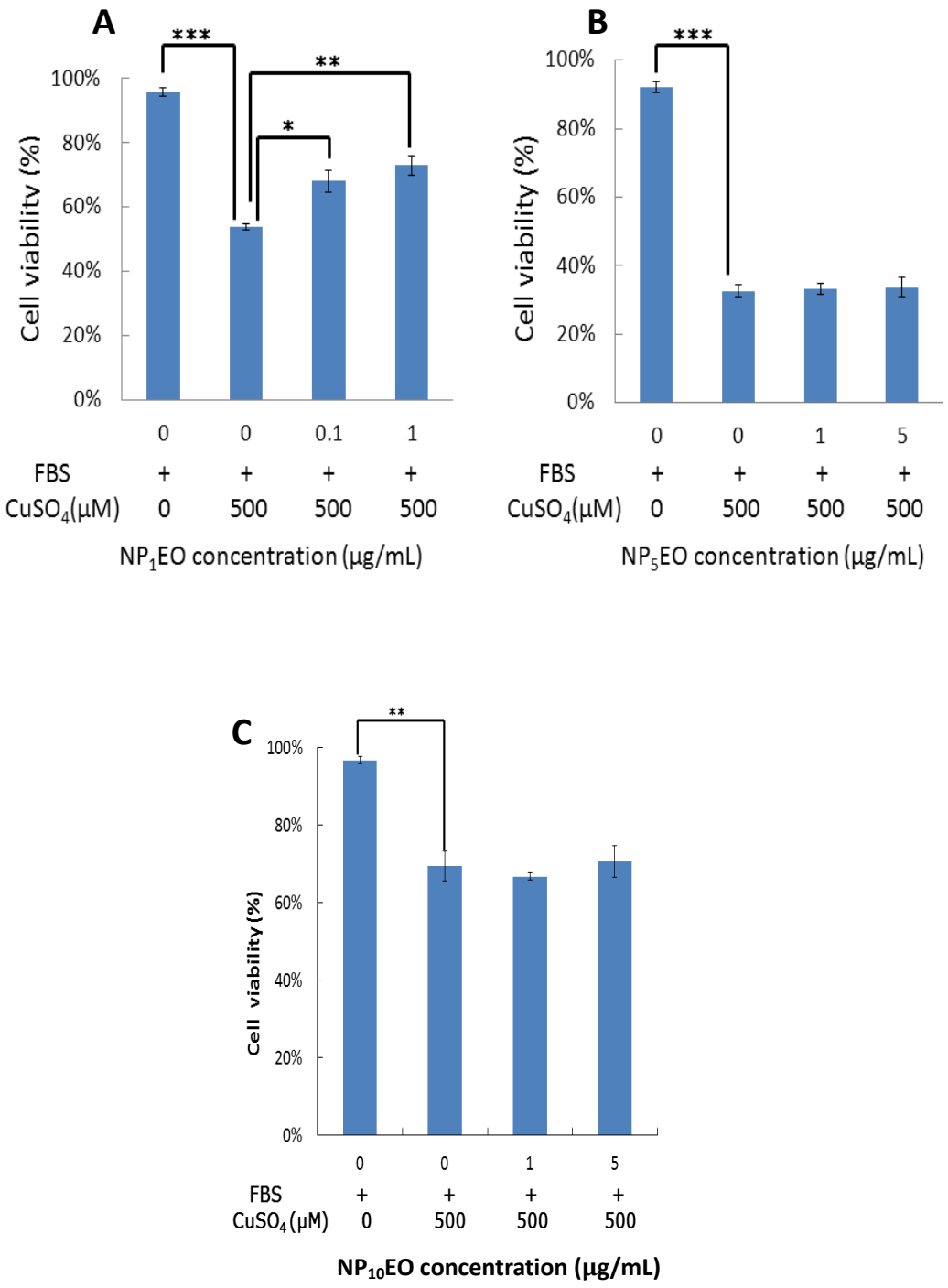
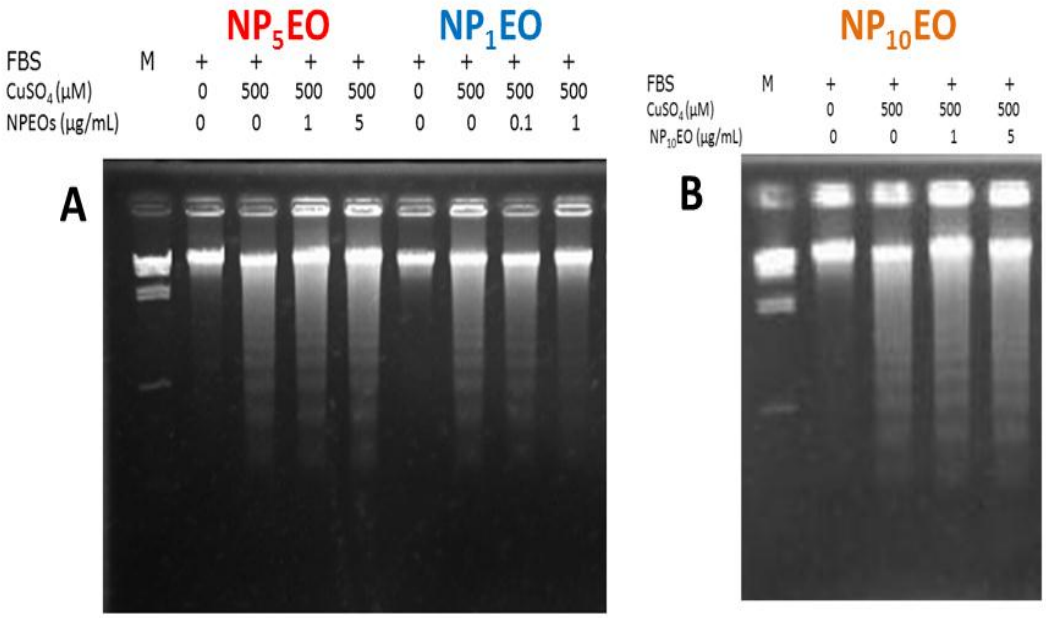


Fig. 4.1 Cell viabilities of PC12 cells exposed to 500 μM $CuSO_4$ containing (A) 0-1 μg/mL NP_1EO, (B) 0-5 μg/mL NP_5EO and (C) 0-5 μg/mL $NP_{10}EO$ for 72 hr. Error bars indicate SEM (n = 3). ***: $p < 0.001$**: $p < 0.01$ *: $p < 0.05$.

4.3.2 DNA Fragmentation by Agarose Gel Electrophoresis

It has been reported that the morphological characteristics of apoptosis are frequently accompanied by multiple cleavage of DNA into fragments 180–200-bp long (Woodgate et al., 1999). The DNA ladders were not observed when cells were cultured in serum medium with or without the target compounds (NP_1EO, NP_5EO, and $NP_{10}EO$ (data not shown). These results indicate that these NPEOs themselves did not induce apoptosis in PC12 cells. At the same time, DNA ladder was observed when apoptosis was induced by copper in the serum for the cells (Fig. 4.2), and the ladder form was decreased in PC12 cells cultured in the serum containing copper medium with 0-1 μg/mL NP_1EO (Fig. 4.2 A). On the other hand, the changes of DNA fragmentation pattern was not observed in the serum medium containing $CuSO_4$ (500 μM) treated with NP_5EO or $NP_{10}EO$ (Fig. 4.2 A and B). It meant that the relative high concentrations of NP_5EO, $NP_{10}EO$ do not affect apoptosis induced by copper exposure.



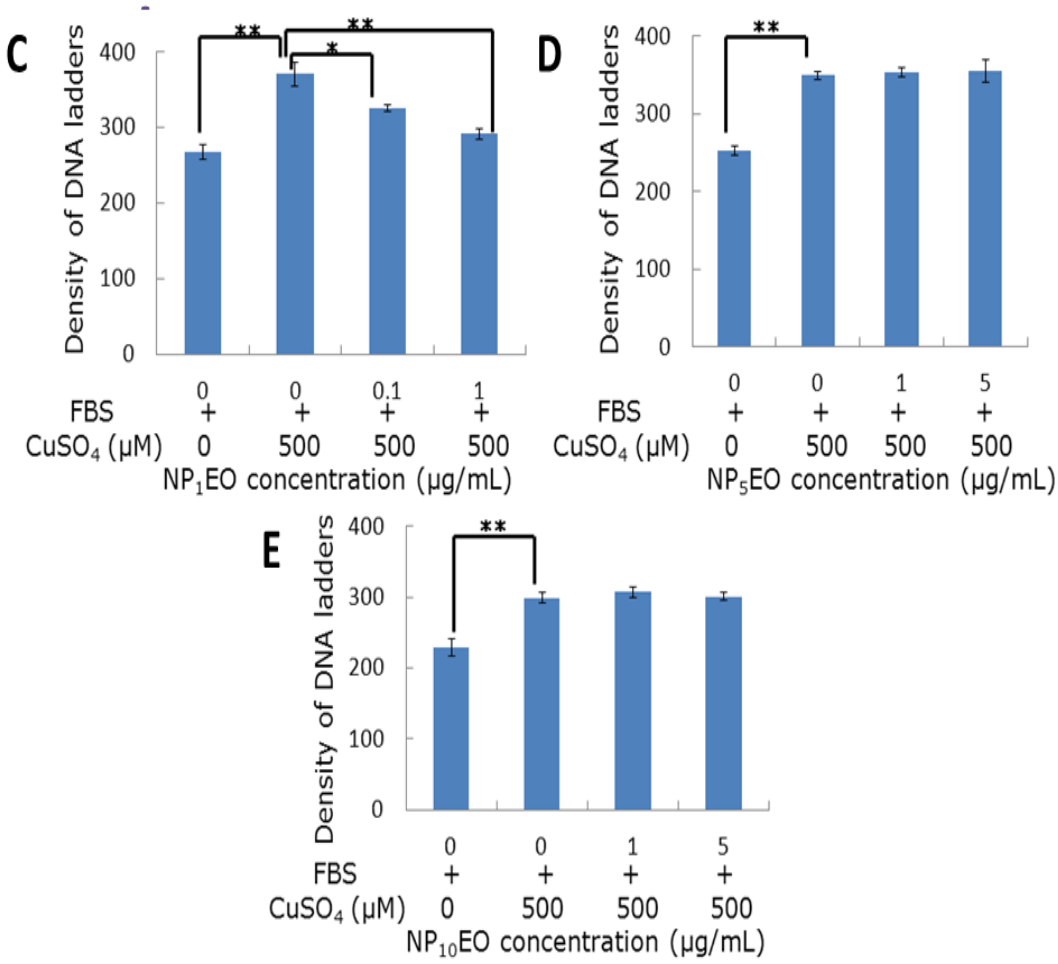


Fig. 4.2 DNA fragmentation of PC12 cells cultured in serum-containing medium with 0-500 μM $CuSO_4$ and with (A) 0-1 μg/mL NP_1EO, 0-5 μg/mL NP_5EO, and (B) 0-5 μg/mL $NP_{10}EO$. Fragmentation was evaluated by measurement of density (C) NP_1EO, (D) NP_5EO (E) $NP_{10}EO$ Error bars indicate SEM (n=3). * and ** mean significant differences of $p < 0.05$ and $p < 0.01$ from the cells incubated in serum containing copper medium without NPEOs, respectively.

4.4 Discussion

In Chapter 3, it has been clarified that NP_2EO inhibits apoptosis under apoptotic conditions through changes of the related apoptotic factors. In addition, it is also confirmed that NP_2EO has no cytotoxicity and cannot induce apoptosis. At the

same time, comparison studies about NPEOs having different EO chain are done to prove the hypothesis presented in chapter III. As compared to NP_2EO, it was expected that NP_1EO could significantly recover the cytotoxicity of cells exposed to $CuSO_4$ (Fig. 4.1 A) although $CuSO_4$ is known to induce apoptosis (Woodgate et al., 1999). In addition, DNA ladder decreased clearly after treatment of NP_1EO (Fig. 3.6). These results indicated that NP_1EO also inhibits apoptosis caused by copper exposure as same as NP_2EO. The short chain NPEOs has the same effects on apoptosis under apoptotic condition in PC12 cells. To further confirm the hypothesis, the relative long chain NPEOs (NP_5EO) and long chain NPEOs ($NP_{10}EO$) are chosen to analyze effects on apoptosis induced in PC12 cells. Teneyck and Markee (2007) had indicated that the strength of toxicity of NPEOs might depend on the length of EO chain.

It the current study, it was confirmed that the relative long chain NPEOs did not impact on apoptosis by detecting cell viability (Figs. 4.1 B and C) and DNA fragmentation (Fig. 4.2). These results indicated that toxicity of NP_1EO was caused by reduction of oxidative stress. EO chain length might influence the difference from the toxicity of NPEOs. However, the related mechanism remains still unclear. To elucidate the mechanism of toxicity of these compounds, some relative studies will be need.

4.5 Conclusion

NP_1EO has the same impacts of NP_2EO on apoptosis under apoptotic condition. It meant that NP_1EO inhibited apoptosis induced by copper. The short chain NPEOs such as NP_1EO and NP_2EO exhibited the opposite effect on apoptosis in PC12 cells as compared with NP. The differences of behavior on apoptosis might be due to the difference in the structure of these compounds. The relative high concentrations of

NP_5EO and $NP_{10}EO$ did not affect apoptosis. It is indicated that the different length of EO chain has different effects on cytotoxicity and apoptosis. Ultimately, further investigation will be required to clarify the precise toxicity mechanism of short chain NPEOs and NP and long chain NPEOs.

References

Crisp M, Clegg D, Cooper L, Wood P, Anderson G, 1998. Environmental endocrine disruption: an effects assessment and analysis. Environ Health Perspect, 106: 11-56.

Di Cocia A, Cavallo R, Crescenzi C, Nazzari M, 2000. Occurrence and abundance of dicar-boxylated metabolites of nonylphenol polyethoxylate surfactants in treatedsewages. Environ Sci Technol, 34: 3914-3919.

EC. 2nd Priority List Volume: 10 European Union Risk Assessment Report.4-Nonylphenol (branched) and nonylphenol 2nd Priority List Volume: 10. Euro-pean Commission; 2002.

EPA. Nonylphenol (NP) and nonylphenol ethoxylates (NPEs) action plan. U.S. Envi-ronmental Protection Agency; 2010.

Giger W, Brunner PH, Schadner C, 1984. 4-nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. Science, 225: 623-625.

Kawakami M, Inagawa R, Hosokawa T, Saito T, Kurasaki M. 2008. Mechanism of apoptosis induced by copper in PC12 cells. Food Chem Toxicol 46: 2157-2164.

Shang Y, Macdonald W, Ikonomou G, 1999. Persistence of nonylphenol ethoxylate surfactants and their primary degradation products in sediments from near a municipal outfall in the Strait of Georgia, British Columbia, Canada. Environ Sci Technol, 33: 1366-1372.

Soares A, Guieysse B, Jefferson B, Cartmell E, Lester JN, 2008. Nonylphenol in the envi-ronment: a critical review on occurrence, fate, toxicity and treatment inwastewaters. Environ Int, 34: 1033-1049.

Teneyck MC, Markee TP, 2007. Toxicity of nonylphenol, nonylphenol

monoethoxylate, and nonylphenol diethoxylate and mixtures of these compounds to pimephalespromelas (fathead minnow) and ceriodaphniadubia. Arch Environ Contam Toxicol, 53: 599-606.

Vallini G, Frassinetti S, D'Andrea F, Catelani G, Agnolucci M, 2001. Biodegradation of 4-(1-nonyl) phenol by axenic cultures of the yeast Candida aquaetextoris: identification of microbial break down products and proposal of a possible metabolic pathway.Intern. Biodet.Biodegr, 47: 133-140.

Woodgate A, MacGibbon G, Walton M, Dragunow M, 1999. The toxicity of 6-hydroxydopamine on PC12 and P19 cells. Brain Res Mol Brain Res, 69: 84-92.

Xuelei H, Jingyun Z, Shunshan D, 2011. Synergistic toxic effects of nonylphenol and nonylphenol ethoxylate on Moina macrocopa. Ecol Environ Sci, 20: 1725-1730.

Ying GG, Williams B, Kookana R, 2002. Environmental fate of alkylphenols and alkylphe-nol ethoxylates – a review. Environ Int, 28: 215-226.

Chapter 5 Total conclusions

Biodegradation is the main process to treat NPEOs and their intermediate metabolites as compared with the other degradation processes. Biological process to remove NPEOs from water because majority of them are adsorbed to solids in primary sewage Treatment plants (STW). Abiotic processes like hydrolysis or photolysis are negligible in degradation of NP and short chain NPEOs in water. Primary NPEOs are transformed to NP_1EO or NP_2EO under anaerobic condition, and NP_1EC and NP_2EC under aerobic condition, before finally degraded into NP under anaerobic conditions.

In this study, in activated sludge process, absorption rates of NP_1EO to NP_3EO were more than 60%, these compounds could be accumulated quickly in sludge at 10 min. But at the same, it was clear that biodegradation process had some roles in treatment of short-chain NPEOs. After 24 hours, NP_2EO and NP_3EO contents were very low, NP_1EO content was relative higher. It was indicated that NP_2EO and NP_3EO were converted to NP_1EO by microorganisms. Furthermore, it could be confirmed that absorption process was more than 60% by inactivated sludge system. Short-chain NPEOs could be accumulated rapidly in sludge in 10 min. Short- chain NPEOs had certain effects on COD removal. Short chain NPEOs could be biodegraded in activated sludge, but it is more difficult than primary NPEOs. Treated wastewater still contains amounts of short chain NPEOs.

Apoptosis has been shown to be induced by serum deprivation or copper treatment. To understand the toxicity of NP_2EO, we investigated the effects of NP_2EO on apoptosis induced by serum deprivation and copper by using PC12 cell system. NP_2EO itself showed no toxicity and recovered cell viability from apoptosis. In addition, NP_2EO decreased DNA fragmentation caused by apoptosis in PC12 cells.

This phenomenon was confirmed after treating apoptotic PC12 cells with NP_2EO, whereas the cytochrome c release into the cytosol decreased compared to that in apoptotic cells not treated with NP_2EO. Furthermore, BAX contents in apoptotic cells were reduced after exposure to NP_2EO. Thus, NP_2EO has the opposite effect on apoptosis in PC12 cells compared to NP, which enhances apoptosis induced by serum deprivation. The difference in structure of the two compounds is hypothesized to be responsible for this phenomenon. These results indicated that NP_2EO has capability to affect cell differentiation and development and has potentially harmful effect on organisms because of its unexpected impact on apoptosis. Present study final conclusion was shown in follow paragraph.

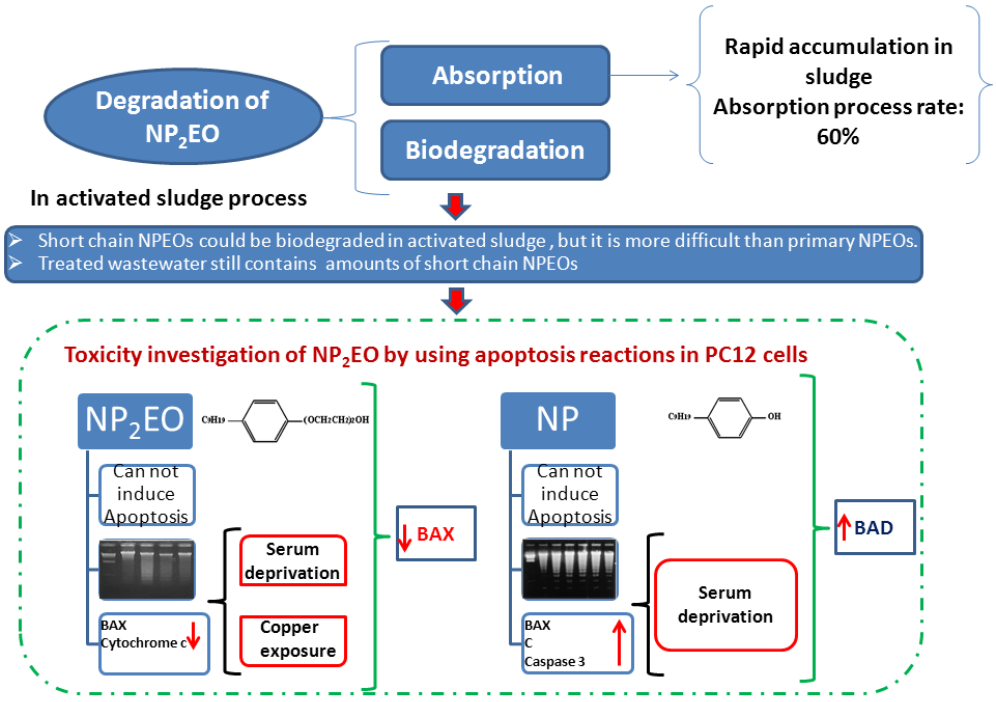


Fig. 5.1 The final conclusions of NP_2EO and NP

To clarify the mechanisms of the effects of short chain NPEOs and long chain NPEOs on apoptotic conditions, cells were exposed to serum containing copper medium with or without NP_1EO, NP_5EO, or $NP_{10}EO$. NP_1EO has the same

impacts of NP_2EO on apoptosis under apoptotic condition. It meant that NP_1EO inhibited apoptosis induced by copper. The short chain NPEOs such as NP_1EO and NP_2EO exhibited the opposite effect on apoptosis in PC12 cells as compared with NP. The relative high concentrations of NP_5EO and $NP_{10}EO$ did not affect apoptosis. It is indicated that the different length of EO chain has different effects on cytotoxicity and apoptosis. Ultimately, further investigation will be required to clarify the precise toxicity mechanism of short chain NPEOs and NP and long chain NPEOs. Final conclusion was shown as follows.

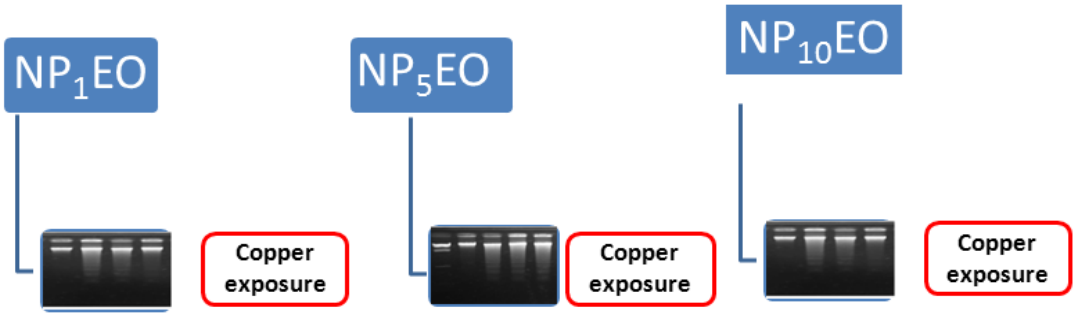


Fig. 5.2 The final conclusions of NP_1EO, NP_5EO and $NP_{10}EO$

Acknowledgements

I would like to express my heartiest gratitude and deepest thanks to my honorable supervisor Dr. Masaaki KURASAKI, for his kind co-operation, continuous guidance, valuable suggestions in all steps of my research and for giving me the opportunity to work autonomous and independent. His generosity, friendly behavior and sincerity are unforgettable to me. His positive attitude, constant encouragement and rapid reviewing of manuscript makes my research easier and completion it within due time.

I also express my sincere gratitude to Prof. Takeshi SAITO for his positive criticisms, kind suggestions and support to my research period.

I would like to thank Prof. Yoshinori KUBOKI for his valuable suggestion and inspiration.

I would like to thank all of my friends, lab mates and well-wishers for their kind help and co-operation in any difficult situation and also for passing enjoyable time in Sapporo.

I acknowledged CSC (China Scholarship Council) for providing fund and for granting my study leave to conduct my higher study in Hokkaido University, Japan.

I acknowledged SOSEIKAWA wastewater treatment plant for providing some materials (activated sludge) and technological support for my study

Most of all, I would like to give special thanks to my beloved wife, Zhang Lifei for her love, patience and continuous inspiration to complete my research.

Finally I thank my parents, parents-in-law, and other relatives for their never-ending interest, support, love and affections.