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고분자량 생분해성 폴리옥살레이트의 합성과 특성분석

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Synthesis and Characterization of High Molecular Weight Biodegradable Polyoxalate

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초록: 생분해성 고분자는 제약 및 생명공학 분야에서 많은 관심을 받고 있는 물질로 특히 나노미립구의 형태로 약물전달체의 개발에 널리 이용되고 있다. 본 연구에서는 cyclohexanedimethanol과 oxalyl chloride를 pyridine의 존재 하에서 반응하여 고분자량의 생분해성 퍼옥살레이트 고분자를 합성하고 그 물리화학적 및 생물학적 특성을 조사하였다. 폴리옥살레이트는 분자량이 약 75000 Da 정도인 반결정성 고분자이며 물에서 가수분해가 일어남을 GPC와 NMR로 확인하였다. 소수성의 폴리옥살레이트는 단일유화법으로 나노미립구로 제조될 수 있으며 약물을 포접할 수 있고 아주 우수한 세포안정성을 가졌다. 용이한 합성과 우수한 물리화학적 및 생물학적 특성을 바탕으로 폴리옥살레이트 나노미립구는 약물전달체 개발에 아주 높은 잠재력이 있음을 확인하였다.

Abstract: Biodegradable polymers have gained enormous attentions in the pharmaceutical and biomedical applications, especially in drug delivery. In this work, we report the synthesis and characteristics of high molecular weight polyoxalate with ~75000 Da. Hydrolytic degradation kinetics and degradation products were characterized by nuclear magnetic resonance and gel permeation chromatography. Polyoxalate is a semicrystalline and thermally stable polymer with a glass transition temperature of ~35 ℃, which is suitable for drug delivery applications. The hydrophobic nature of polyoxalate allows it to be formulated into nanoparticles and encapsulate drugs using a conventional oil-in-water emulsion/solvent displacement method. Polyoxalate nanoparticles also exhibited excellent cytotoxicity profiles. It can be suggested that polyoxalate has great potential for numerous biomedical and pharmaceutical applications.

Keywords: polyoxalate, biocompatibility, biodegradation, nanoparticles.

Introduction

There has been considerable interest in the development of biodegradable polymers for the achievement of clinical applications over the past two decades.[1] Degradation of polymer means that physical properties change from chemical reactions including the polymer backbone and side chains. Heat, photoelectron, microbial metabolism are well known as causes of physical property changes or chemical reactions. The polymer which has biodegradable properties has been utilized to deliver drugs effectively to a target site and improve the pharmacological and therapeutic activity of drugs while minimizing adverse side effects and reducing the dose.[2] Biodegradable polymers have been widely used for controlled drug delivery vehicles and devices owing to these advantages.[3]

The polymers used in drug delivery vehicles can be divided into two categories. One is a synthetic polymer group such as poly(lactide-*co*-glycolide) (PLGA), polycaprolactone (PCL) and polyanhydride.[4,5] The other is a natural polymer group such as keratin, chitosan, fibrin, silk, collagen and so on. Among them, PLGA is approved by the United State-Food and Drug Administration and has been most commonly used in pharmaceutical applications.[6,7] PLGA is also capable

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of controlling degradation kinetics by varying the ratios of lactide and glycolide component.[8] For these reasons, PLGA has been widely applied for drug delivery vehicles and porous scaffolds in tissue engineering.[4] However, its clinical applications are sometimes impeded by its acidic products, which can lead to an inflammation response. Additionally, the use of PLGA has a limitation for the fast drug delivery because of slow hydrolysis kinetics.[3,9]

Polyoxalate has been previously developed for biomedical applications such as drug delivery devices and sutures.[10,11] It was synthesized from a complicate two-step reaction of ester interchange reaction of diols with ester of oxalic acid, preferably diethyl oxalate in the presence of catalyst such as stannous octoate. Polyoxalate was known to degrade by water hydrolysis into small compounds that can be easily removed from a body. There have been, however, no implications on the nanoparticles formulation based on polyoxalate. In our laboratory, we developed polyoxalate (POX) from a simple reaction of oxalyl chloride and 1,4-cyclohexanemethanol in the presence of triethylamine. The polyoxalate showed a molecular weight of ~15000 Da and favorable physicochemical properties for the formulation of nanoparticles with a mean diameter of ~500 nm.[10] They also exhibited excellent biocompatibility and biodegradation profiles.

In order to broaden the pharmaceutical and biomedical applications of polyoxalate and overcome the limitations of PLGA, we have developed a high molecular weight polyoxalate. In this study, we synthesized polyoxalate using oxalyl chloride and cyclohexanedimethanol in the presence of pyridine. We could obtain biodegradable polyoxalate with a molecular weight, ~75000 Da using a weaker base pyridine. Herein, we report the physicochemical, thermal and biological properties of biodegradable high molecular weight polyoxalate.

Experimental

Materials. Oxalyl chloride, 1,4-cyclohexanedimethanol, dichloromethane (DCM), and poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich (St. Louis, MO. USA). Pyridine was obtained from Junsei Chemical (Japan). The reagents were used without further purification.

Synthesis of Polyoxalate. 1,4-Cyclohexanedimethanol (16.6 mmol) was diluted in 14 mL of dry dichloromethane (DCM) containing pyridine(43 mmol) under nitrogen atmosphere and cooled down to 4 ℃. Oxalyl chloride (16.6 mmol) in 4 mL of dry DCM was added to the mixture dropwise at 4 ℃. The reaction was kept under a nitrogen atmosphere at room temperature for 8 h. The reaction was terminated by the addition of a saturated brine solution and the products were extracted with additional DCM. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The obtained polymer was obtained after precipitation in cold hexane and solvent evaporation under high vacuum. The precipitation process was repeated for 3~4 times to remove pyridine completely. The obtained polymer was completely dried under vacuum and stored at 4 ℃ until the use.

Characterization of Polyoxalate. The chemical structure of polyoxalate was investigated with a 400 MHz ^{1}H NMR spectrometer (JNM-EX400 JEOL) using $CDCl_3$ as a solvent. The molecular weight of polyoxalate was determined using a gel permeation chromatography (GPC) with polystyrene standards. For degradation kinetic studies, polymer was incubated in pure water at 37 ℃ and the hydrolyzed polymers were collected and lyophilized at appropriate time points. GPC were employed to determine the molecular weight of hydrolyzed polyoxalate.

Thermal and Physical Analysis of Polyoxalate. Differential scanning calorimetry (DSC) was carried out on a DSC 3100 (TA instruments, USA) in the range of 0~250 ℃ at a scanning rate of 5 ℃/min under nitrogen as a purge gas. The second run of quenched samples was used for interpretation. Thermogravimetric analysis (TGA) was carried out using TA Q50 (TA Instrument, USA) from 20 to 500 ℃ at a scanning rate of 10 ℃ /min under the flow of dry nitrogen. Polyoxalate was dissolved in DCM and films were cast by solvent evaporation at room temperature. Polyoxalate films were subjected to the X-ray diffraction (XRD) study using a diffractometer (D/MAX-2200 V, Rigaku, Japan) with CuKα at the scanning rate was 4°/min.

Preparation of Polyoxalate Nanoparticles. Fifty milligrams of polymers was dissolved in 500 μL of DCM was then the solution was added into 5 mL of 10 (w/v)% PVA solution. The mixture was sonicated using a sonicator (Fisher Scientific, Sonic Dismembrator 500) for 30 sec and homogenized (PRO Scientific, PRO 200) for 1 min to form a fine oil/water emulsion. The emulsion was added into 20 mL PVA (1w/w%) solution and homogenized for 2 min. The remaining solvent was removed by rapid stirring for at least 3 h. Nanoparticles were obtained by centrifuging at 11000 g for 5 min at 4 ℃ and washing the recovered pellet with deionized water twice, followed by lyophilization. The SEM images of peroxalate nanoparticles were made using a scanning electron microscope (S-3000 N, Hitachi, Japan) under an accelerating voltage of 15.0 kV.

Cell Toxicity of Polyoxalate Nanoparticles. RAW 264.7 cells

(1.5×10^5 cells/well) were seeded and incubated at 37 ℃ with a humidified atmosphere containing 5% CO_2 for 1 day. Various amount of polyoxalate nanoparticles (10, 50, and 100 μg) were added to each well and incubated for 1 day. Each well was treated with 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubation for 4 h. Two hundred microlitters of dimethyl sulfoxide (DMSO) was added to cells to dissolve the resulting formazan crystals. After 10 min of incubation, the absorbance at 570 nm was measured using a microplate reader (Thermolex, Molecular Device Co.). The cell viability was obtained by comparing the absorbance of nanoparticles-treated cells to that of control cells.

Results and Discussion

Synthesis and Characterization of Polyoxalate. Polyoxalate was synthesized from a one-step reaction of oxalyl chloride and 1,4-cyclohexanedimethanol in the presence of pyridine, as shown Figure 1. 1,4-Cyclohexanedimethanol was used due to excellent toxicity profile with the LD_{50} of 3200 mg/kg for an oral dosage.[12] The reaction proceeded for 6 h in dry DCM under nitrogen atmosphere. Polyoxalate was obtained as pale solids after precipitation in cold hexane and drying under high vacuum, with ~70% yield. Polymerization of oxalyl chloride and 1,4-cyclohexanedimethanol was confirmed by the NMR (Figure 2). Cyclohexanedimethanol has methylene protons at 3.5 ppm (data not shown). The large peaks at 4.1~4.5 ppm are attributed to the methylene protons adjacent oxalate ester groups. Disappearance at 3.5 ppm and the appearance at 4.1~4.5 ppm demonstrate the condensation reaction to form peroxalate ester linkages. Multiplet peaks below 2.0 ppm are observed because 1,4-cyclohexanedimethanol used is a mixture of *cis* and *trans* isomers. Each peak assignment was not attempted because it did not provide further significant information. However, the chemical structure of polyoxalate was further confirmed by the ^{13}C NMR spectrum showing oxalate carbons at 157 ppm and methylene carbons next to oxalate esters at ~70 ppm. The NMR spectra clearly support the successful synthesis of polyoxalate.

Polyoxalate obtained from this reaction was determined to have a molecular weight of ~75000 Da, corresponding to a degree of polymerization of ~350 repeating units (Figure 3), with a polydispersity index of ~1.8 and therefore has potential for formulation into nanoparticles. Previously, we synthesized polyoxalate using triethylamine as a base, which had a molecular weight ~15000 Da. The significant increase in the molecular weight of polyoxalate synthesized using pyridine may be due to less degradation during the polymerization reaction. Peroxalate ester groups are known to undergo degradation which is accelerated under a basic condition.[13,14] We speculate that the use of pyridine, a weak base resulted in the less degradation and therefore significant increase in the molecular weight.

Polyoxalate is known to degrade via hydrolysis under physiological conditions.[10,15] The degradation kinetics of polyoxalate was investigated by measuring its molecular weight using GPC. Figure 4 shows that polyoxalate degrades hydrolytically, having a half-life of ~5 days. Peroxalate ester linkages in the backbone readily become cleaved although

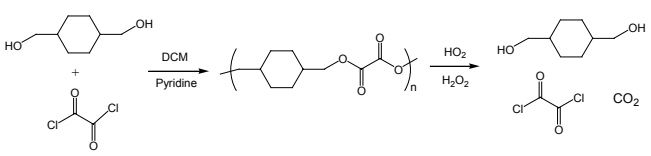

Figure 1. Synthesis of polyoxalate and its degradation in the presence of water and hydrogen peroxide.

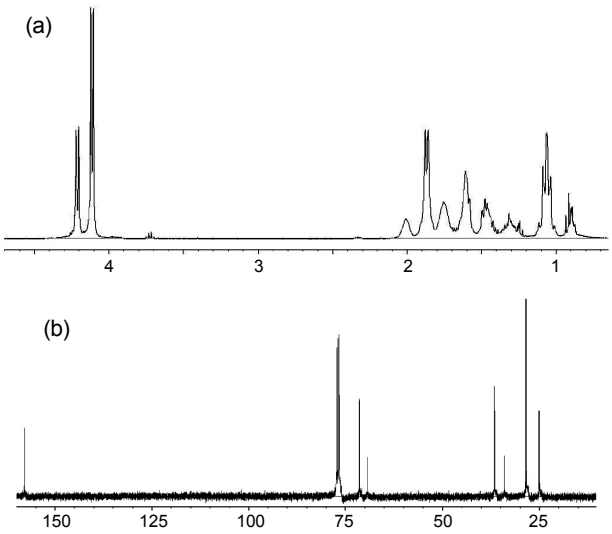

Figure 2. NMR spectra of polyoxalate synthesized from the reaction of 1,4-cyclohexanedimethanol and oxalyl chloride: (a) ^1H NMR spectrum; (b) ^{13}C NMR in $CDCl_3$.

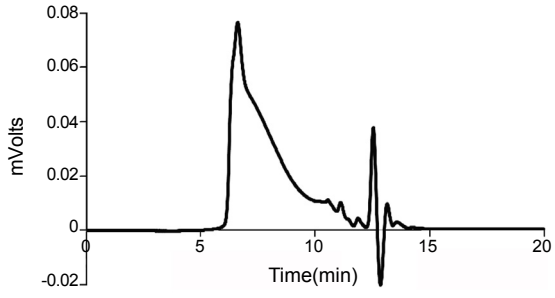

Figure 3. GPC trace of polyoxalate in THF.

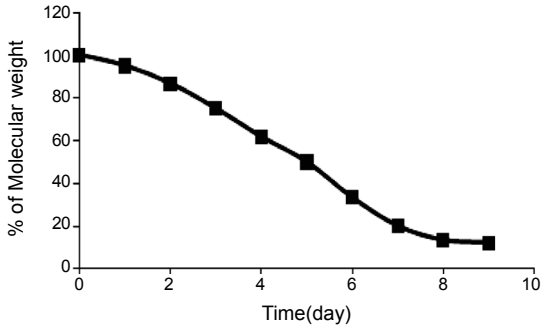

Figure 4. Hydrolysis kinetics of polyoxalate under physiological conditions.

polyoxalate has a hydrophobic cycloalphatic chain in its backbone. PLGA is known to have a half-life of several weeks, depending on the molecular weight and ratios of lactide and glycolide and therefore polyoxalate seems to degrade faster than PLGA.[16,17] It can be expected that polyoxalate nanoparticles will have fast drug release kinetics, suitable for the treatment of acute inflammatory diseases such as acute liver failure and acute lung injury.

Thermal and Physical Properties of Polyoxalate. The thermal stability of polyoxalate was studied using TGA. The thermogram is illustrated in Figure 5(a). A slight weight loss was observed at ~120 ℃. The onset of major weight loss took place at polymer decompose at ~300 ℃. The DSC thermogram of quenched polyoxalate is represented in Figure 5(b). The glass transition temperature (T_g) is well defined at ~35 ℃. The low T_g may be attributed to the linear and flexible aliphatic chain of the backbone. It was also found that polyoxalate is semicrystalline, with crystallization temperature and melting temperature at 90 and 160 ℃, respectively. Low molecular weight polyoxalate was also subjected to DSC study. However, no difference was observed in thermal behaviors between high molecular and low molecular polyoxalate (data not shown). It can be anticipated that polyoxalate is useful for drug delivery applications because T_g is close to a body temperature. Hydrophobic and semicrystalline polymers have been widely used for drug delivery applications in the formation of nano/microparticles and 3-dimensional tissue engineering scaffolds. Semicrystalline polymers with T_g around 37 ℃ are soft and flexible in a body. In addition, their hydrolysis is accelerated in a body because water easily diffuses into the amorphous regions of polymers.

X-ray diffraction of polyoxalate films is shown in Figure 6. As evidenced in DSC showing a crystallization of polyoxalate, a large distinct diffraction peak is observed at ~20°, indicating a spacing of 4.5 Å, which is characteristic of and average distance between two neighboring chain molecules. It seems that polyoxalate has less crystallinity than PLGA because

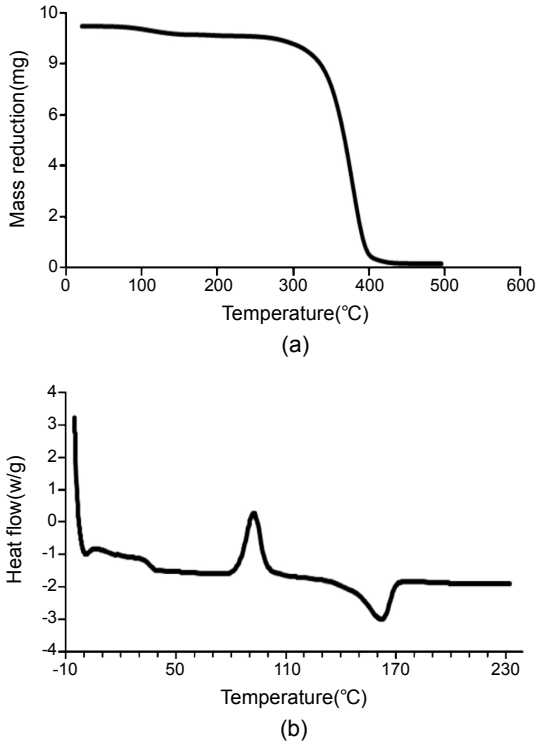

Figure 5. Thermal analysis of polyoxalate: (a) TGA thermogram; (b) DSC thermogram of polyoxalate.

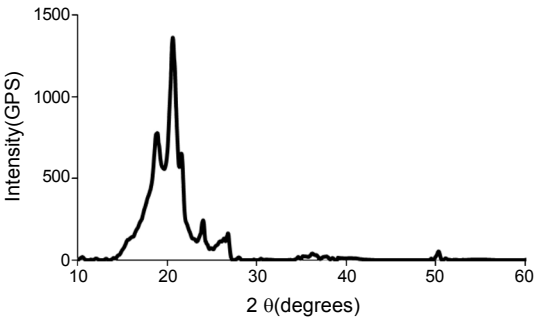

Figure 6. X-ray diffraction of polyoxalate films.

bulky aliphatic cyclic rings in its backbone reduce the molecular mobility and packing capability.

Properties of Polyoxalate Nanoparticles. Polyoxalate is hydrophobic and semicrystalline polymer and suitable for the nanoparticle formulation. The polyoxalate nanoparticles were prepared using a single emulsion/solvent displacement method and observed using SEM. They were round spheres with smooth surface and had a mean diameter of ~550 nm (Figure 7). Their particle size was similar to that of polyoxalate with a molecular weight of 15000 Da. The surface morphology of polyoxalate nanoparticles was also observed as a function of degradation time. As the nanoparticle degradation progressed, the nanoparticles deformed from the spherical or ovoid shapes into irregular particles with

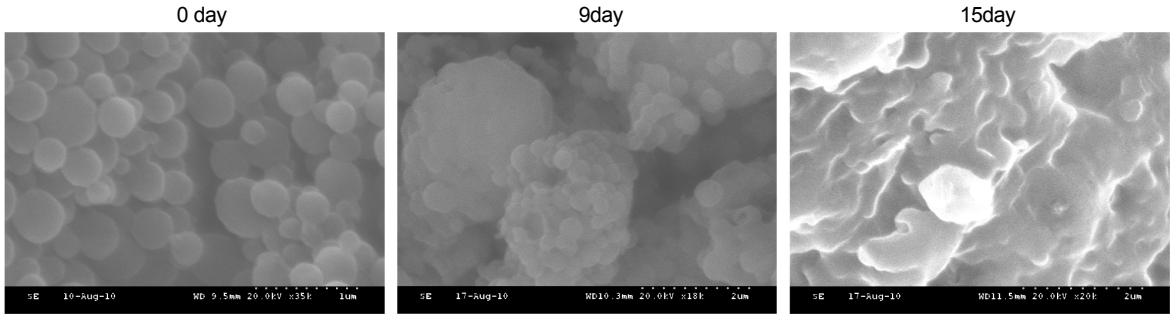

Figure 7. Representative SEM images of polyoxalate nanoparticles.

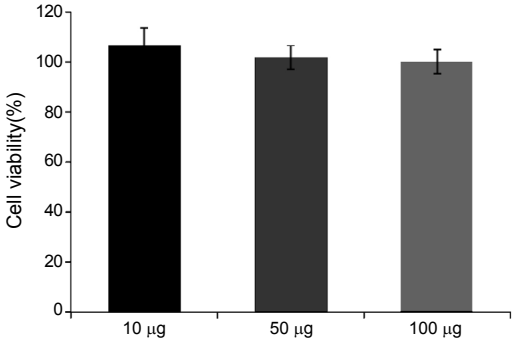

Figure 8. Cytotoxicity of polyoxalate nanoparticles based on the MTT assay.

aggregation. By the ninth day, the nanoparticle collapsed and showed extensive changes in morphology. Upon the further degradation, the nanoparticles continued to lose mass and progressively decreased in size. At the fifteen day, nanoparticles have completely degraded.

An MTT assay was performed to evaluate the cytotoxicity of polyoxalate nanoparticles using RAW264.7 cells. Cells were incubated with a various amount of polyoxalate nanoparticles for 24 days. Figure 8 represents the cell viability after the incubation with polyoxalate nanoparticles. No change in the cell viability was observed with the studied concentration, demonstrating that polyoxalate exhibits an excellent cytotoxicity profile and prominent biocompatibility. The nanoparticle forming ability and excellent cytotoxicity profile make polyoxalate as a potential drug carrier for various acute inflammatory diseases.

Conclusions

A polymerization reaction of oxalyl chloride and 1,4-cyclohexanedimethanol was conducted in the presence of pyridine to generate a high molecular weight biodegradable polymer. The obtained polymer had a molecular weight of 75000 Da, which might be due to the less degradation during the polymerization under weak basic conditions. Thermal analysis revealed that polyoxalate was thermally stable and semi-crystalline with T_g of 35 ℃. The polyoxalate hydrolytically degraded into two small compounds with a half-life of 5 days. Polyoxalate was hydrophobic and could be formulated into nanoparticles with smooth surface and ~500 nm diameter. MTT assay showed that polyoxalate nanoparticles exhibited excellent cytotoxicity. Given their appealing features such as the ease of synthesis and excellent biocompatibility, polyoxalate nanoparticles may have great potential for pharmaceutical and biomedical applications.

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