Original Article

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Preparation and *in vitro* tumor growth inhibitory effect of oligo (L-lactate) nanoparticles

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SUMMARY Oligo L-lactates (oligolactates) that have low molecular weights less than 2000 have been reported to inhibit tumor growth and extend the survival of experimental animals. Because oligolactates are scarcely soluble in water, they require a solvent or a solubilizing agent, such as a surfactant, to be dissolved in water. However, these agents are generally cytotoxic, an *in vitro* assay appropriate to evaluate the inhibitory effect on tumor growth has not been developed yet. Here, we prepared a solid nanodispersion of oligolactates using an oil-in-water emulsion solvent evaporation method to evaluate its tumor inhibitory activity *in vitro* without a solvent or surfactant. Polyol solutions containing polyvinyl alcohol (PVA) were used as a continuous phase. The formation of nanoparticles depended on the concentrations of polyol and PVA in the continuous phase. The nanoparticles with a particle size of approximately 100 nm were obtained using 10-15% PVA and 60% propylene glycol. The obtained aqueous nanodispersion of oligolactates inhibited the growth of B16-BL6 melanoma cells *in vitro*, whereas the medium alone did not affect tumor cell growth. Therefore, oligo(L-lactate) nanoparticles may be useful in the research and development of oligolactates as a remedy for cancer.

Keywords oligo(L-lactate), solid nanodispersion, polyol, solvent evaporation method, tumor growth inhibition, melanoma

1. Introduction

Polylactides are linear lactic acid polymers with high molecular weights which are inert; thus, they are used as green plastics or inactive biodegradable ingredients. A collegium on L-lactic acid oligomers (oligolactates), which was established in 1996 and sponsored by Tokai Education Instruments Co., Ltd. (Kanagawa, Japan) and Amato Pharmaceutical Products, Ltd. (Osaka, Japan), suggested that cyclic and linear oligolactates of molecular weights below 2000 exhibit various physiological activities such as antihyperglycemic and anti-allergic activities, suppression of anaerobic glycolysis, and mitochondrial proliferation (not published). The substances were originally discovered in a conditioned medium harvested from cultured HeLa cells (not published). Some fractions of the conditioned medium showed growth inhibition or tumoricidal effects against HeLa cells and other tumor cells. Subsequent investigations revealed that oligolactates from the conditioned medium showed a tumor static effect. Moreover, the collegium developed a method for synthesizing oligolactates with a degree of polymerization from 3-13 (*1*) and confirmed that these oligolactates show *in vivo* tumor static effects in mice. The oligolactates inhibit the anaerobic activity of tumor cells. Thus, they effectively inhibit the growth of FM3A ascites tumors (*2*). Other studies have also reported that an extract containing cyclic poly-L-lactates inhibited the cell growth of leukemic (*3,4*) and carcinoma cells (*5*). However, oligolactates are water-insoluble substances and require a water-miscible organic solvent such as dimethyl sulfoxide or a solubilizing agent such as a surfactant to be dissolved in water; unfortunately, these agents are generally cytotoxic or affect cell proliferation. Consequently, an *in vitro* assay to evaluate the inhibitory effect of oligolactates on tumor growth has not been developed yet.

Macromolecular compounds and microparticles are generally membrane-impermeable, and their cellular uptake can occur *via* endocytosis, *i.e.*, pinocytosis and phagocytosis. For the uptake of particles in non-phagocytes, the necessity for keeping particles between 10 and 100 nm to enter endocytic vesicles was postulated and became the foundation for the current definition of nanomedicine by various agencies worldwide (*6*). Thus, it can be a strategy for formulating nanoparticles by increasing the cellular uptake of water-

insoluble macromolecules.

Several techniques for reducing particle size have been proposed, including micro/nanomization (*7-10*), solubilization (*11,12*), and self-emulsification (*13-16*). Among the size reduction methods, the solvent diffusion method is often used for preparing polymeric nanoparticles (*17-21*). A dispersed phase of an organic polymer solution is injected into a continuous phase of a miscible solvent. Being miscible, the interfacial tension between dispersed and continuous phase is reduced, and the deposition of the polymer occurs as nano-sized particles. In general, acetone and water are used as the dispersed and continuous phase, respectively. However, because commercially available oligolactates are insoluble in acetone, it is difficult to apply the solvent diffusion method to fabricate oligo(L-lactate) nanoparticles. In addition, this method typically generates nanoparticles of around 200 nm, and it is difficult to obtain nanoparticles around/below 100 nm. Thus, the particles are in a size range that promotes cellular uptake.

Because oligolactates are soluble in methylene chloride, the emulsion-based solvent evaporation method is suitable to fabricate oligolactates particles. For further size reduction using this method, the interfacial tension must be reduced between the dispersed phase containing the polymer and the continuous phases. Additionally, the hydrophobicity between the two phases must be closer. The addition of polyols such as propylene glycol and butylene glycol to water as a continuous phase can increase the hydrophobicity of the solution. Moreover, polyol aqueous solution can dissolve PVA at high concentrations. PVA, a synthetic water-soluble polymer, is very less toxic and commonly used for producing polymer emulsions in the preparation of nano/microparticles, which is advantageous to reduce the interfacial tension between dispersed and continuous phases. Hence, in this study, to obtain nanoparticles of oligolactates around 100 nm or smaller, we used the oil-in-water (o/w) type emulsion solvent-evaporation method with a polyol aqueous solution as the continuous phase and a polymeric organic solvent as the dispersed phase.

The objectives of this study were to develop an alternative fabrication method for oligo(L-lactate) nanoparticles, which are difficult to fabricate using conventional methods for polymeric nanoparticles, such as a solvent diffusion method, and confirm the tumor toxicity of oligolactates *in vitro*. We investigated the feasibility of implementing an o/w type emulsion solvent evaporation method using a polyol aqueous solution to obtain oligo(L-lactate) nanoparticles of various sizes. The aqueous nanodispersion can apply to *in vitro* cell culture without an organic solvent or surfactant. Our study will lead to a reasonable evaluation of the activity of oligolactates *in vitro* and help in developing oligolactates and their derivatives as a new type of antitumor agent in the future.

2. Materials and Methods

2.1. Materials

CPL, a commercial oligolactate mixture synthesized using a decompression and dehydration condensation method, was kindly gifted by Shumeido Co., Ltd. (Yamaguchi, Japan). Polyvinyl alcohol (PVA; JP03) was obtained from Japan VAM & POVAL Co., Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (D-MEM) were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Dulbecco's phosphate-buffered saline (D-PBS), non-essential amino acids, penicillin, streptomycin, and Cell Count Reagent SF were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). All other chemicals used were of reagent grade. There are no ethical aspects to declare in this study.

2.2. Cell lines and culture

Murine B16-BL6 melanoma cells were kindly provided by Dr. Y. Hayakawa (University of Toyama) and routinely incubated and maintained in complete medium (DMEM containing 10% FBS, non-essential amino acids, 100 U/mL penicillin, and 100 U/mL streptomycin, pH 7.4) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

2.3. Synthesis of low molecular weight oligolactates

Oligolactates were synthesized by dehydration polycondensation to obtain oligolactates with a lower molecular weight than CPL. L-Lactic acid (500 mL) was placed into a three-necked flask fitted with a distillation head and condenser, a thermometer for monitoring the temperature of the solution, and an inlet for inert nitrogen gas (300 mL/min). The vessel was heated at 145°C for 3 h using a mantle heater. The pressure and temperature were then changed stepwise to 150 mmHg at 145°C for 3 h, 3 mmHg at 155°C for 3 h, and 3 mmHg at 185°C for 1.5 h. The obtained mixture was mixed well with ethanol (100 mL) and methanol (400 mL) at 100°C, and then cooled to room temperature overnight. The obtained suspension was filtered to remove participants, dried under reduced pressure, and filtered to remove participants once again before the dry substance was dissolved in acetonitrile (200 mL). The solution was purified using reverse phase Octa decyl silyl (ODS) column (Biotage® sfär C18 Duo 100Å 30 µm, Biotage AB, Uppsala, Sweden) through stepwise elution of 0.01 M hydrochloride -30% acetonitrile, 0.01 M hydrochloride -50% acetonitrile, and 0.01 M hydrochloride -100% acetonitrile. The elute collected with 100% acetonitrile was dried under reduced pressure to obtain oligolactates. Hereinafter,

the obtained oligolactates are referred to as "synthesized oligolactates".

To prepare the oligo(L-lactate) nanoparticles used in the *in vitro* tumor growth inhibition study, we used oligolactates washed with water to remove water-soluble components. Namely, 1 g of oligolactates was dissolved in 2 mL of methylene chloride, washed with 10 mL water by shaking for 30 min, and dried under reduced pressure.

2.4. Preparation of commercial or synthesized oligo(L-lactate) nanoparticles

CPL- or synthesized oligo(L-lactate) nanoparticles were prepared using the o/w type emulsion solvent evaporation method. CPL or the synthesized oligolactates were dissolved in methylene chloride to obtain the oil phase. The oil phase was emulsified in 5 mL of PVA solution containing propylene glycol at 20,000 rpm using a homogenizer (ULTRA-TURRAX T18, IKA®-Werke GmbH & Co., KG, Staufen, Germany) for 5 min at 20-23°C. The resulting o/w type emulsion was added to 100 mL of water, and the diluted emulsion was stirred at 20-23°C for 180 min to remove the solvent. The aggregates generated during the manufacturing process were removed using a 20-μm stainless mesh or 0.45-μm membrane filter if necessary. For the *in vitro* tumor growth inhibition study, the obtained nanoparticle suspension was washed and replaced with a complete medium by ultrafiltration using a 100 kDa filter at 4°C.

2.5. Characterization of nanoparticles

The size and zeta potential of the nanoparticles were measured using dynamic light scattering (DLS) with a zeta (ζ) potential analyzer (ELSZ-2, Otsuka Electronics Co. Ltd., Osaka, Japan). The cumulant size obtained by DLS was used as the hydrodynamic diameter.

2.6. Characteristics of CPL and synthesized oligolactates

CPL and the synthesized oligolactates were dissolved in tetrahydrofuran, mixed with 3-indoleacrylic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and characterized by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). The mass spectrometry was performed using an AXIMA® Confidence™ running LAUNCHPAD 2.8.4 (Shimadzu Co., Ltd., Kyoto, Japan). The wavelength of the laser was 337 nm.

2.7. Transmission electron microscope imaging

The nanoparticles were absorbed onto formvar film-coated copper grids and negatively stained by treating them with 2% phosphotungstic acid solution (pH 7.0) for a few seconds. The samples were observed using a transmission electron microscope (TEM; JEM-1400Plus, JOEL Ltd., Tokyo, Japan) at 100 kV. Digital images were captured with a CCD camera (EM-14830RUBY2, JOEL Ltd., Tokyo, Japan).

2.8. *In vitro* tumor growth inhibition study

The influence of CPL and the synthesized oligo(L-lactate) nanoparticles on *in vitro* B16-BL6 cell growth was evaluated using the water-soluble tetrazolium (WST) method. In brief, 1×10^4 cells/mL of the exponentially growing B16-BL6 cells (100 μL) in a complete medium was plated in a 96-well plate. After a 24-h incubation at 37°C in a humidified atmosphere containing 5% carbon dioxide to allow cell attachment, the cells were treated with varying concentrations of the synthesized oligo(L-lactate) nanoparticles dispersed in the complete medium and incubated for 48 h under the same conditions. After 4 h of incubation under the same conditions, followed by the addition of 10 μL of Cell Count Reagent SF, the supernatant was measured spectrophotometrically at 450 nm using a hybrid multimode microplate reader (Synergy H4; BioTek Instruments, Winooski, VT, USA).

2.9. Statistical analysis

Experiments were performed at least three times. The results are presented as the mean ± standard deviation. The differences between the means for the two groups were statistically analyzed using Williams' test for multiple comparisons. P-values < 0.05 indicated significant differences.

3. Results

3.1. Characteristics of CPL and synthesized oligolactates based on MALDI-TOF-MS analysis

Figure 1 shows the mass spectra of CPL and the synthesized oligolactates, and Table 1 lists the detected molecular weights. The measurement in positive mode provided molecular ion peaks corresponding to protonated, sodium, and ammoniated oligolactates ions. For CPL, the peaks corresponding to polymerization degree 4-31 indicating linear structure and 5-12 indicating cyclic structure were detected. For the synthesized oligolactates, peaks corresponding to polymerization degrees 2-12 (linear) and 2-8 (cyclic) were detected.

The complicated stepwise change in pressure and temperature in the dehydration polycondensation method for synthesizing oligolactates was aimed at increasing the yield of cyclic lactides. However, some of the major molecular weight peaks were identified as linear oligolactates through MALDI-TOF-MS. This indicates that a large quantity of linear oligolactates

is among the oligolactates synthesized in this study as well as in CPL.

3.2. Influence of fabrication conditions on the characteristics of CPL nanoparticles

Table 2 summarizes the particle sizes of CPL nanoparticles in various continuous phases during the emulsification process. The increase in PVA concentration resulted in smaller CPL nanoparticles, and the particle sizes were below 200 nm in continuous phases containing more than 5% PVA concentration in the case of 60% propylene glycol (Figure 2a). With the addition of polyol, an increase in propylene glycol concentration up to 60% resulted in a smaller particle size (Figure 2b); however, 80% propylene glycol-10% PVA solution could not be used as a continuous phase because of its gelation at room temperature. The reduction in particle size by the addition of polyols was in the order of 1,3-butylene glycol = propylene glycol > glycerin, and butylated glycol and propylene glycol provided particle sizes below 150 nm in the case of 10% PVA. When glycerin was used as a polyol, the aggregates

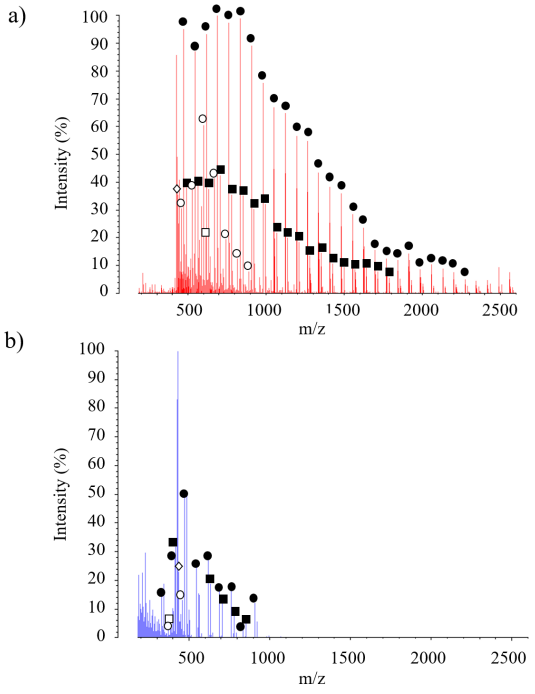

Figure 1. MALDI-TOF-MS spectra. a) CPL and **b)** synthesized oligolactates. The molecular weight of peaks corresponds to ●: [linear oligolactates-Na]$^+$, ■: [linear oligolactates-K]$^+$, ○: [cyclic oligolactates-Na]$^+$, □: [cyclic oligolactates-K]$^+$, ◊: [cyclic oligolactates-H]$^+$.

Table 1. List of detected cyclic oligolactates in CPL and synthesized oligolactates

Molecular weight	Degree of polymerization	Adduction	Area percentage of cyclic oligolactates*	
			CPL	Synthesized oligolactates
144	2	-NH_4^+	-	1.84
216	3	-H^+	-	2.48
		-NH_4^+	-	0.56
		-Na^+	-	0.95
		-K^+	-	3.00
288	4	-H^+	-	0.19
		-NH_4^+	-	0.24
		-Na^+	-	1.04
		-K^+	-	1.64
360	5	-NH_4^+	0.45	0.07
		-Na^+	-	1.09
		-K^+	-	0.06
432	6	-H^+	1.77	5.23
		-Na^+	1.52	2.93
		-K^+	1.42	1.16
504	7	-H^+	0.18	0.07
		-Na^+	1.85	-
		-K^+	0.70	-
576	8	-H^+	-	0.19
		-Na^+	3.02	-
		-K^+	1.01	-
648	9	-Na^+	2.05	-
		-K^+	0.59	-
720	10	-Na^+	0.97	-
792	11	-Na^+	0.62	-
864	12	-Na^+	0.40	-
Total (%)			16.57	22.75

*[cyclic oligolactates]/[linear and cyclic oligolactates] × 100. CPL: brand name of commercial oligolactates.

Table 2. Composition of continuous phase and hydrodynamic diameter of the fabricated nanoparticles

Number	Composition of continuous phase	Mean size	Polydispersity index
#1	10% PVA	435.0 ± 48.5 nm	0.202 ± 0.030
#2	10% PVA-60% glycerol	1609 ± 220 nm	0.648 ± 0.088
#3	10% PVA-60% propylene glycol	117.1 ± 4.3 nm	0.098 ± 0.018
#4	10% PVA-60% 1,3 butylene glycol	137.1 ± 7.5 nm	0.075 ± 0.013
#5	15% PVA-60% propylene glycol	99.1 ± 3.3 nm	0.131 ± 0.027
#7	5% PVA-60% propylene glycol	162.7 ± 1.9 nm	0.062 ± 0.013
#8	2.5% PVA-60% propylene glycol	200.8 ± 6.5 nm	0.066 ± 0.017
#9	0.5% PVA-60% propylene glycol	278.7 ± 9.5 nm	0.136 ± 0.031
#10	10% PVA-80% propylene glycol	-	-
#11	10% PVA-50% propylene glycol	123.3 ± 4.6 nm	0.114 ± 0.012
#12	10% PVA-40% propylene glycol	165.5 ± 12.9 nm	0.111 ± 0.041
#13	10% PVA-20% propylene glycol	245.2 ± 3.5 nm	0.107 ± 0.018

Data are represented as mean ± S.D. (n = 3 batches). PVA, polyvinyl alcohol; S.D., standard deviation.

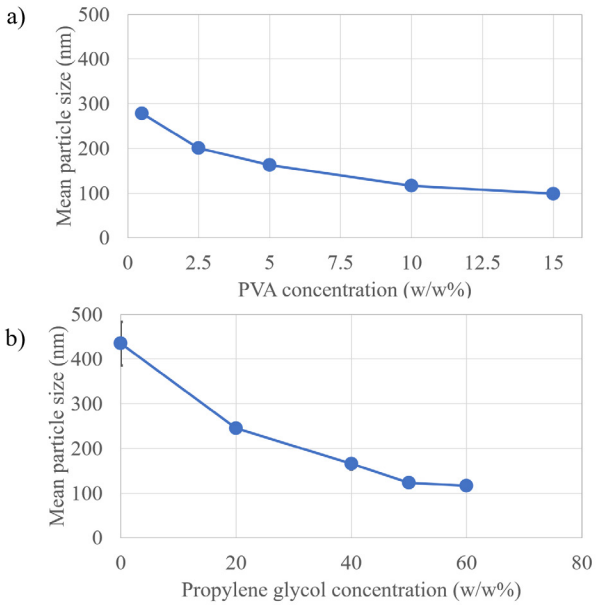

Figure 2. Effect of the continuous phase composition on the hydrodynamic diameter of nanoparticles. a) Effect of PVA concentration in 60% propylene glycol solution, **b)** Effect of propylene glycol concentration in 10% PVA solution. Data are represented as mean ± S.D. (n = 3 batches)

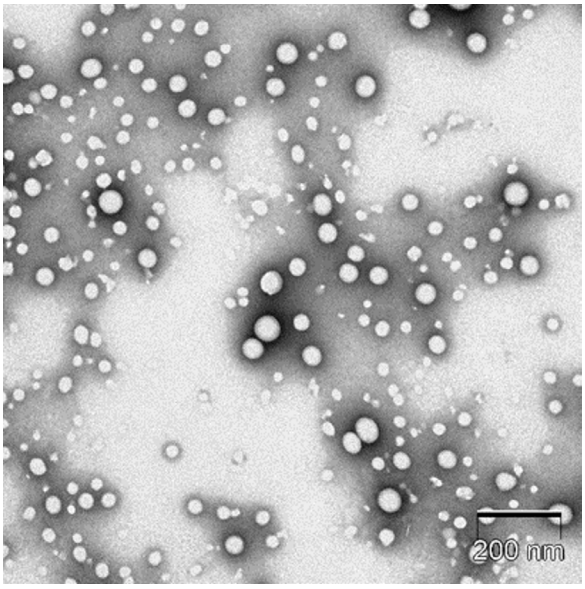

Figure 3. Transmission electron micrographs of the typical CPL nanoparticles. The nanoparticles were fabricated using 15% PVA-60% propylene glycol as a continuous phase.

of 55.2 ± 14.1% over a size of 20 μm were generated. Figure 3 shows the transmission electron micrographs of the typical CPL nanoparticles (hydrodynamic diameter: 117.5 nm). Transmission electron microscopy images confirmed spherical nanoparticles in the size range of 1.7 nm to 66.4 nm (number mean size 25.6 nm).

3.3. Evaluation of tumor growth inhibition

The oligo(L-lactate) nanoparticles were evaluated for their growth inhibition by the WST assay at various concentrations. The nanoparticles used in this study were well-dispersed at the size of 126.3 nm (polydispersity index: 0.129) and ζ-potential of -4.64 mV for CPL, and 149.4 nm (polydispersity index: 0.181) and ζ-potential of -16.78 mV for the synthesized oligolactates in a D-MEM culture medium without aggregates (Figures 4a and 4b). The results of the inhibitory effect on the *in vitro* growth of B16-BL6 melanoma cells are shown in Figure 4c. The synthesized oligo(L-lactate) nanoparticles significantly induced growth inhibition at a concentration of 5 mg/mL with an IC_{50} of 9.7 mg/mL. On the other hand, CPL nanoparticles showed no growth inhibition at concentrations up to 10 mg/mL.

4. Discussion

To fabricate oligo(L-lactate) nanoparticles, we employed an o/w emulsion solvent evaporation method. We used the detergent (D) phase emulsification technique using a polyol-surfactants solution as continuous phase (*22*) to produce fine emulsions, which involves the formation of an oil-in-surfactant gel emulsion by dispersing oil in the surfactant solution. Consequently, we obtained nanoparticles of around 100 nm in hydrodynamic diameter from DLS analysis.

For the D phase emulsification method, a ternary system composed of water, polyol, and surfactant was used as the continuous phase. The role of polyol in this method is as follows: 1) the enhancement of the interaction between water and surfactant and the effective attachment of surfactant on oil droplets, and 2) the suppression of the liquid crystalline formation of a surfactant such as a hexagonal liquid crystal and increase in the region of the detergent phase (*22*). In the case of the ternary system of water, propylene glycol, and PVA used in this study, the effective attachment of PVA on oil droplets is indicated by the result that the influence of the PVA concentration on the size of the obtained particles was less sensitive than that of the propylene glycol concentration. On the other hand, the mechanism of the suppression of liquid crystalline formation in the D phase emulsification method may not be applied to the ternary system of water, propylene glycol, and PVA. However, the association between PVA is suppressed by propylene glycol. PVA has intermolecular hydrogen bonding, which leads to reduced efficiency as an emulsifier, especially at high concentrations. Polyols are used as plasticizers in PVA (*23*). It is known that hydrogen bonds between plasticizer and PVA chains are formed. It has been reported that the intermolecular interactions in the PVA solutions were notably reduced by propylene glycol because of its hydrogen bond breaking effects (*24*). This indicates that PVA in propylene glycol can effectively act as a monomeric emulsifier. This can account for the fact that nanoparticles were obtained by the addition of propylene glycol concentrations.

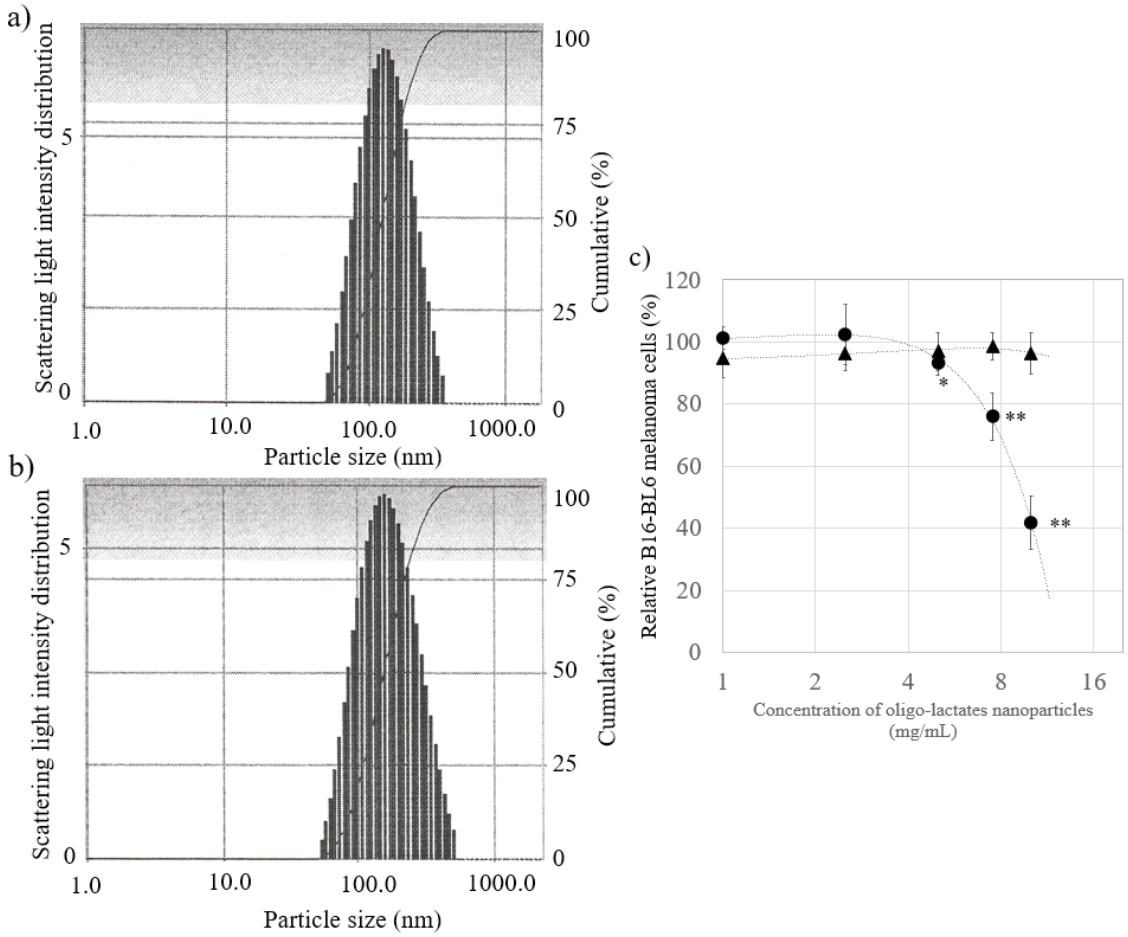

Figure 4. ***In vitro*** **growth inhibition of B16-BL6 melanoma cells by CPL/synthesized oligo(L-lactate) nanoparticles.** The graphs show the size distribution of **a)** CPL-nanoparticles and **b)** synthesized oligo(L-lactate) nanoparticles applied to *in vitro* growth inhibitory study. **c)** The graph shows *in vitro* dose inhibitory curve of CPL/synthesized oligo(L-lactate) nanoparticles to B16-BL6 melanoma cells. B16-BL6 melanoma cells were grown in 96-well microplates in a complete medium (10% FBS D-MEM, pH 7.4). Nanoparticles of various concentrations were added to each well and the cells were incubated for 48 h. Data are represented as mean ± S.D. (n = 7) of the relative B16-BL6 melanoma cells, percentages of treated group (▲: CPL nanoparticles, ●: synthesized oligo(L-lactate) nanoparticles) to non-treated group. * and ** represent significant differences between non- and nanoparticle-treated group at $p < 0.05$ and $p < 0.025$, respectively.

It has been reported that the hydrogen bond breaking effects of propylene glycol on PVA became dominant between 20 and 30 wt% in a PVA/propylene glycol film (*23*). Moreover, propylene glycol bonded to PVA may increase its affinity to the surface of oil droplets due to its hydrophobicity. This hypothesis explains why glycerol did not provide nanoparticles. The effective attachment of PVA on the surface of the oil phase by the inhibition of intermolecular interactions and the hydrophobicity of propylene glycol and butylene glycol contributed to the production of nanoparticles.

The data from the WST assay demonstrated that the synthesized oligolactates could inhibit the growth of B16-BL6 cells when administered as the nanoparticles. On the other hand, CPL did not show growth inhibition up to 10 mg/mL. CPL and synthesized oligolactates can be degraded to produce lactic acid by hydrolysis and may show cytotoxic effect by the acidification of a medium; however, the nanoparticle-dispersed D-MEM medium was confirmed to remain at a low pH of 6.9 (initial pH 7.4) after incubation at 37°C for 2 days. Hence, the growth inhibition is not due to the acidification of the medium. The synthesized oligolactates contain lower molecular weight components than CPL, based on MALDI-TOF-MS analysis, although cyclic oligolactates were also detected in both CPL and the synthesized oligolactates, indicating that the observed growth inhibition can attribute to the lower molecular weight components. It is difficult to identify the ratio of the lower molecular weight cyclic structure in the synthesized oligolactates and CPL from MALDI-TOF-MS analysis because the detection sensitivity of MALDI-TOF-MS may be different between linear and cyclic oligolactates and between molecular weights. However, it could be supposed that the synthesized oligolactates have higher contents of growth inhibition components than CPL. The growth inhibition activity of the synthesized oligo(L-lactate) nanoparticles was weak in comparison with that of doxorubicin, which showed approximately 9.5% at a concentration of 10 μg/mL in the relative percentage of cells in treated versus non-treated groups. Considering this weak activity of oligolactates, the active fraction and,

furthermore the active substance, should be separated from the mixtures to improve the tumor inhibitory effect.

It seems difficult for oligolactate molecule to pass through cell membrane by diffusion as a solute. The growth inhibition activity of the nanodispersion may be related to the cellular uptake of the nanoparticles by endocytosis, which is mainly determined by particle size and surface properties. For the size, it is reported that relative to internalized 50 nm beads, the uptake of the 100 nm beads was diminished by approximately 3-4-fold, whereas the internalization of the 200 and 500 nm beads was reduced by approximately 8-10 times in non-phagocytic B16 cells (*25*). In the present study, we obtained nanoparticles of around 150 nm in hydrodynamic diameter, although the median volume and number size from DLS were calculated to be 68.6 nm and 52.8 nm for the synthesized oligo(L-lactate) nanoparticles, respectively (data not shown). This suggests that further downsizing in hydrodynamic diameter may be effective to enhance the tumor growth inhibitory effect of oligo(L-lactate) nanoparticles. For the surface properties, endocytosis is also influenced by the particle's charge besides the size. In this study, we used negatively charged nanoparticles without surface modification. In general, positively charged particles show better interaction with the cellular membrane and internalization properties than negatively charged particles because the cellular membrane possesses negative charge. Therefore, we should investigate the fabrication of oligo(L-lactate) nanoparticles with surface charge modification further to improve their affinity to cells.

There are two more limitations on this study. One is that the long-term *in vitro* effects of oligolactates on tumor cell growth remains to be investigated. CPL was found to induce morphological changes of tumor cells during long-term administration *in vivo* (*2*). Another is that energy metabolism in tumor cells was not evaluated in this study. Tumor growth inhibition is related to energy metabolism and the suppression of anaerobic glycolysis by oligolactates and thereby mitochondrial proliferation are indicated. Thus, further investigation is needed to clarify the relationship between tumor growth inhibition and energy metabolism changes over time during incubation with oligolactates *in vitro*.

In conclusion, we developed a new method to produce oligo(L-lactate) nanoparticles of around 100 nm hydrodynamic diameter through o/w emulsion solvent evaporation using PVA-polyol aqueous solution as a continuous phase. Moreover, it was demonstrated that tumor growth is inhibited by the synthesized oligo(L-lactate) nanoparticles in an *in vitro* study using B16-BL6 melanoma cells. Thus, it was suggested that the nanoparticle is useful for an *in vitro* assay of effects of oligolactates and in development of various dosage forms for their clinical use, which may also help in developing oligolactates and their derivatives as a new type of antitumor agent in the future.

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