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잠재적 종양 치료를 위한 전분-라우르산 나노 입자의 제조

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Fabrication of Starch-Lauric Acid Nanoparticles for Potential Tumor Therapy

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초록: 라우르산의 양을 변화시킴으로써 잠재적인 암 요법을 갖는 전분-라우르산(starch-lauric acid; St-LA) 나노 입자를 제조하고 최적화된 St-LA 나노 입자를 발견하였다. 또한 주사 전자 현미경(SEM) 및 푸리에 변환 적외선 분광법(FTIR) 분석을 통해 St-LA 나노 입자가 성공적으로 제조되었음을 확인하였다. 세포 실험에서, St-LA 나노 입자는 A549 및 Caco-2 세포에서 세포 독성을 나타내지만 NIH/3T3 세포는 나타내지 않았음이 LIVE/DEAD 염색 및 형광 이미징에 의해 확인되었다. 따라서 St-LA 나노 입자는 잠재적으로 암 화학 요법에 적용될 수 있을 것으로 판단된다.

Abstract: This manuscript reports on the fabrication of starch-lauric acid (St-LA) nanoparticles having potential cancer therapy. St-LA nanoparticles were fabricated by varying the amount of LA, and the optimized St-LA nanoparticles were found. In addition, it was confirmed that the St-LA nanoparticles were successfully fabricated through scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analysis. *In vitro*, the St-LA nanoparticles showed cytotoxicity in A549 and Caco-2 cells but not NIH/3T3 cells. These results were confirmed by LIVE/DEAD staining and fluorescence imaging. Our next goal is to evaluate the St-LA nanoparticles containing various anticancer drugs through *in vitro* and *in vivo* studies. St-LA nanoparticles can potentially be applied to cancer chemotherapy.

Keywords: lauric acid, nanoparticles, antitumor effect.

Introduction

Nanotherapy is attracting attention as a promising technology for the treatment, diagnosis, and prevention of disease using nanoscale materials. Nanoparticles are nano-sized particles measuring less than 1000 nm and are used primarily in nanotherapy of cancer, and in bioimaging and drug delivery systems.[1-4] Various nanoparticles include polymer nanoparticles [poly(lactide-*co*-glycolide), polylactic acid, gelatin, and chitosan],[5-7] liposomes,[8] micelles,[9] dendrimers,[10] and inorganic nanoparticles (gold, carbon nanotube, SiO_2, and Fe_3O_4).[11] These nanoparticles circulate in blood vessels and are able to penetrate cancer tissues most effectively with enhanced permeability and retention (EPR) effect (passive targeting).[12] Grafting cancer target molecules onto nanoparticles increases intracellular drug concentration in cancer cells while preventing damage to normal cells (active targeting).[13] Nanoparticle-based cancer therapy has been actively studied based on these characteristics.[14]

Lauric acid (LA) is a saturated fatty acid with a 12-carbon atom chain and properties of medium-chain fatty acids.[15] LA is also present in fruits, vegetable oils, and breast milk.[16] LA is reported to exhibit antimicrobial, antifungal,[17] and anti-inflammatory activities,[18] in addition to preventing prostatic hyperplasia,[19] and triggering apoptosis of colon cancer cells.[20] In particular, LA induces apoptosis in various cancer cells and therefore, used in cancer chemotherapy.[21] Cancer chemotherapy via induction of cellular apoptosis is mediated via increased oxidative stress.[22] The oxidative stress occurs when the glutathione (GSH) level is lowered or the reactive oxygen

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species (ROS) level is increased, leading to an imbalance in cell proliferation and apoptosis.[23] LA produces a substantial amount of ROS at the beta-oxidation stage, one of the metabolic degradation mechanisms, leading to cancer cell death.[24] The increased ROS also trigger the epidermal growth factor receptor/extracellular signal-regulated kinase (EGFR/ERK) transduction pathway referred to as the cell proliferation regulatory pathways and altered gene expression, leading to apoptosis.[21]

In this study, we fabricated starch-conjugated lauric acid (St-LA) nanoparticles for cancer chemotherapy. Starch encapsulating LA is biodegradable cost-effective, and nontoxic.[25] We believe that the St-LA nanoparticles have significant potential in enhancing the anticancer effect.

Experimental

Materials. Starch, LA, capric acid (CA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was ordered from Tokyo Chemical Industry Co., Ltd (TCI, Tokyo, Japan). Dialysis membranes (MWCO 6–8 kDa) were supplied by CelluSep (Sequin, TX, USA). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (containing penicillin and streptomycin). Cell Counting Kit-8 (CCK-8, Dojindo Co. Ltd., Tokyo, Japan) was used to quantitatively measure the number of cells.

Synthesis and Characterization of Starch-Lauric Acid Nanoparticles. Starch (100 mg) was mixed with 20 mL of DMSO and stirred for 3 h. LA (10, 25, 50, and 70 mg). DMTMM (0.2 g) was added into the starch dispersion and stirred for another 24 h to obtain St-LA, which was dialyzed in DMSO for 1 day to remove DMTMM. Subsequently, the St-LA solution was dialyzed in deionized water (DW) for 2 days to obtain St-LA nanoparticles, which were lyophilized for 2 days for further use. Starch-conjugated capric acid (St-CA) nanoparticles were also synthesized according to the same protocol. The size and zeta potential were measured via a dynamic light scattering method (Zetasizer nano ZS, Malvern Instruments, Malvern, UK). The morphology was observed by scanning electron microscopy (SEM, S-4800, Hitachi, Tokyo, Japan). Fourier transform infrared spectroscopy (FTIR, IRAffinity-1, Shimadzu, Tokyo, Japan) was used to characterize the St-LA nanoparticles.

Cell Viability. Mouse embryonic fibroblast (NIH/3T3), adenocarcinomic human alveolar basal epithelial cells (A549), and human epithelial colorectal adenocarcinoma cells (Caco-2) purchased from the Korea Cell Line Bank (Seoul, Korea) were selected as model cells to evaluate the cell viability of St-LA and St-CA nanoparticles. Aqueous dispersions of St-LA and St-CA nanoparticles at concentrations of 50, 75, 100, 200, and 500 μg/mL were added to each well containing 5×10^3 cells in a 96-well plate and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 48 h. The cells were counted using the CCK-8 assay. Cell viability was calculated as the number of live cells divided by the number of total cells. The cells were stained with a LIVE/DEAD Viability/Cytotoxicity Kit and observed with fluorescence microscopy (FM, IX71, Olympus Co. Ltd., Tokyo, Japan).

Results and Discussion

Preparation and Characterization of Starch-Lauric Acid Nanoparticles. Figure 1 shows the synthesis of St-LA nanoparticle. St-LA was synthesized between the LA and starch by DMTMM.[26] DMTMM activates carboxyl groups of LA and induces esterification reaction with the hydroxyl groups of starch. DMTMM was used as a conjugation agent due to its non-toxicity and high conjugation efficiency.[27] The synthesized St-LA solution with the DMSO phase was dialyzed in the DMSO phase to remove DMTMM. The St-LA solution loaded on the dialysis membrane was transferred into the aqueous phase of DW to obtain nanoparticles. In the aqueous phase, hydrophobic aggregation of LA particles occurred with starch as the hydrophilic component surrounding LA to form spherical nanoparticles.[28]

St-LA nanoparticles were prepared by varying the amount of LA (10, 25, 50, and 70 mg) to determine the optimized St-LA nanoparticles. Figure 2(A) and 2(B) show the size of St-LA and St-CA nanoparticles. The St-LA nanoparticles containing 10 and 25 mg of LA measured 472.0±15.0 and 356.0±13.1 nm, respectively. However, the sizes of St-LA nanoparticles carrying 50 and 70 mg of LA were 163.7±31.2 and 120.2±1.2 nm, suggesting that the size of St-LA nanoparticles decreased with an increased amount of LA. The reduction in size is due to the stronger hydrophobic interaction between nanoparticle cores as the amount of LA increases.[29] The size of St-CA nanoparticles also showed a similar tendency as St-LA.

As shown in Figure 2(C), the zeta potentials of St-LA nanoparticles with 50 and 70 mg of LA were -23.7±3.5 and

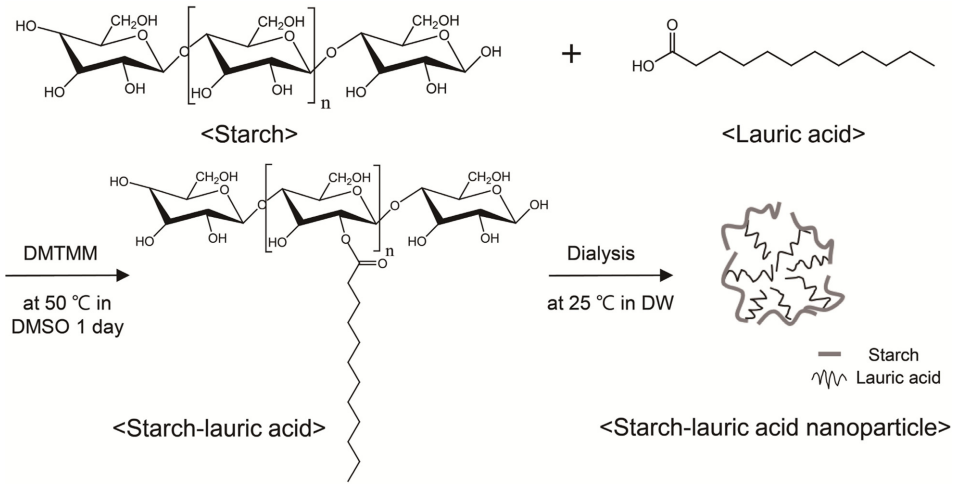

Figure 1. Schematic illustration of the synthesis of St-LA nanoparticle.

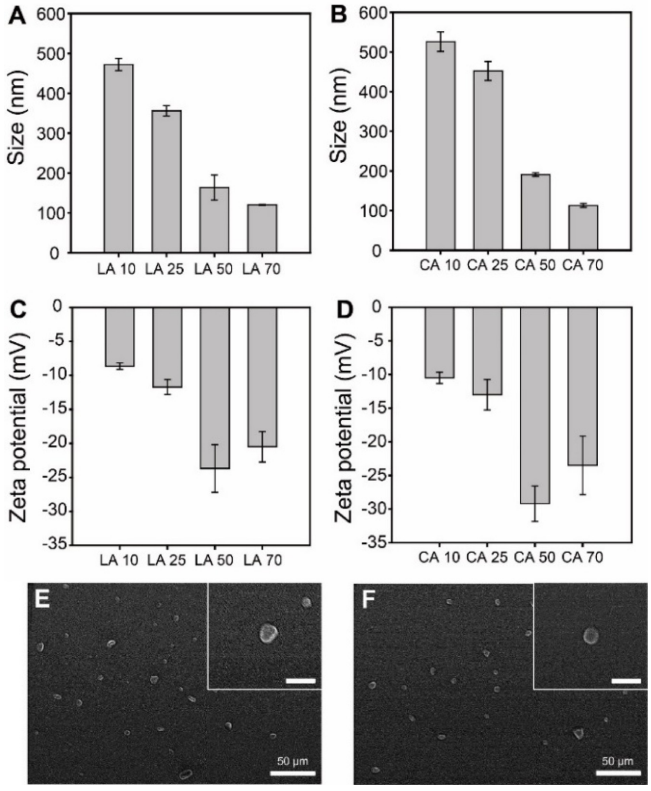

Figure 2. Size (A, B) and zeta potentials (C, D) of St-LA (A, C); St-CA nanoparticles (B, D). SEM images of (E) St-LA (LA 70); (F) St-CA (CA 70) nanoparticles. The inserts are magnified SEM images and scale bars represent 200 nm.

-20.5±2.2 mV, higher than the zeta potential of starch. In the synthesis, the hydroxyl group of starch was decreased and the zeta potential was increased.[30] The zeta potentials of St-LA nanoparticles with 10 and 25 mg of LA were -8.6±0.5 and -11.7±1.1 mV similar to the zeta value of LA, indicating the incomplete formation of the nanoparticles.[31]

The zeta potentials of St-CA nanoparticles also showed a similar tendency as St-LA. Therefore, the St-LA nanoparticles with 70 mg of LA were selected for the subsequent study because of their high LA content and appropriate size for cellular uptake. He *et al*. demonstrated that the smaller size of nanoparticles facilitated efficient cellular uptake because larger nanoparticles require stronger driving force for cellular internalization.[32] Studies *in vitro* showed that nanoparticles measuring 100 nm in size showed optimal uptake.[33,34] Figure 2(E) and 2(F) represent the SEM images of St-LA and St-CA nanoparticles with 70 mg LA and CA. Both nanoparticles exhibit spherical morphology.

Figure 3 presents the FTIR spectrum of starch, LA, and St-LA nanoparticles highlighting the esterification between hydroxyl groups in starch and carboxyl groups in LA. The region 3200 to 3600 cm^{-1} corresponds to the peak of hydroxyl groups (-OH stretch) in starch.[35] However, the hydroxyl groups in St-LA nanoparticles disappeared due to the reduction of hydroxyl groups in the esterification reaction. The decreased peaks of carboxylic groups in C=O (1700 cm^{-1}) and -OH (3200-3600 cm^{-1}) indicate that the carboxylic groups were reduced in the esterification reaction.[36] These results demonstrate the successful synthesis of St-LA nanoparticles.

In Vitro Experiment of Starch-Lauric Acid Nanoparticles. Figure 4 shows the viabilities of NIH/3T3, A549, and Caco-2 cells treated with St-LA, and St-CA nanoparticles during 48 h at different nanoparticles concentrations. In the case of NIH/3T3 cells (Figure 4(A)), a minor variation in cell viability was

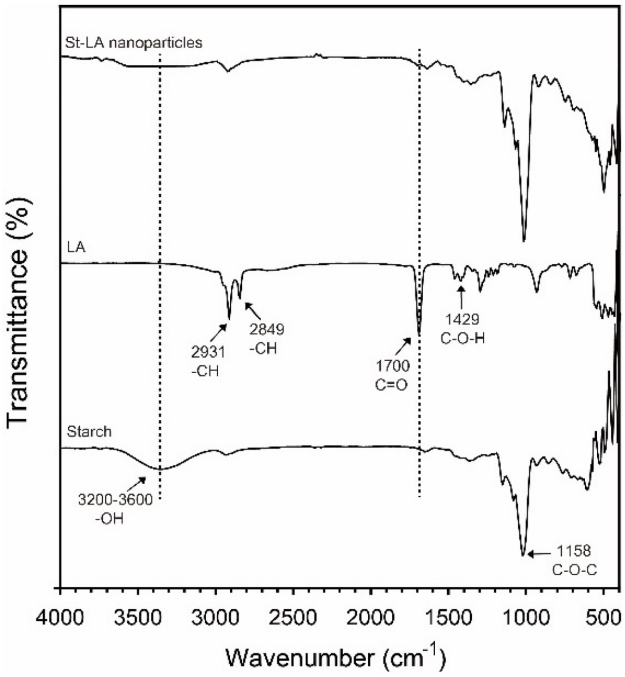

Figure 3. FTIR spectra of starch, LA, and St-LA nanoparticles.

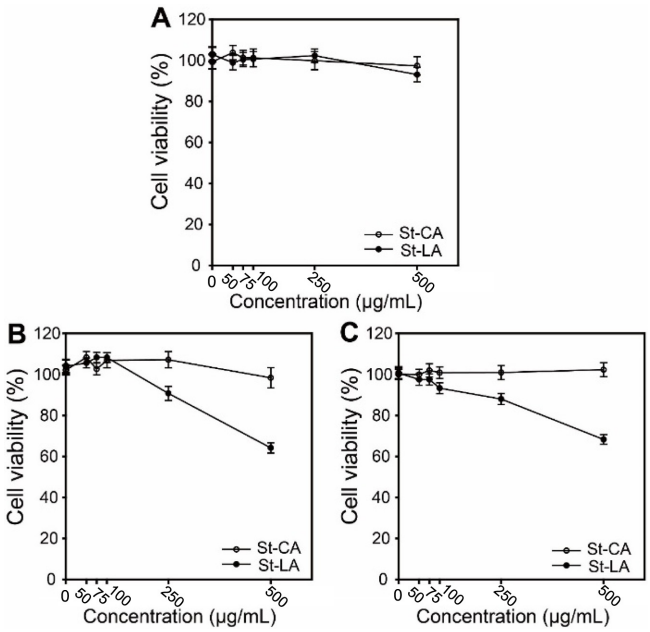

Figure 4. Viability of (A) NIH/3T3; (B) A549; (C) Caco-2 cells after treatment with St-CA and St-LA nanoparticles during 48 h of incubation at different concentrations.

observed depending on the concentration of nanoparticles, indicating that the St-LA nanoparticles were non-toxic in normal cells. EGF-induced apoptosis in the EGFR/ERK transduction pathway also occurred in normal cells as a 'normal' physiological event, and the apoptosis of St-LA nanoparticles was insignificant to affect normal cell viability.[37] As shown in Figures 4(B) and 4(C), the viabilities of A549 and Caco-2 cells treated with St-LA nanoparticles at 500 μg/mL were reduced to 64.1% and 66.9%, respectively. However, St-CA nanoparticles did not affect either cell viability because of the different metabolism of LA in CA oxidation in mitochondria.[38] St-LA nanoparticles specifically target cancer cells due to their EGFR overexpression resulting in increased apoptotic signals compared with normal cells. EGFR mainly plays a role in regulating cell proliferation, migration, and differentiation, but it has been demonstrated that EGFR may 'paradoxically' mediate apoptosis signals in some cell types. EGFR overexpression increases the apoptotic effect by mechanism impairing Akt activation by blocking Ras signaling. In EGFR-overexpressing cells, the apoptotic effect can reach a threshold, and the balance beyond that is tipped from proliferation to apoptosis due to misregulation of the signaling circuitry.[37]

Figure 5 shows a visual representation of cell death following treatment with 500 μg/mL St-LA nanoparticles. The cells were stained with the live and dead assay kit, with green and red colors indicating live and dead cells. The fluorescence images matched the cell viability data closely.

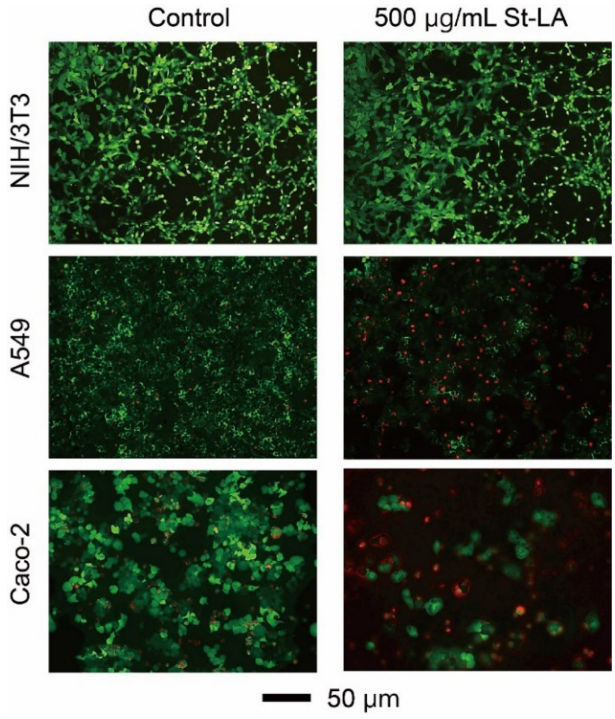

Figure 5. Fluorescence microscopy images of NIH/3T3, A549 and Caco-2 cells treated with St-LA nanoparticles for 48 h, followed by LIVE/DEAD staining (Green: live cells, Red: dead cells).

Conclusions

St-LA nanoparticles were fabricated for potential application in cancer chemotherapy. Since St-LA nanoparticles were fabricated based on their hydrophobic interaction in dialysis, hydrophobic anticancer drugs such as doxorubicin can be encapsulated together. Although the anticancer effect of St-LA nanoparticles alone is about 65% *in vitro*, the effect is increased when the nanoparticles are used as carriers of various anticancer drugs. Our next goal is to combine St-LA nanoparticles with various anticancer agents and cancer target moieties to generate highly efficient anticancer nanoparticles. We believe that the St-LA nanoparticles carry great potential for anticancer therapy.

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