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Research Article

Nanoliposomal L-Asparaginase and Its Antitumor Activities in Lewis Lung Carcinoma Tumor-Induced BALB/c Mice

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Although L-Asparaginase (L-ASP) is an effective chemotherapeutic agent, it has side effects such as fever, skin rashes, chills, anaphylaxis, and severe allergic reactions. Moreover, the short half-life of L-ASP reduces its antitumor activity. To reduce its side effects and broaden its pharmaceutical applications, L-ASP obtained from *Pectobacterium carotovorum* was subjected to liposomal conjugation. The enzyme was then loaded into liposomes using the hydrated thin-film method. The *in vitro* cytotoxic activity of liposomal L-ASP was evaluated with the MTT assay using cancerous cell lines, and its antitumor effects were examined in Lewis lung carcinoma (LLC) tumorized mice. The average size of the liposomes containing purified L-asparagine was 93.03 ± 0.49 nm. They had a zeta potential of –15.45 ± 6.72 mV, polydispersity index of 0.22 ± 0.02, and encapsulation efficiency of 53.99 ± 5.44%. The *in vitro* cytotoxic activity of liposomal L-ASP was less effective against LLC, MCF-7 (human breast carcinoma), HepG2 (human hepatocellular carcinoma), SK-LU-1 (human lung carcinoma), and NTERA-2 (pluripotent human embryonic carcinoma) cells than that of free L-ASP. However, the antitumor activity of liposomal L-ASP was significantly greater than that of untrapped L-ASP at the same doses (6 UI/mouse) in terms of tumor size (6309.11 ± 414.06 mm^3) and life span (35.00 ± 1.12 days). This is the first time the antitumor activities of PEGylated nanoliposomal L-ASP have been assessed in LLC carcinoma tumor-induced BALB/c mice and showed significantly improved pharmacological properties compared to those of free L-ASP ($P < 0.05$). Thus, nanoliposomal L-ASP should be considered for its widening applications against carcinoma tumors.

1. Introduction

L-Asparaginase (L-ASP) hydrolyzes L-asparagine into L-aspartate and ammonia and is used to treat acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma [1]. Although the enzyme has been a mainstay in the treatment of ALL [2–4], it can induce fever, skin rashes, chills, anaphylaxis, and severe allergic reactions [5, 6]. Continued treatment leads to frequent hypersensitivity reactions in the enzyme as a foreign protein [7, 8]. To minimize the incidence of systemic immunological reactions and other limitations of L-ASP, liposomal encapsulation of the enzyme using different techniques has been adopted. The main components of liposomes are phospholipids, which are similar to cell membranes [9]. The phospholipids form a bilayer membrane, which allows drugs with different physicochemical properties to be loaded into the liposome or conjugated into the biolayers and then delivered to a lesion [10]. When liposomes are loaded with enzymes, they can be targeted to organs such as the spleen, liver, and bone marrow [11], prolonging the circulation time without inhibiting enzymatic activities [12]. L-ASP was clarified for its antitumor activities in breast tumor bearing mice by Shiromizu et al. [13]. However, liposomal L-ASP has been

only reported for its improvement *in vivo* anticancer activities in lymphomatic mice [11]. Therefore, we developed PEGylated nanoliposomal L-ASP and first time evaluated its antitumor efficacy in Lewis lung carcinoma (LLC) tumorized BALB/c mice for broadening its pharmaceutical applications.

2. Materials and Methods

2.1. Materials. The bacterial strain *Pectobacterium carotovorum* was provided by Prof. Gilles Truan (Axe Biocatalyse/Ingénieries Métabolique et Moléculaire, LISBP, INSA, Toulouse, France). The strain was used for the production of L-ASP enzyme. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG-2000) was purchased from Avanti polar lipids Inc. (Alabaster, Alabama, USA). Cholesterol, soybean lecithin, fetal bovine serum (FBS), and gentamicin was obtained from Sigma Chemical Co. (St. Louis, MO., USA). Dulbecco's modified Eagle's medium and nonessential amino acid (NAA) and L-glutamine was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Animals. Male and female albino BALB/c mice (8–10 weeks old) were obtained from the Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST, Hanoi, Vietnam). All mice were housed in a temperature-controlled room on a 12-hour light/12-hour dark cycle with food and water ad libitum. Experiments were performed in accordance with Vietnamese Ethical Laws and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

2.3. Isolation, Extraction, and Purification of L-Asparaginase. The bacterium was cultivated in the optimal medium to produce L-asparaginase as reported by Gulati et al. [14]. In details, the inoculum was prepared by adding a loop full of 250 ml cultivated medium in a 1 L flask and incubated at 30°C, 180 rpm in a shaking incubator for 12 h (to reach the culture OD at 600 nm = 0.6 to 0.8). The continued 2% of the inoculum was continuously incubated in a shaking incubator at 30°C, 180 rpm for additional 24 h. Cells were harvested by centrifugation at 10,000 g for 10 min at 4°C. Cells were washed with 50 mM Tris-HCl buffer (pH 8, 6) and resuspended in the same buffer. The cells were cooled on ice and ultrasonicated at 20 MHz, 35% amplitude, 20 min. The lysate was centrifuged at 20,000 g for 10 min at 4°C. The clear supernatant was loaded on DEAE cellulose and Sephadex G-100 chromatography for purification. The obtained eluted fractions were protein-quantitated using Pierce BCA Protein Assay Kit (Thermo Scientific) and qualified using SDS-PAGE [15].

2.4. Determination of L-Asparaginase Activity. L-ASP activity was measured by the modified method of Wriston [16] in which the rate of ammonia formation will be detected by Nessler' reagent at 37°C. One unit of L-ASP activity was defined that liberates the amount of enzyme that released 1 μM of ammonia (with 10 μM–10 mM ammonium sulfate as the standard) per minute under the assay conditions.

2.5. Procedure for the Preparation of L-ASP Nanoliposomes. L-ASP was entrapped into liposomes by thin film dehydration-rehydration method, as Cruz et al. [17] with slight modification. Briefly, soybean lecithin (40 μmol), cholesterol (4 μmol), and DSPE-PEG-2000 (8 μmol) were dissolved in chloroform : methanol (9 : 1 v/v) and stirred mechanically to form homogeneous mixture in round bottom flask. The solvent was removed using a rotary evaporator under an aspirate vacuum (25 mmHg) and a water bath with the temperature maintained at 25°C. The thin film which formed on the walls of the flask was dispersed in 2 ml of 5 mM potassium phosphate buffer (pH 7.5) containing L-ASP (150 I.U.) and rotated without vacuum at 100 rpm, 25°C. Then, the multilamellar vesicles were sonicated three times at 30 seconds intervals for resizing before filtered through 0.22 μm membrane to receive the L-ASP liposomal mixture. Finally, the liposomal mixture was washed twice with normal saline.

The particle size, zeta potentials, and size distribution of liposomes were determined using a Zetasizer Nano-Z (Malvern Instruments, UK). The conjugates were also observed by high-solution transmission electronic microscopy (TEM) (Jeol 1200EX TEM, Jeol Company, Tokyo, Japan). The liposomal samples were mounted on metal stands and coated with gold to thickness of 200–500 A°. Then, the plates were magnified 200x to capture the morphology of the prepared liposomes.

2.6. Determination of Encapsulation Efficiency (EE). After preparation, liposomal solution was loaded to Vivaspin column and centrifuged at 3000 RCF for 20 min, 25°C to remove the free L-ASP. The liposome pellet was lysed with 10% Triton X-100 to disrupt the liposomal bilayer and to release L-ASP. The Bradford assay was used to determine the amount of released L-ASP. Encapsulation efficiency of L-ASP into liposomes is determined as the following formula [18]:

$$\text{EE\%} = \frac{C_f}{C_i} \times 100, \quad (1)$$

where C_f is the encapsulated amount of L-ASP into liposomes measured after lysing with 10% Triton-X 100 and C_i is the L-ASP amount added to the lipid mixture.

2.7. Antiproliferative Activities of L-ASP Encapsulated Nanoliposomes. The antiproliferative assays were carried out in triplicate in 96-well microtiter plates against LLC (Lewis lung carcinoma), MCF-7 (human breast carcinoma), HepG2 (human hepatocellular carcinoma), SK-LU-1 (human lung carcinoma), and NTERA-2 (pluripotent human embryonic carcinoma) cells. Those cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO),

1% antibiotic-antimycotic (Thermo Fisher), 1% nonessential amino acid, and 2 mM of L-glutamine. For MCF-7, insulin was added to the growth medium at 10 μg/ml concentration. All cells were incubated in a humidifier with 5% CO_2 at 37°C and subcultured every 2-3 days. The cancer cells were seeded into a 96-well plate at a density of 1×10^4 cells/well. Then nanoliposomal conjugates were added to reach the final concentration ranging from 0.01–2.5 UI/mL and incubated for further 72 h. To assess effect of liposomes on cell viability, the MTT assay was performed as previously described [19].

2.8. Antitumor Efficiency of L-ASP Encapsulated Liposomes. The experiment was carried out using BALB/c mice at 20–25 gr weight. Mice were inoculated by subcutaneously injection with 1×10^6 LLC cells to induce tumor. On the day 7th of LLC cell injection, mice were randomly divided into 4 groups (6 mice per group) including a control group, two treated groups of liposomal L-ASP (6 UI/mouse and 3 UI/mouse), and the free L-ASP treated group (6 UI/mouse). The drugs were administrated intravenously every 2 days. Growth of tumor was determined by caliper measurements in two dimensions, length (L) and width (W), every 7 days. The tumor volume (V) was calculated using the formula $V = 1/2 \times L \times W^2$ [20].

The survival time of mice in all experimental groups were recorded. It was calculated from the day of LLC cell inoculation to the day of death, and percentage increase in average life span (ILS) was calculated by the formula % $\text{ILS} = (A/B - 1) \times 100$ in which A means survival time of treated, B is mean survival time of control group, and ILS is increase in average life span group.

2.9. Statistical Analysis. Statistical analyses and significance, as measured by two-way analysis of variance (ANOVA), were performed using GraphPad PRISM 5.0 software (GraphPad Software, USA). In all comparisons, $P < 0.05$ was considered statistically significance.

3. Results and Discussion

3.1. Isolation, Purification, and Determination of L-ASP Activity. First, L-ASP crude extract was purified in three steps with a final yield of 23.5% and a purification fold of 9.38 (Table 1). The estimated molecular weight of the obtained enzyme was 36 kDa (Figure 1). This purified enzyme was used for further liposomal conjugation.

3.2. Physicochemical Characteristics of Liposomal L-ASP. Generally, the components of a bilayer have strong effects on the rigidity, fluidity, and toxicity of the phospholipid bilayer [21]. Nontoxic phospholipids are usually selected to reduce toxicity and to strengthen drug-liposomal conjugates, including natural and synthetic phospholipids such as soybean lecithin and DSPE-PEG-2000 [10]. Soybean lecithin is a natural phospholipid that is often used for large-scale industrial applications to reduce production costs [22]. DSPE-PEG-2000 is reported elsewhere as important breakthrough in the liposomal development pertaining stealth behavior [23]. Cholesterol is often used in liposomal formulation because it facilitates complex interactions with phospholipids and other lipids in cellular membranes [24]. Therefore, we used soybean lecithin, DSPE-PEG-2000, and cholesterol to make liposomal L-ASP, encapsulating the purified L-ASP using the rehydrated thin film method. The resulting liposomes were 93.03 ± 0.49 nm in size with a polydispersity index of 0.22 ± 0.02, indicating that the size distribution was quite homogeneous. The zeta potential is a measure of the magnitude of electrostatic or charge repulsion between particles, which affects liposome stability [25–27]. The liposomes harboring L-ASP had a zeta potential of -15.45 ± 6.72 mV, the opposite of liposomes reported by Bahreini et al. that were made from chitosan and tripolyphosphate using ionotropic gelation [28]. The entrapment efficiency was determined as the ratio of encapsulated L-ASP to the amount used to prepare the liposomes. The encapsulation efficiency was 53.99%, demonstrating that L-ASP was effectively loaded into the liposomes (Table 2).

3.3. High-Resolution Transmission Electron Microscopic Analysis. High-resolution transmission electron microscope (TEM) was used to evaluate the size, shape, and morphology of the L-ASP liposomes [26]. On TEM, the L-ASP liposomal particles were spherical and evenly dispersed (Figure 2). The obtained liposomes were nanometers in size (Figure 2).

3.4. Cytotoxicity Activities. The effects of free and liposomal L-ASP on the survival of LLC (Lewis lung carcinoma), MCF-7 (human breast carcinoma), HepG2 (human hepatocellular carcinoma), SK-LU-1 (human lung carcinoma), and NTERA-2 (pluripotent human embryonic carcinoma) cell lines were evaluated using the MTT assay. The cytotoxic activity of the liposomal L-ASP was dependent on the cancer cell line and concentrations tested (Table 3 and Figure 3). SK-LU-1 cells were the most sensitive to liposomal L-ASP and to the unloaded enzyme. The conjugates had similar activity on SK-LU-1 and LLC cells, with IC_{50} values of 0.21 ± 0.03 and 0.23 ± 0.02 UI/mL, respectively. MCF-7 cells were the least sensitive to both free and liposomal L-ASP. A decrease in the L-ASP concentration led to reduced cell death in all cell lines tested. Generally, malignant cells have a high division rate and need more nutrients than normal cells. Asparagine is important in cell growth and function, as it coordinates protein and nucleotide synthesis [29]. L-ASP induces cytotoxicity by breaking asparagine into L-aspartate and ammonia. According to Moharib, the cytotoxic activity of L-ASP is closely related to asparagine, and the asparagine level differed among various cell lines [30]. Therefore, L-ASP induced cancer cell death to varying degrees. However, the cytotoxic activity of the liposomal L-ASP was 3.00–5.68 times lower than that of free L-ASP after 72 h of treatment. In general, and with L-ASP, liposomal formulations are significantly less toxic than the unconjugated forms because of slow uptake. In our study, the L-ASP liposomes were less active against all cancer cells, in agreement with other studies

Table 1: Purification profile of isolated L-ASP.

Steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude extract	5972	276.5	21.6	0	100
Ammonium sulfate precipitation	2204	65.4	33.7	1.56	36.9
Sephadex G-100 chromatography	1403	6.9	202.6	9.38	23.5

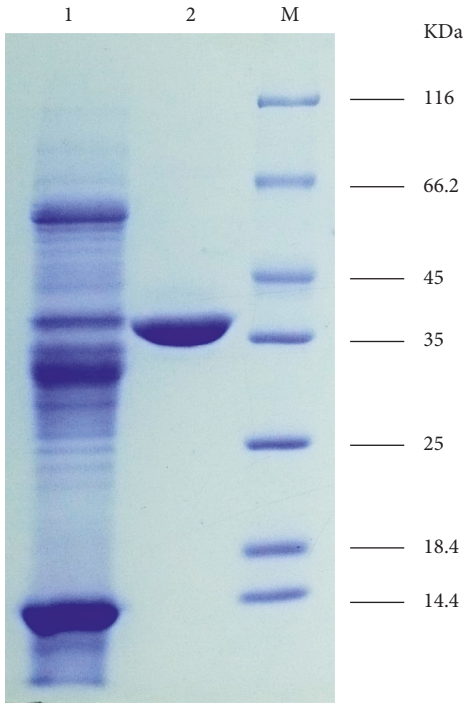
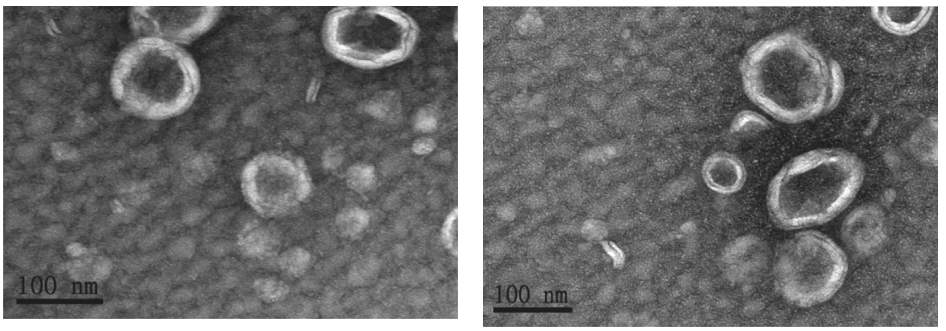


Figure 1: Molecular weight analysis of purified L-ASP obtained from *P. carotovorum* using SDS-PAGE on 12.5% gel of stained with coomassie blue; M-Fermentas ladder; 1-crude extract of L-ASP; 2-purified L-ASP.

Table 2: Determined physiochemical characteristics of liposomes loaded L-ASP.

Samples	Size (nm)	PDI	Zeta potential (mV)	EE (%)
Blank liposome*	97.53 ± 22.17	0.24 ± 0.02	−22.80 ± 0.00	—
L-ASP-liposome	93.03 ± 0.49	0.22 ± 0.02	−15.45 ± 6.72	53.99 ± 5.44

*Blank liposome containing soy lecithin, cholesterol, and DSPE-PEG2000.



(a) (b)

Figure 2: The TEM images of nanoliposomes (Jeol 1200EX TEM system, Japan) (a) DSPE-PEG2000-soy lecithin-cholesterol-loaded L-ASP nanoliposomes; (b) blank liposomal nanospheres.

Table 3: Cytotoxic activities of L-ASP-encapsulated liposomes and free L-ASP on different cell lines.

Samples	Values of IC_{50} (UI/ml)				
	LLC	HepG2	SK-LU-1	MCF-7	NTERA-2
Loaded L-ASP liposomes	0.23 ± 0.02	0.28 ± 0.01	0.21 ± 0.03	0.36 ± 0.05	0.23 ± 0.02
Free L-ASP	0.079 ± 0.001	0.044 ± 0.004	0.037 ± 0.001	0.12 ± 0.01	0.038 ± 0.003

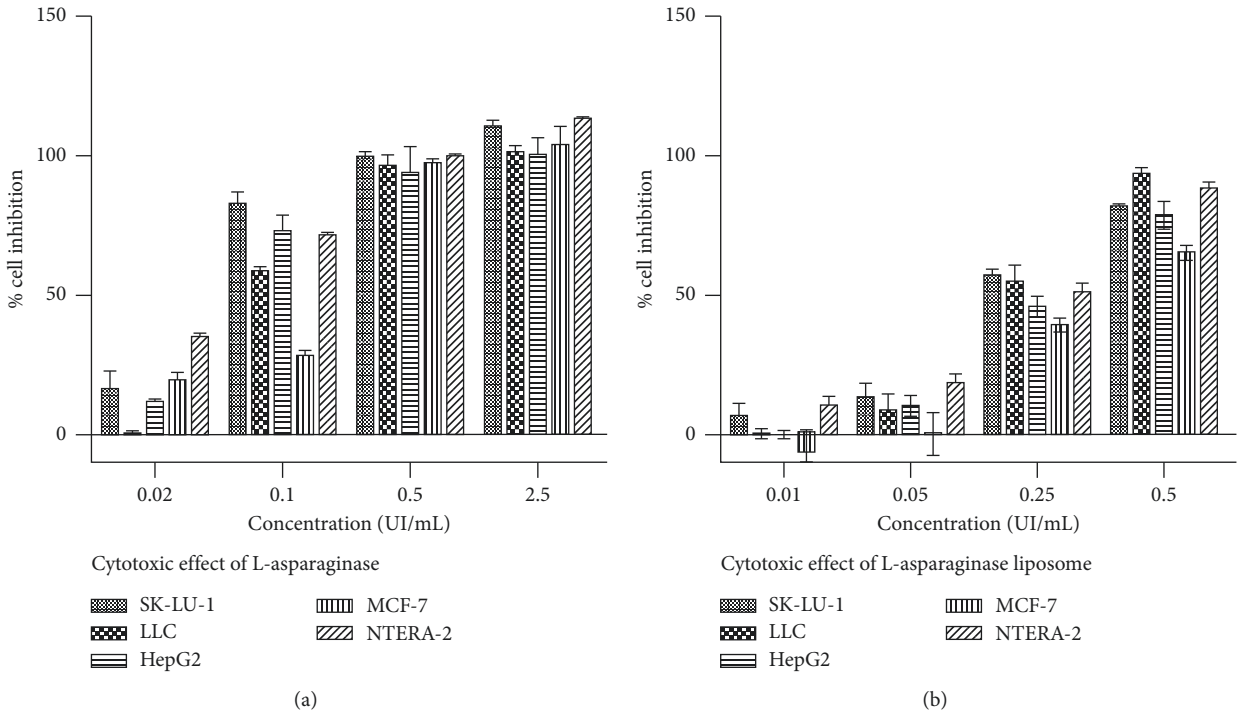


Figure 3: Effectiveness of free L-ASP (a) and L-ASP-encapsulated liposomes (b) on different cell lines. Cultured cells (1×10^4 cells/well) were treated with different concentrations of either liposomal (a) or free L-ASP (b). Normal saline was served as the negative control. Each value represents the mean ± SD.

[17]. The reduction in L-ASP cytotoxicity with liposomal encapsulation might be due to the slower release rate and slower substrate depletion, allowing cells to adapt and synthesize asparagine themselves.

3.5. In Vivo Antitumor Activity of Liposomal L-ASP. The antitumor activity of liposome-L-ASP was examined in BALB/c mice harboring tumors induced by LLC cells (Figure 4). Groups of mice were treated with two doses of liposomal L-ASP (6 or 3 UI/mouse) or free L-ASP (6 UI/mouse) intravenously. The tumor volumes of the mice were analyzed at different time points. Dose-dependent antitumor activity of liposomal L-ASP was observed. Twenty-eight days after LLC inoculation, the tumors of the group treated with 6 UI liposomal L-ASP measured $6309.11 \pm 414.06\ mm^3$, which was significantly smaller than in the negative control ($9{,}319.35 \pm 469.58\ mm^3$, $P < 0.01$). Liposomal L-ASP suppressed tumor growth more strongly and significantly at a dose of 6 UI than at a dose of 3 UI ($7{,}885.80 \pm 824.36\ mm^3$) ($P < 0.05$). The tumors in the group treated with free L-ASP ($7{,}544.94 \pm 284.05\ mm^3$) were larger than those in the group treated with liposomal L-ASP at the same dose (6 UI/mouse), and this difference was significant ($P < 0.05$) (Figure 4). The mice treated with 6 UI/mouse untrapped L-ASP showed smaller volumes of tumors in comparison with the 3 UI/mouse liposomal treated group but no significance was observed ($P > 0.05$). The mean body weight of the mice in the liposomal L-ASP groups was similar to that of the negative controls at the end of the experiment, indicating that the encapsulation did not induce toxicity (data not shown).

Furthermore, the administration of liposomal L-ASP prolonged the lives of the mice (Table 4). The median survival of mice treated with 6 UI liposomal L-ASP was 35.00 ± 1.13 days, which was 12.30% longer than that of control mice ($P < 0.05$). The life span also increased slightly in the free L-ASP group, but not significantly.

Since the early 1970s, L-ASP has been used to treat ALL. Currently, L-ASP is also used to treat ovarian cancer, hepatocellular carcinoma, and gastric carcinoma [31]. However, L-ASP can induce fever, thrombosis, and impaired liver, kidney, and central nervous system function, which may be related to the glutaminase activity of L-ASP [32].

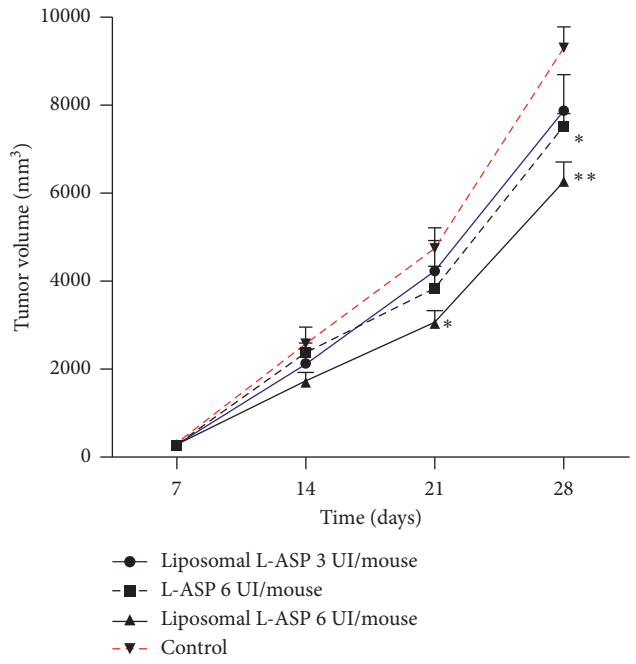


Figure 4: Tumor growth after treatment with free L-ASP or liposomal L-ASP. BALB/c mice harboring tumors induced by LLC cells were treated with either free L-ASP (6 UI/mouse) or encapsulated L-ASP liposomes at either 6 UI/mouse or 3 UI/mouse ($n = 6$). Liposome-entrapped L-ASP at both doses 6 UI/mouse and 3 UI/mouse significantly inhibited tumor growth after 28 days compared with the negative control (blank liposome) ($^{**}P < 0.01$ and $^{*}P < 0.05$, respectively). Error bars represent standard error (SE).

Table 4: Effects of liposome-L-ASP and free L-ASP on survival time of LLC cells bearing mice from different experimented groups (mean ± SE) ($n = 6$).

Groups	Mean survival time (days)	% ILS
Control group (blank lipsosome)	31.17 ± 1.28	—
Liposomal-L-ASP (3 UI/mouse)	33.33 ± 0.92	6.95
Liposomal-L-ASP (6 UI/mouse)	35.00 ± 1.13*	12.30
Free L-ASP (6 UI/mouse)	32.17 ± 0.79	3.21

*$P < 0.05$ in comparison with the control group (blank liposome).

According to Kumar et al. [15], L-ASP from *Pectobacterium carotovorum* is free of glutaminase activity, which might avoid the disadvantages of L-ASP isolated from bacteria such as *Escherichia coli* and *Erwinia chrysanthemi*. In our study, both free and liposomal L-ASP isolated from *P. carotovorum* showed strong activity against various solid cancer cell lines, including lung, liver, and breast carcinomas and pluripotent human embryonic carcinoma cells. Shiromizu et al. reported similar activity of L-ASP in colon cancer (C-26), a murine sarcoma cell line (S-180), and murine breast cancer (4T1) [13].

Other limitations of L-ASP treatment include its short biological half-life ($T_{50} = 2.88$ h), which necessitates increasing the dosage and intervals of L-ASP treatment [33]. Liposomes are considered an effective solution to these obstacles. Since their discovery in the 1960s, liposomes have been used as drug carriers to enhance the potency and to reduce the toxicity of therapeutic agents. Liposomes also improve the delivery of therapeutic agents to specific sites in the body [18, 34]. As reported elsewhere, solid tumors show enhanced permeability by and retention of lipids and macromolecules [35, 36]. Some effects in some solid tumors are not observed in normal tissues or organs, such as extensive angiogenesis and impaired lymphatic drainage/recovery [37]. These effects lead to higher delivery of a liposome-encapsulated drug to tumors compared with other sites. However, the size of liposomes affects the circulation of drug-loaded liposomes, especially liposomal L-ASP. Larger liposomes are easily opsonized and then rapidly removed from the blood by the mononuclear phagocytic system [38], whereas small liposomes (range 50–200 nm) increase the circulation time of loaded molecules, such as enzymes [39]. With the thin film method, simplified dehydration-rehydration vesicles produce large liposomes. However, our use of bath sonication produced liposomes smaller than 200 nm. This suitable average size helps to prolong the retention time of circulating liposomal L-ASP *in vivo* and its accumulation in tumors.

Additionally, the PEGylation of the liposomes helped them to avoid recognition by the mononuclear phagocytic system. Other outstanding features of PEG-coated liposomes include avoiding aggregation between liposomal particles and reduced clearance and immunogenicity [40]. These result in higher antitumor efficacy of liposomal L-ASP *in vivo*. However, the drug release rates of PEGylated formulations were lower than those with no PEGylation [41, 42], which may be an additional explanation for the lower *in vitro* cytotoxic activity of liposomal L-ASP.

4. Conclusion

Using the dehydrate-rehydrate thin film method, L-ASP was effectively encapsulated into liposome carriers at an efficiency of 53.99 ± 5.44%. PEGylated nanoliposomal L-ASP first showed significantly improved antitumor activities compared to those of free L-ASP ($P < 0.05$) in LLC induced BALB/c mice but showed lower activity in an *in vitro* assay. Our PEGylated nanoliposomal L-ASP exhibited improved pharmacological properties and bioavailability and should be considered for further clinical applications against carcinoma tumors.

Data Availability

The physicochemical data of blank and liposomal L-ASP used to support the findings of this study are included within the supplementary information files.

Conflicts of Interest

The authors report no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: concentration of L-ASP in nanoliposomes. Supplementary Table 2: relative concentration of L-ASP with concentration of liposomes in Figure 3(B). Supplementary Figure 1: normalized tumor volume change to body weight after treatment with free L-ASP or liposomal L-ASP at different times. BALB/c mice harboring tumors induced by LLC cells were treated with either free L-ASP (6 UI/mouse) or encapsulated L-ASP liposomes at either 6 UI/mouse or 3 UI/mouse ($n = 6$). Liposome-entrapped L-ASP at both doses 6 UI/mouse and 3 UI/mouse significantly inhibited tumor growth after 28 days compared with the negative control (blank liposome) ($^{**}P < 0.01$ and $^{*}P < 0.05$, respectively). Error bars represent standard error (SE). (*Supplementary Materials*)

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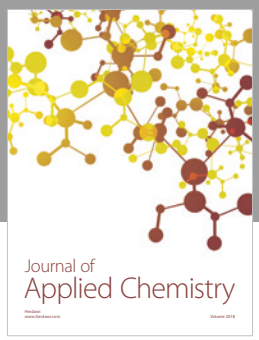
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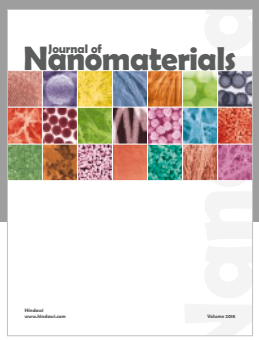
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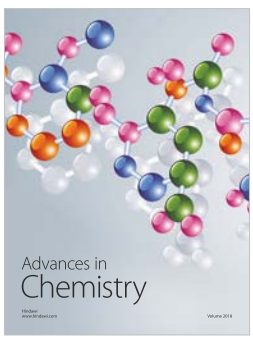
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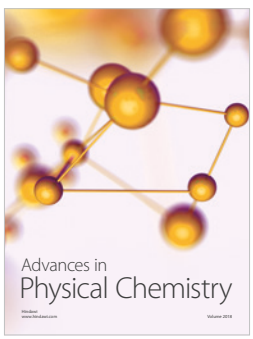
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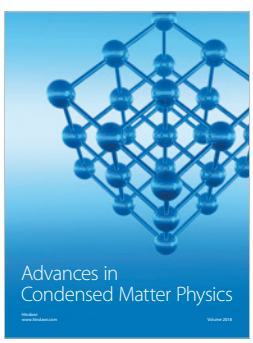
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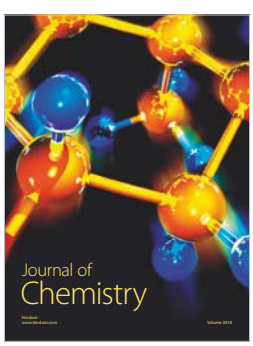
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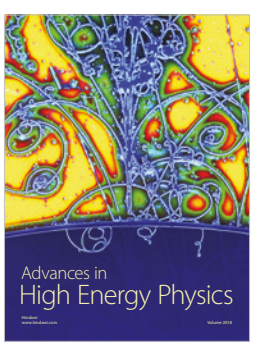
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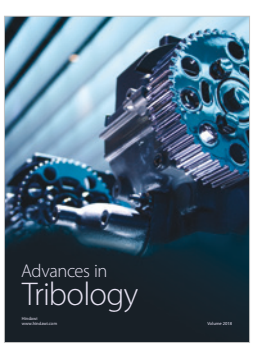
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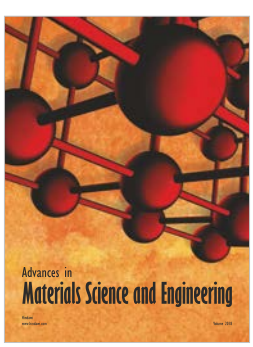
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