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Preliminary study for the preparation of fatty acid bioconjugated L-asparaginase micellar nanocarrier as a delivery system for peptide anti-cancer agents

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Abstract

In the present study, the mixing behavior of different surfactants and fatty acid bioconjugated L-asparaginase was investigated. The amphiphilic macromolecules were achieved by covalent linkage of fatty acids with different chain lengths (C_{12}, C_{16}, and C_{22}) to the native enzyme, L-asparaginase. The amino group of L-asparaginase lysine residue was conjugated to the carboxylic group of fatty acids, using a carbodiimide activator. The particle sizes of resulted micellar nanocarrier before and after lyophilization and enzyme activity was investigated. Among all surfactants, pluronic F-127 with fatty acid bioconjugated L-asparaginase presented more plasma and PBS half-life and Higher activity value after lyophilization. These findings from L-asparaginase modification by fatty acid and surfactants indicate a promising stabilized product that may serve as a new candidate for medical purposes.

Keywords: L-asparaginase, Lipid-protein conjugation, Micellar Nanocarrier, Pluronic F-127

1. Introduction

L-asparaginase (L-ASNase) is an antitumor agent that catalysis the hydrolysis of amino acid L-asparagine to L-aspartic acid and ammonia (1). This enzyme alone or in combination with other antitumor drugs has excellent efficacy against leukemia and lymphosarcoma in the pharmaceutical industry and clinical trials (2, 3). The main drawback to the use of L-asparaginase is short in vivo half-life ($t_{1/2}$=1.2 days), immunogenicity, and low stability to proteolysis (4, 5). Until recent years, numerous strategies have evaluated in an attempt to improve *in vitro* and *in vivo* efficacy of L-ASNase (1, 6). As a result, attention paid to prolonging the duration of action of L-ASNase, improve its stability in the body, minimize the immunogenic effect, and lowers the affinity to natural inhibitors (7).

Chemical modification of L-ASNase is one of these approaches which lead to the increase in enzyme stability and prolonging its half-life (8, 9). The poly (ethylene glycol) [PEG] conjugated L-ASNase from *E. coli* is an effective alternative to the native enzyme for clinical anti-leukemia therapy (10, 11). Pegaspargase should not routinely substitute for the native protein. A recent report shows a possible higher incidence of pancreatitis associated with pegaspargase (12, 13). Results suggest that the covalent coupling of polysialic acids reduces the antigenicity of L-ASNase and, as a result, extends its circulation in the blood even in the presence of anti-ASNase antibodies (14). In another research presents by Tabandeh et al., the effect of oxidized inulin conjugation on the physicochemical and immunological properties of L-ASNase was investigated (15). The modified enzyme increased the thermostability, *in vitro*

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half-life, resistance to protease digestion, reusability after repeated freezing-thawing, and widened the range of optimum pH. Recently, our research group offers a fatty acid bioconjugated L-ASNase with different chain lengths of fatty acid (C_{12}, C_{16}, and C_{22}) (16) which presented excellent proteolysis stability, prolong in vitro half-life and higher affinity to its substrate.

Regarding our investigation, the hydrophobic/hydrophilic structure achieved. As a new plan in this study, we proposed a micellar nanocarrier in which surfactants added to fatty acid bioconjugated L-ASNase. In recent decades, the development of micelles among the variety of surfactants (17), fatty acids/ surfactants (18) and other amphiphilic polymers in combination with such compounds have attracted much attention.

In this study the effect of surfactants on the size and activity of enzymes investigated to find suitable surfactants for the next step and evaluate the future experimental design (19). In this study, the impact of micellization on enzyme activity and particle size of the bioconjugate along with different surfactant considered. This structure is supposed to present physicochemical properties different from the native form, which investigated in this experiment.

2. Material and Methods

2.1. Materials

L-Asparaginase with 140 KDa molecular weight and 10,000 IU activity was obtain from Medac®, Germany. A fatty acid used for conjugation (palmitic and lauric acid) was from Merck Co. (Darmstadt, Germany). Behenic acid was purchased from Fluka Co. Ltd. (Switzerland). Surfactants used for micellar nanocarrier were from Merck Co. (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). Other chemical reagents such as L-asparagine, trypsin, and reagents were of analytical grade and were purchased locally.

2.2. Synthesis of bioconjugates

Fatty acid bioconjugated L-ASNase achieve through the conjugation of an amine group of L-ASNase with the carboxylic group of different lipids (behenic acid, palmitic acid, and lauric acid) using carbodiimide activator (20). Briefly, 3.57 µmole of fatty acids (behenic acid, palmitic acid, and lauric acid) was dissolved in 5mL of DMSO by subsequent addition of 3.93 µmole EDC and 3.57 µmole NHS. The mixture then stirred for 3 hours at 25 °C. Later, the 0.0714 µmole of L-ASNase in 5mL of water was added to the mixture dropwise. After the reaction time, unreacted lipid, EDC, NHS, and DMSO were removed by dialysis against water for 24 hours. The separations by dialysis performed using cellulose acetate membranes (12 KDa). Differential scanning calorimetry (DSC) and colorimetric spectroscopy methods (21) were used to approve the purifying of bioconjugates. All experiments performed in triplicate.

2.2.1. Morphology of bioconjugates

The particle morphology examined by transmission electron microscopy (TEM) (Zeiss, model EM10C, Germany) to evaluate the effect of lipidation on enzyme structure. For imaging, bioconjugated samples were immobilized on copper grids, dried at room temperature and, then, examined using a TEM without being stained.

2.3. Preliminary study for surfactant selection of micellar nanocarrier

For the preparation of nano-micellar structures of the fatty acid bioconjugated L-ASNase, at a preliminary step, an appropriate amount of various types of surfactants were dispersed in about 10 mL of bioconjugates suspension to prepare a concentration above the CMC value of that surfactant, while stirring at room temperature for 60 minutes. Finally, samples were lyophilized for 48 h.

2.4. Lyophilization of the bioconjugated enzyme and micellar nanocarrier

The total quantity of sample materials (bioconjugates and micellar nanocarrier) after dialysis has divided into several sample containers. The control sample (the native enzyme without fatty acid) kept under the same conditions. Each sample is being frozen at -70 °C for a minimum of 12 hrs. In the primary drying, the shelf temperature and chamber pressure adjusted at -45 °C and 0.07 mbar, respectively, for 30 hrs. After that, within the secondary drying, bound water was removed via desorption by the chamber pressure of 0.04

mbar for 18 h. Finally, samples were lyophilized for 48 hrs using a freeze drier.

2.5. Determination of particle size

The mean diameters of all nanoparticles were measured in triplicate by laser-diffraction instrument using a particle size analyzer (Shimadzu, model SALD-2101, Japan). Samples reconstituted to appropriate concentrations (0.1 mg/ml) with distilled/filtered water. Measurements conducted at 25 °C. The instrument parameters set as follows: refractive index of the material of 1.45 (i.e., protein), dispersant viscosity of 0.8872 (i.e., water), and dispersant refractive index of 1.33.

2.6. Determination of time-activity profile in PBS and human plasma

The catalytic activity and half-life of the micellar nanocarriers investigated *in vitro* in PBS and plasma. For this experiment, equivalent preparations containing 1 mg of formulation loaded in 2 mL of plasma or PBS following by mild mixing. Then, samples were incubated at 37 °C for 0, 1, 2, 4, 8, 10, 12, 24, 48, 72, 96, 168 and 336 h. For the determination of enzyme activity, 0.1 mL of each sample taken at mentioned time intervals. All experiments performed in triplicate(8, 22).

2.7. L-ASNase-activity assay method

In this study, ammonia liberated from the L-ASNase reaction was quantify using Nessler reagent (3, 23). Briefly, 0.1 mL of L-ASNase solution with different concentrations in the range of 1-10 µg/ml added to the mixture of 0.1 mL of L-asparagine (186 mM), 1.0 mL of 0.05 M tris buffer (pH 8.5), 0.9 mL deionized water. The mixture then incubated at 37 °C for 30 min. Subsequently, 0.1 ml of 1.5 M trichloroacetic acid added to stop the enzymatic reaction and centrifuged for 5 min at 10,000 g to separate the precipitated protein from the solution. After that, 0.2 ml of supernatant, 4.3 ml deionized water and 0.5 ml Nessler's reagent added to a suitable container and the absorbance value of the solution at 436 nm recorded on a UV-visible spectrophotometer (24).

3. Results and Discussion

3.1. Synthesis of bioconjugates

In this study, three different chain lengths of fatty acid like lauric acid, palmitic acid, and behenic acid (C_{12}, C_{16}, and C_{22}) used for conjugation with L-ASNase. The bioconjugates prepared by the direct reaction of the carboxyl group of fatty acids to amino groups of the enzyme. Conjugation of lysine group has been reported for the modification of L-ASNase using oxidized dextran (25), oxidized inulin (15), silk sericin and fibroin (8, 12). The improved properties of fatty acid bioconjugated L-ASNase characterized as reported previously by the authors (16).

3.1.1. Morphology

The morphological studies demonstrated the particle size and size distribution of the nanostructured carriers while showing a spherical shape (Figure 1). This morphological structure was under the results of the light scattering method that

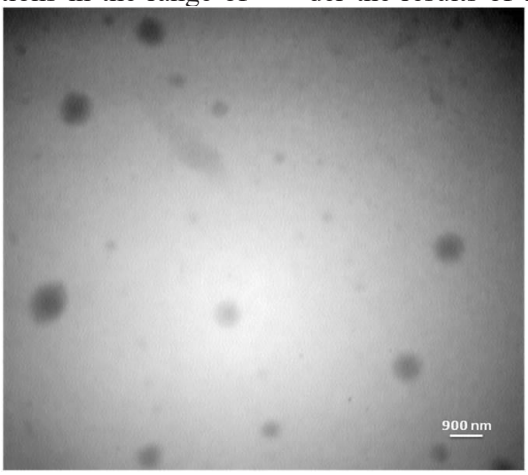

Figure 1. TEM images of behenic acid bioconjugated L-ASNase.

Table 1. The particle size of bioconjugates with or without surfactant, before lyophilization

		Particle size (µm)		
	Conc. mM	Lauric acid bioconjugate	Palmitic acid bioconjugate	Behenic acid bioconjugate
Without surfactant		0.491±0.04	0.671±0.03	0.827±0.07
Brij 35	0.417	2.158±0.23	8.551±0.82	4.350±2.43
Brij 52	0.435	84.82±0.55	14.798±7.66	10.22±0.24
Brij 72	0.5	0.866±0.14	55.861±7.90	5.256±1.91
Brij 92	0.5	2.219±0.93	4.001±0.43	3.806±2.46
Span 20	1.44	0.412±0.01	0.578±0.01	0.559±0.02
Span 40	1.24	0.744±0.03	0.770±0.07	1.684±0.70
Span 60	1.16	1.700±0.69	8.214±0.55	4.471±0.18
Span 83	0.721	0.832±0.06	0.696±0.03	0.497±0.18
Span 85	0.522	0.615±0.02	13.03±0.25	8.212±0.49
Sodium cholate	23.26	0.459±0.06	12.77±0.27	9.545±0.16
Pluronic F-127	0.79	0.502±0.01	13.47±0.38	0.765±0.07
Pluronic F-68	0.8	2.921±0.85	99.12±0.53	3.80±0.28
Tween 20	0.443	0.524±0.06	12.15±2.62	8.595±0.09
Tween 40	0.389	6.857±0.77	8.679±2.53	11.20±8.68
Tween 60	0.381	10.26±0.12	8.593±0.36	78.56±7.97
Tween 80	0.382	0.861±0.52	9.835±1.55	5.97±0.63
Triton X-100	1.6	9.514±6.07	8.589±0.34	6.634±0.74
Sodium lauryl sulphate	3.47	27.25±3.54	10.81±0.38	10.01±0.66
Polyvinyl alcohol	0.037	0.618±0.02	16.21±1.01	13.89±0.18
Soybean Lecithin	0.65	27.66±2.11	5.286±4.48	5.097±0.53
Isopropyl myristate	1.85	1.861±0.65	5.578±1.61	1.316±0.14
Isopropyl myristate/Lecithin	0.925/0.324	0.437±0.03	3.143±0.23	0.314±0.00
Sodium cholate/Lecithin	11/0.324	0.349±0.00	0.752±0.04	0.386±0.03
Pluronic F-127/Lecithin	0.4/0.324	0.685±0.01	0.910±0.04	0.417±0.02
Cetyl pridinium chloride	1.4	7.148±0.21	1.647±0.12	0.694±0.01

demonstrated the nanostructured carrier after bioconjugation.

3.2. Preliminary study for surfactant selection of micellar nanocarrier

According to the change in the molecular structure of L-ASNase and conjugation of the hydrophilic protein with hydrophobic hydrocarbon chains, the new structure has the amphiphilic moieties. Similar works on bioconjugation of macromolecules showed those bioconjugates could create self-assembled supramolecular aggregate in water and reported for protein such as insulin (26), other therapeutic agents like heparin (27, 28) and salmon calcitonin (29). Until recent years micellar nanocarriers have gained immense attraction (30-32). The drug is well protected in a micellar form against possible inactivation under the influence of the biological environment and does not cause undesirable side effects on non-target organs and tissues (33). A variety of agents have identified to protect proteins from aggregating *in vitro*. These agents fall into several categories: amino acids, sugars, polyols, polymers, and surfactants (34). Surfactants are known to stabilize proteins and often employed as additives in protein formulations (35). After that, in this study, surfactants and other agents were considered to be used along with fatty acid bioconjugated L-ASNase to prepare a micellar carrier while preserving enzyme activity in an acceptable range. The particle size and enzyme activity of the micellar nanocarriers of fatty acid

Table 2. The particle size of bioconjugates with or without surfactant, after reconstitution of the lyophilizates.

		Particle size (µm)		
	Conc. mM	Lauric acid bioconjugate	Palmitic acid bioconjugate	Behenic acid bioconjugate
Without surfactant		0.961±0.051	0.790±0.19	0.905±0.062
Brij 35	0.417	1.313±0.242	7.110±0.34	8.142±2.72
Brij 52	0.435	0.780±0.240	2.705±0.06	0.806±0.119
Brij 72	0.5	7.150±0.238	7.118±0.22	6.830±0.92
Brij 92	0.5	0.691±0.230	0.808±0.03	0.392±0.01
Span 20	1.44	0.577±0.241	0.483±0.12	0.404±0.01
Span 40	1.24	6.735±0.937	1.751±0.57	6.751±0.02
Span 60	1.16	0.657±0.028	0.697±0.10	6.039±1.36
Span 83	0.721	0.399±0.040	0.414±0.02	0.418±0.031
Span 85	0.522	1.003±0.010	0.445±0.03	0.878±0.105
Sodium cholate	23.26	0.537±0.199	0.399±0.01	0.603±0.127
Pluronic F-127	0.79	0.397±0.015	0.570±0.16	0.451±0.091
Pluronic F-68	0.8	0.615±0.069	0.696±0.03	5.425±0.43
Tween 20	0.443	1.051±0.107	1.101±0.407	0.712±0.163
Tween 40	0.389	4.981±0.689	6.995±1.99	1.760±0.40
Tween 60	0.381	7.340±1.193	19.994±7.39	7.525±0.42
Tween 80	0.382	2.266±0.097	8.036±1.18	2.228±0.46
Triton X-100	1.6	9.175±0.717	8.277±0.28	2.907±0.40
Sodium lauryl sulphate	3.47	7.644±0.563	14.748±3.96	6.886±1.991
Polyvinyl alcohol	0.037	1.142±0.243	1.024±0.13	1.014±0.278
Soybean Lecithin	0.65	0.429±0.034	0.392±0.02	0.649±0.199
Isopropyl myristate	1.85	1.789±0.814	2.485±0.59	35.031±5.98
Isopropyl myristate/Lecithin	0.925/0.324	0.405±0.010	1.144±0.18	0.501±0.17
Sodium cholate/Lecithin	11/0.324	0.850±0.026	0.528±0.02	0.429±0.02
Pluronic F-127/Lecithin	0.4/0.324	0.411±0.001	0.785±0.128	0.413±0.01
Cetyl pridinium chloride	1.4	2.089±0.282	0.668±0.18	0.576±0.14

bioconjugated L-ASNase summarized in Tables 1 to 3.

Surfactants consist of a hydrophilic (water compatible) head group and a hydrophobic (water-repellent) hydrocarbon moiety (36). Surfactants classified into four main categories depending on the nature of the charge carried by the hydrophilic part of the surfactant: anionic, cationic, nonionic, and ampholytic surfactants (37, 38).

In this part of the study, different groups of the surfactants including nonionic, cationic and anionic ones with a wide range of HLB from 1.8 (i.e., span 85) to 40 (i.e., SDS) and mixtures of them utilized to evaluate their effects on enzymatic activity retention of fatty acid bioconjugated L-ASNase and particle size of the resultant micellar structure. As shown in Tables 1 to 3, we examined the mostly nonionic surfactants.

Generally non-ionic surfactants do not denature or destabilize proteins (39), e.g. apo-α-lactalbumin (40) and cutinase (41). Nonionic surfactants preferred in protein stabilization. Due to the relatively low critical micelle concentrations (CMC) of nonionic surfactants, they can prevent or reduce protein surface adsorption and aggregation at low concentration (37). Researchers suggested stabilization mechanisms of nonionic surfactants are ice crystal size modification during freezing and surface tension reduction (42). Therefore, there are more desirably that the surface-active agent is nonionic (43).

Polyoxyethylene alkyl ethers (Brijs) are

Table 3. Enzyme activity of bioconjugates with or without surfactant, after reconstitution of the lyophilizates.

		Enzyme activity %*		
	Conc. mM	Lauric acid bioconjugate	Palmitic acid bioconjugate	Behenic acid bioconjugate
Without surfactant		80.74±1.70	77.08±2.23	74.30±3.24
Brij 35	0.417	16.67±1.17	36.17±0.73	63.61±0.86
Brij 52	0.435	68.24±2.63	54.82±1.86	63.17±2.15
Brij 72	0.5	82.91±0.86	82.71±1.67	80.26±2.28
Brij 92	0.5	26.39±1.71	49.78±1.10	95.67±2.24
Span 20	1.44	58.02±1.95	65.46±4.09	63.79±1.25
Span 40	1.24	71.32±0.74	74.16±1.45	66.66±0.93
Span 60	1.16	72.26±1.09	80.81±1.67	68.81±1.58
Span 83	0.721	75.86±1.35	86.67±0.95	80.27±2.05
Span 85	0.522	34.06±3.25	59.72±1.95	53.55±7.91
Sodium cholate	23.26	91.19±2.19	93.51±1.53	89.14±2.16
Pluronic F-127	0.79	112.64±4.66	106.34±2.42	107.31±3.48
Pluronic F-68	0.8	58.17±1.84	68.62±1.26	74.00±0.88
Tween 20	0.443	71.03±2.26	73.50±1.82	76.19±7.90
Tween 40	0.389	39.85±2.03	29.05±2.78	37.99±3.84
Tween 60	0.381	7.91±2.99	36.96±2.14	19.18±16.93
Tween 80	0.382	33.90±1.08	4.99±1.90	22.78±3.34
Triton X-100	1.6	40.50±6.07	38.55±2.44	24.05±9.34
Sodium lauryl sulphate	3.47	0.00±0.00	0.00±0.00	0.88±0.58
Polyvinyl alcohol	0.037	88.58±2.63	89.16±2.70	93.61±4.09
Soybean Lecithin	0.65	67.68±5.63	64.25±4.06	69.23±2.74
Isopropyl myristate	1.85	59.73±1.37	54.69±1.20	57.35±2.28
Isopropyl myristate/Lecithin	0.925/0.324	66.63±3.83	59.60±1.26	62.18±1.56
Sodium cholate/Lecithin	11/0.324	8.10±1.54	0.00±0.00	0.00±0.00
Pluronic F-127/Lecithin	0.4/0.324	1.93±1.08	0.55±1.31	0.00±0.00
Cetyl pridinium chloride	1.4	0.00±0.00	0.00±0.00	2.90±1.61

*Enzyme activity %= (activity of modified enzyme /activity of native enzyme) ×100

one of the nonionic surfactant groups (44). It reported that Brij 35 and Brij 30 protected lactate dehydroganase (LDH) from freezing denaturation to various degrees (45, 46). The stability of recombinant human growth hormone (rhGH) has been also investigated by the influence of surface-active agents such as Brij (47, 48). Herein, the enzymatic activity of the fatty acid bioconjugated L-ASNase with Brijs was about 16.67% to 95.67%, while the particle sizes varied from 0.392 to 8.142 µm (see Tables 1-3).

The second group of nonionic surfactant that used in this experiment is sorbitan fatty acid esters (Spans) (44). As reported, Span 20, 60, 40, and 80 were used with insulin to prepare nonionic surfactant vesicles (49). In this study, the particle size of the micellar structures had ranging from 0.399 to 6.751 µm and enzyme activity in the range from 34.06% to 86.67%. Within this group, Sorbitan sesquioleate (Span 83) presented small particle size, good cryoprotective effect (Tables 1 and 2), and high enzyme activity (Table 3).

Polyester oxyethylene sorbitan (Tweens) fatty acid esters are another group of surfactants that are widely used along with proteins, and we also try to investigate their effect on fatty acid bioconjugated L-ASNase (44). Several proteins protected from freezing denaturation with Tween 80 at concentrations of 0.005 to 0.01% such as LDH, tumor necrosis factor binding protein (TNFbp),

Table 4. The *in vitro* half-lives of the native and modified L-ASNase in PBS (0.150 mM, pH 7.4) and human plasma. The highest activity of native and modified L-ASNase has been set as 100%.

L-ASNase/	Human Plasma		PBS	
L-ASNase modified	t ½ (h)	K (h^{-1})	t ½ (h)	K (h^{-1})
Native L-ASNase	19.14±1.71	0.036±0.003	22.12±4.76	0.032±0.008
L-ASNase-Lauric acid bioconjugate	28.42±9.62	0.026±0.008	23.25±6.64	0.032±0.011
L-ASNase-Palmitic acid bioconjugate	40.65±12.63	0.018±0.005	20.40±3.17	0.035±0.006
L-ASNase-Behenic acid bioconjugate	47.76±8.22	0.015±0.003	27.42±3.89	0.026±0.003
L-ASNase-Lauric acid bioconjugate/Span 83	13.26±2.11	0.053±0.008	20.46±2.25	0.034±0.004
L-ASNase-Palmitic acid bioconjugate/Span 83	8.18±1.31	0.086±0.013	13.82±2.49	0.051±0.009
L-ASNase-Behenic acid bioconjugate/Span 83	15.02±0.85	0.046±0.003	12.68±0.68	0.055±0.003
L-ASNase-Lauric acid bioconjugate/Sodium cholate	10.54±0.96	0.066±0.006	10.56±0.76	0.066±0.005
L-ASNase-Palmitic acid bioconjugate/Sodium cholate	9.97±1.35	0.070±0.009	11.66±1.58	0.060±0.009
L-ASNase-Behenic acid bioconjugate/Sodium cholate	11.23±1.35	0.062±0.008	9.54±0.20	0.073±0.002
L-ASNase-Lauric acid bioconjugate/Pluronic F-127	48.26±3.14	0.014±0.001	20.41±2.15	0.034±0.004
L-ASNase-Palmitic acid bioconjugate/Pluronic F-127	49.81±11.52	0.014±0.004	40.55±10.07	0.018±0.004
L-ASNase-Behenic acid bioconjugate/Pluronic F-127	60.00±16.86	0.012±0.004	44.90±2.59	0.015±0.001

interleukin-1a (IL-1a), primary fibroblast growth factor (bFGF), malate dehydrogenase (MDH), aldolase, and phosphofructokinase (PFK) (42, 50). The particle size of fatty acid bioconjugated L-ASNase with Tweens were higher than 0.712 µm and the enzyme activity was in the range of 4.99% to 76.19% (Table 1 and 2). The enzyme activities decreased, and particle sizes were large, with Tweens (Table 3).

Other agents used with fatty acid bioconjugated L-ASNase include fatty acid ester (e.g., Isopropyl myristate), phospholipids (e.g., soybean lecithin) and bile salt (e.g., sodium cholate) (51, 52). Among these groups, sodium cholate also presents small particle size, and fatty acid bioconjugated L-ASNase had accepted enzyme activity in the presence of sodium cholate.

There were several reports that some anionic and cationic surfactants have a destructive effect on protein (53). In this regard, we examined sodium lauryl sulfate (as an anionic surfactant) and cetyl pyridinium chloride (as a cationic surfactant) to know whether they have destructive effects on L-ASNase or not. The enzyme activity in this condition was decreased significantly.

The poloxamer polyols (pluronics®) are a series of closely related block copolymers of ethylene oxide and propylene oxide. It investigated that pluronic® F-127 reduces the tendency of peptide unfolding relative to their low CMC and lack of binding (54). Furthermore, in the presence of pluronic F-127, the three-dimensional structure of proteins is better preserved (54). Moreover, the enzymatic activity of both a model enzyme (urease) and a model recombinant-derived protein (interleukin-2) not reduced, and secondary structures of both were also preserved when each protein incubated with pluronic F-127 (43, 55). Pluronic F-127 with fatty acid bioconjugated L-ASNase shows the unexpected result on small particle size and increasing the enzyme activity more than the related bioconjugates. It may propose that along with micellization, this surfactant helps the active site of the enzyme to be more accessible for its substrate. Pluronic F-68 is another nonionic surfactant from this group, which utilized to control shear forces in suspension cultures (44, 56). Pluronic F-68 could induce a small particle size in combination with fatty acid bioconjugated L-ASNase, while the enzyme activity decreased to 58% (Tables 2 and 3).

As reported in the literature, nonionic surfactants, in combination with lecithin, presents small particle size and excellent stability for proteins (57, 58). Thus, we try to investigate the effect of the mixtures in this study with lecithin along with fatty acid bioconjugated L-ASNase. The enzyme activity and particle size were not in an acceptable range when bioconjugates mixed with

Table 5. The effect of freeze-drying on the enzyme activity of the native L-ASNase and the micellar nanocarriers in comparison with relevant fatty acid bioconjugated L-ASNase.

L-ASNase/ L-ASNase modified	Conjugation degree %	Enzyme activity %before lyophilization	Enzyme activity %after lyophilization	Enzyme activity recovery** %	Enzyme activity recovery*** % (Compared to the control)	Enzyme activity recovery*** % (compared to the native form)	Enzyme activity recovery*** % (compared to the related fatty acid bioconjugate)
Control*		74.19±4.19	69.49±1.55	93.79±3.59		71.65±1.82	
L-ASNase -Lauric acid bioconjugate	52.3±1.97	78.92±3.16	78.92±2.93	100.08±4.58	111.01±4.13	81.43±4.62	
L-ASNase-Palmitic acid bioconjugate	54.4±2.10	81.96±2.25	76.83±1.04	93.79±2.66	108.07±1.46	79.22±1.32	
L-ASNase-Behenic acid bioconjugate	52.2±2.34	77.02±3.66	74.96±2.04	97.40±2.30	105.44±2.87	77.31±2.84	
L-ASNase-Lauric acid bioconjugate/ pluronic F-127(0.79 mM)		80.17±2.79	79.71±2.95	99.42±0.27	114.81±6.80	82.24±4.62	101.00±0.11
L-ASNase-Palmitic acid bioconjugate/ pluronic F-127 (0.79 mM)		82.13±1.25	81.21±1.06	98.88±0.63	116.92±3.80	83.78±3.60	105.72±2.79
L-ASNase-Behenic acid bioconjugate/ pluronic F-127 (0.79 mM)		92.96±0.72	92.39±1.21	99.38±0.57	132.98±2.65	95.26±1.63	123.30±3.63

* Control sample is the native enzyme under the reaction condition without fatty acid.

** Enzyme activity recovery= (Activity after lyophilization/Activity before lyophilization)×100

*** Enzyme activity recovery=(Activity after lyophilization/Activity After lyophilization)×100

lecithin mixtures.

Alkylphenol hydroxypolyethylene (Triton X-100) (45, 46) is another nonionic surfactant. It has a particle size of more than 2 µm and enzyme activity about 30% with fatty acid bioconjugated L-ASNase.

Polyvinyl alcohol (PVA) is a water-soluble polymer used as a polymeric surfactant, and its effect on particle size reduction was investigated (59, 60). Fatty acid bioconjugated L-ASNase presents a large particle size (more than 1 µm) but acceptable enzyme activity in the presence of PVA.

Among these kinds of surfactants, pluronic F-127 (HLB value of 18-23), sodium cholate (HLB value of 18), and Span 83 (HLB=3.7) presented the smaller particle size and higher enzyme activity than other agents through the procedure. Sodium cholate, with a reduction in interfacial tension, decrease the particle size and by forming a steric barrier on the particle surface, protects the particles from coagulation and thus, make the nanoparticles more stable (61). Also, it investigated that pluronic F-127 reduces the tendency of peptide unfolding relative to their low CMC. On the other hand, Span 83 has recently identified as an essential allergen (62, 63). Therefore, in a subsequent experiment, the effect of these three surfactants on *in vitro* half-life in PBS and Human plasma was investigated.

3.3. Determination of time-activity profile in PBS and human plasma

To obtain more clinical effectiveness for L-ASNase, a longer in vitro half-life is desirable by maintaining higher activity in human plasma. The first-order model was used to determine the in vitro half-life in PBS and human plasma for native L-ASNase, fatty acid bioconjugates, and mi-

cellar nanocarriers (Table 4). The half-life of the native L-ASNase was 22.12±4.76 and 19.14±1.71 hrs in PBS and human plasma, respectively. 68.97±6.27% of enzyme activity reduced after 10 hrs of native enzyme incubation in human plasma while the micellar nanocarrier with pluronic F-127 retained its activity for 96 hr incubated in the same manner.

The presence of nonspecific antibodies or proteases in human plasma may explain this fact. These results demonstrate that the human plasma already contains natural antibodies and proteases capable of inactivating L-ASNase. The fatty acid bioconjugated L-ASNase pluronic F-127, sodium cholate, and span 83 mixed to the same human plasma samples to verify if the enzyme-modified with fatty acid and surfactant results in more excellent enzyme stability. The modified enzyme retained its activity for 96 hr when it was incubated in the same manner as the physiological solution at 37 °C. These results suggest that the modification with fatty acid chain and pluronic F-127 protects L-ASNase by a steric hindrance to the binding of non-specific antibodies or at enzymatic hydrolysis caused by proteases in human plasma (16, 64, 65). Optimized formulation (behenic acid bioconjugated L-ASNase/pluronic F-127) because of the sterical stabilization effect of pluronic F-127 (54), can circulate in the blood for more extended periods (2.1 fold greater half-life) in comparison to the native enzyme. According to the results of the experiments mentioned above, this modification with pluronic F-127 is capable of extending biological half-life and improves therapeutic properties (66-68).

3.4. Lyophilization of the native and modified L-ASNase

As shown in Table 5, the fatty acid bioconjugated L-ASNase could maintain their activity during lyophilization better than the control samples (i.e., native enzyme). Lipid modification of protein plays an essential role in stabilizing the proteins and hindering the protein degradation (69, 70). Higher activity value after lyophilization for bioconjugate forms could result from this effect of lipidation. Superior to the impact of lipid modification in enzyme stabilization during freeze-drying the cryoprotective influence of pluronic F-127, which are supposed to be used in the following step, is also considered. Protein stabilizers can also stabilize proteins during freezing. Several stabilization mechanisms postulated for surfactants, including modification of the size of ice crystals, reduction of surface tension, and restriction of diffusion of reacting molecules. It seems that the decrease in surface tension is the most mechanism of protein stabilization during freezing (42).

Surfactants can have potent effects on both the stabilities and activities of enzymes (71). It also shows that fatty acid bioconjugated L-ASNase/pluronic F-127 could also retain enzyme activity after lyophilization better than the control and even related fatty acid bioconjugated L-ASNase (Table 5).

4. Conclusion

Today, micellar nanocarrier owing to their properties such as smaller particle size, higher stability, and drug loading efficiency superior to those of the mono-micellar system, emulsion, and nanoparticles have gained immense attraction. In this study, the effect of different kinds of surfactants on particle size and enzyme activity of fatty acid bioconjugated L-ASNase was evaluated. Although sodium cholate and span 83 with fatty acid bioconjugates present a smaller particle size and higher enzyme activity, *in vitro* half-life in PBS and plasma were not in acceptable value. While pluronic F-127 has the longer in vitro half-life compares to the native and bioconjugated enzyme, and also has a better recovery after lyophilization. After that, modification of L-ASNase with fatty acid and surfactants also indicates a promising stabilized product for in vivo experiments. These studies explain the new delivery candidate for the medical purposes of L-ASNase in the future.

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Conflict of Interest

None declared.

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