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Historical Perspective

L-Asparaginase delivery systems targeted to minimize its side-effects

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ABSTRACT

L-asparaginase (L-ASP) is one of the key enzymes used in therapeutic applications, particularly to treat Acute Lymphocytic Leukemia (ALL). L-asparagine is a non-essential amino acid, which means that it can be synthesized by the body and is not required to be obtained through the diet. The synthesis of L-asparagine occurs primarily in the liver, but it also takes place in other tissues throughout the body. In contrast, leukemic cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase and should obtain it from circulating sources for protein synthesis and cell division processes to ensure their vital functions. L-ASP catalyzes the deamination process of L-asparagine amino-acid into aspartic acid and ammonia, depriving leukemic cells of asparagine. This leads to decreased protein synthesis and cell division in tumor cells. However, using L-ASP has side effects, such as hypersensitivity or allergic reaction, antigenicity, short half-life, temporary blood clearance, and toxicity. L-ASP immobilization can minimize the side effects of L-ASP by stopping the immune system from attacking non-human enzymes and improving the enzyme's performance. The first strategy includes modification of enzyme structure, such as covalent binding (conjugation), adsorption to the support material and cross-linking of the enzyme. The chemical modification of residues, often nonspecific, changes the enzyme's hydrophobicity and surface charge, lowering the enzyme's activity. Also, the first strategy exposes the enzyme's surface to the environment. This eliminates its performance and does not allow targeted delivery of the enzyme. The second strategy is based on the entrapment of the enzyme inside the protecting structure or encapsulation. This strategy offers the same benefits as the first. Still, it also enables reducing toxicity, prolonging *in vivo* half-life, enhancing stability and activity, enables a targeted delivery and controlled release of the enzyme. Compared to the first strategy, encapsulation does not modify the chemical structure of the enzyme since L-ASP is only effective against leukemia in its native tetrameric form. This review aims to present state of the art in L-ASP formulations developed for reducing the side effects of L-ASP, focusing on describing improvements in their safety. The primary focus in the field remains to be improving the overall performance of the L-ASP formulations. Almost all encapsulation systems allow reducing immune response due to screening the enzyme from antibodies and prolonging its half-life. However, the enzyme's activity and stability depend on the encapsulation system type. Therefore, the selection of the right encapsulation system is crucial in therapy due to its effect on the performance parameters of the L-ASP. Biodegradable and biocompatible materials, such as chitosan, alginate and liposomes, mainly attract the researcher's interest in enzyme encapsulation. The research trends are also moving towards developing formulations with targeted delivery and increased selectivity.

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1. Introduction

L-asparaginase (L-ASP) is one of the key enzymes used in therapeutic applications, mainly to treat Acute Lymphocytic Leukemia (ALL) and Lymphosarcoma cancer. In food industries, especially for the reduction of acrylamide (carcinogen) from starch-rich foods which are cooked at temperatures above 90°C [1,2]. It is produced by cells as protein to catalyze the deamination process of L-asparagine amino acid into aspartic acid and ammonia [3,4].

It has been almost 70 years since the L-ASP is known for anti-tumor activity, particularly ALL. In 1953, L-ASP was first reported that the guinea pig serum had activity against rat lymphoma [4]. Later, in 1961, Broome identified that the anti-tumor activity of guinea pig serum was due to the presence of L-ASP [5].

The differences in metabolism between healthy cells and tumor cells explain the mode of action of L-ASP. Healthy cells can independently synthesize many amino acids, including asparagine, while leukemic cells cannot synthesize asparagine, but they need a large amount for protein synthesis and cell division processes to ensure their vital functions. L-Asparaginase, an amidohydrolase, catalyzes the breakdown of L-asparagine into aspartic acid and ammonia, thereby depriving leukemic cells of asparagine (Fig. 1). This leads to decreased protein synthesis and cell division in tumor cells [6,7].

So far, various organisms, including bacteria, fungi, actinomycetes, plants, microalgae, and animals, have been used to extract L-ASP [8]. As the L-ASP gene is present in all types of life, researchers have been continuously screening for L-ASP with superior features [9,10].

Recently Sobat et al. [11] used next-generation sequence and in-silico screening for L-ASP with anti-leukemia properties from the Caspian Sea using 27,000 publicly available prokaryote genomes. The most commonly used bacterial sources include *Erwinia caratovora, Pseudomonas stutzeri, Bacillus sp, Corynebacterium glutamicum* and *E. coli etc.* However, only *Erwinia* and *E. coli* were used on a manufacturing scale. For example, Elspar®, isolated from *E.Coli* was the first L-ASP available on the market since 1978 and was approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with acute lymphoblastic leukemia (ALL) [3].

Enzymes as therapeutic agents for cancer treatment have a low risk of lysine resistance due to selective interactions with cell wall components like enzymes acting on bacterial cell wall peptidoglycan and therefore unlikely to affect mammalian cells negatively [12].

However, using L-ASP has side effects, such as hypersensitivity or allergic reaction, antigenicity, temporary blood clearance, and toxicity [13]. The main limitation of L-ASP from *E. coli* is hypersensitivity, which is reported in 15 to 73% of cases [14]. Additional side effects, such as hepatotoxicity, thrombosis, hyperglycemia, pancreatitis, and abnormalities of lipid metabolism, are commonly linked with using L-ASP [15]. Furthermore, the activity of most of the L-ASPs is limited by high temperatures, narrow pH range, and by its short half-life [16].

Therefore, there is a trend in minimizing or circumventing its side effects and improving the performance parameters of the native L-ASP, such as selectivity, stability against degradation, shelf-life and affinity [7,17]. The enrichment of L-ASP and their therapeutic properties can be achieved by various immobilization techniques within several supports with consideration of quality aspects of the product [18]. Enzyme immobilization can improve the L-ASP activity, stability, and catalytic half-life, allow their reusing at a broad range of temperatures and pH, and improve enzyme pharmacokinetics with minimum side effects [19,20].

To date, five types of L-ASP immobilization techniques are used to stop the immune system from attacking nonhuman enzymes and improve the performance of the enzyme [18,21–23]. Generally, they can be divided into two strategies [24]. The first strategy is based on the chemical or physical modification of the enzyme molecule. It includes the technique of covalent binding (conjugation) with polymers, such as polyethylene glycol (PEG) [25–27]; physical adsorption to the support material *via* van der Waals forces, ionic interactions and hydrogen bonds [28]; and cross-linking of enzyme molecules [29,30], which often improves its stability [31].

For example, the Michaelis-Menten constant (Km) for immobilized L-ASP was 1.6-fold lower than the free form, indicating a higher affinity for the asparagine substrate. In addition, immobilized L-ASP exhibited superior stability against elevated temperature, freeze-thaw cycles, and proteolysis, yielding a novel stabilized preparation with promising biomedical applications [25]. For example, PEG-L-ASP was approved for the treatment of acute lymphoblastic leukemia (ALL) patients allergic to L-ASP by the US Food and Drug Administration (FDA) in 1994, and it has been used as the first-line treatment for adult and pediatric ALL since 2006 [32]. Additionally, in 2019 Young et al. [26] based on wide pieces of evidence from literature reported that intramuscular (IM) or intravenous (IV) administration of PEG L-ASP is an effective first-line treatment for pediatric and adult patients with ALL those who are hypersensitive to *E. coli* L-ASP.

In a recent systematic review, Camile et al. 2020 [27] compared the efficacy and safety of PEG-L-ASP with native *E. coli* L-ASP for the treatment of ALL in both children and adults using the wide range of literature data (PubMed, Web of Science, Science Direct, Cochrane Library, Scopus, LILACS (Latin American and Caribbean Health Sciences Literature) and EMBASE databases). They used the Cochrane recommendation tool and the GRADE system and their results show that evidence is inadequate to evaluate the effects of PEG-L-asp due to the limited number and power of studies and essential flaws in their design or conduct in classifying PEG-L-ASP as a superior drug or not, in the therapy of ALL.

Nevertheless, some side effects of PEG have been identified, such as the presence of PEG antibodies in the blood due to immune response [33]. In addition, PEGylated L-ASP, which has been approved for treating Acute Lymphoblastic Leukemia (ALL), can give allergic reactions, and reports hypersensitivity reactions to Oncaspar® have also been attributed to anti-PEG antibodies that develop CARPA [34].

Also, the chemical modification of residues, often nonspecific, changes the enzyme's hydrophobicity and surface charge, lowering the enzyme's activity [35]. In this strategy, the enzyme's surface is exposed to the environment. This eliminates its performance and does not allow targeted delivery of the enzyme.

The second strategy is based on the entrapment of the enzyme inside the protecting structure or encapsulation [22]. This strategy offers the same benefits as the first but also enables a targeted delivery and

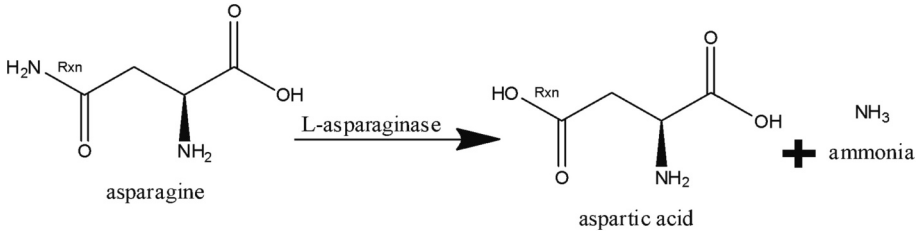


Fig. 1. Hydrolysis of asparagine into aspartic acid and ammonia by L-ASP.

controlled release of the enzyme [36]. It also resulted in a longer circulation half-life, reducing toxicity and antigenicity, enhancing stability, activity, *etc.* Furthermore, compared to the first strategy, encapsulation does not modify the chemical structure of the enzyme, since L-ASP is only effective against leukemia in its native tetrameric form and very significantly doesn't disrupt the L-ASP conformation [37].

Among other techniques, an encapsulation technique is one of the easiest to implement and shows high enzyme activity at a low cost of immobilization [21]. Encapsulation can be commonly implemented *via* three basic procedures: solvent extraction/evaporation, phase separation (coacervation) and spray-drying [38–40]. Numerous encapsulating systems include polymers, polyelectrolytes, solid inorganic nanoparticles, liposomes, phytosomes *etc.* (Fig. 2) [41]. However, it is worth noting that not many reports are available on L-ASP immobilization. (Tables 1–11.)

During immobilization, the support materials can change the enzyme properties; therefore the activity of immobilized L-ASP should be thoroughly studied and evaluated before therapeutic use to know that the steric availability of the enzyme binding site did not change after immobilization [43]. Hence, the following section focuses on the encapsulation systems used for L-ASP delivery to improve L-ASP performance and reduce its side effects.

This review will mainly discuss the second approach for delivering L-ASP, including encapsulation and entrapment techniques. Both these techniques will be generally called encapsulation. The study of encapsulation of asparaginase in different protective structures, its effect on release kinetics and the enzyme activity is of great interest to researchers since this approach brings crucial benefits for asparaginase performance.

2. L-ASP encapsulation systems

We present the classification of the L-ASP encapsulation system based on the different materials used for its incorporation and delivery. This includes polymers, solid lipid nanoparticles, liposomes and red blood cells. In the Section 2.1 describing polymer based systems we included different polymeric structures such as polymersomes, gels, polyelecrolyte multilayers and complexes, as well as systems based on natural polymers such as chitosan and alginate. The information about methods was described in the Section 2, however our review mainly devoted to materials and structures used for L-ASP incorporation and delivery. The side effects that were minimized were described in the text of this section as well.

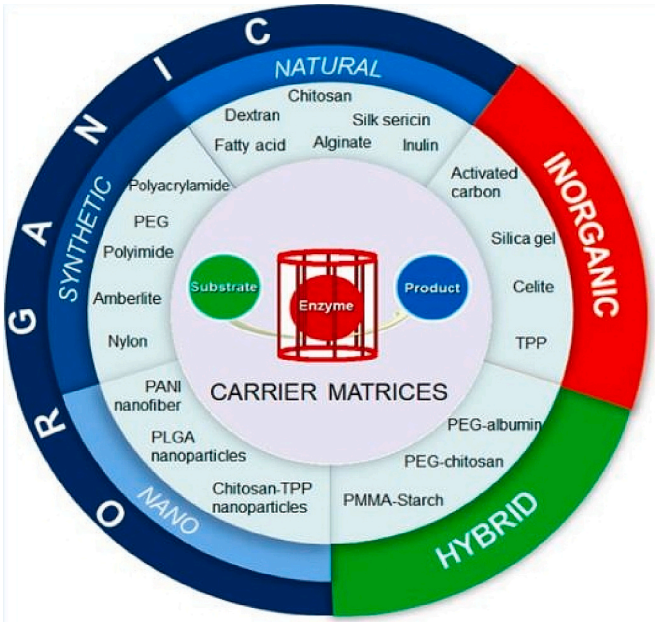


Fig. 2. The chemical/physical methods for the immobilization of L-ASP onto the natural or synthetic carriers [42].
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2.1. L-ASP encapsulation in (or via) polymeric systems

One of the first materials used for studying the delivery of L-ASP is biodegradable polymers. Polymers are widely studied for human drug delivery due to their biodegradability and biocompatibility. To prolong the half-life of the L-ASP, reduce immune reaction and improve other properties of polymers, such as polyacrylamide [44], poly(lactide-co-glycolic) acid (PLGA) [45–48], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) [49,50], pillar[5]arene-based amphiphiles [51], polyaniline [52], were utilized.

Edman and his colleagues were among the first to propose using microparticles (1 μm) of polyacrylamide as carriers for immobilising 1:1 L-asparaginase-L-glutaminase [44]. The immobilized enzyme possessed higher stability than the free enzyme when stored at 4°C, improving the enzyme's biological half-life. However, the *in vivo* studies revealed that whereas enzymes immobilized in microspheres significantly decreased L-asparagine and L-glutamine levels for up to 5 days, soluble L-asparaginase-L-glutaminase administered intraperitoneally to rats had no effect on the amino acids measured. The authors attributed the reason for the ineffectiveness of the free enzyme *in vivo* to its instability in biological fluids and to the fact that the enzyme is rapidly removed from circulation [44].

Later Gaspar et al. [45] proposed L-ASP encapsulation into poly (lactide-*co*-glycolide) (PLG) nanospheres using a water-in-oil-in-water solvent evaporation technique. Since it has a long history of medical usage and is biocompatible, biodegradable, and has a wide variety of degradation rates, poly(lactic-*co*-glycolic acid) (PLGA) is widely utilized for encapsulation.

Authors [45] showed that several factors, such as PLG molecular weight and the structure of the PLG chain, affect the nanoparticles' physicochemical and *in vitro* release properties. Notably, it was determined that PLG molecular weight influences the size distribution, encapsulation efficiency (EE) and *in vitro* release properties. Regarding particle size, it's essential to mention that while nanoparticles can also be injected intravenously, micron-sized particles can only be injected subcutaneously and intramuscularly. The increase in the PLG molecular weight led to the larger size of nanoparticles, a higher loading capacity and a slower release rate. The presence of free carboxyl-end groups in the PLG chain significantly affects the entrapment and release of L-ASP from PLG nanoparticles (high protein loading (4.86%, w/w), delivery of the active enzyme for 20 days, lower enzyme loading (2.65%, w/v) and no active enzyme in the release medium after a 14-day incubation [45].

Authors PEG-ylated L-ASP before encapsulation to overcome the denaturation and formation of insoluble aggregates and to protect the L-ASP enzyme [46]. Double emulsion solvent evaporation techniques were used to obtain L-ASP-loaded PLG nanocapsules. After PEG-ylation, around 63% of the specific activity was still present, and 82.7% was also recovered from the particles. The release profile for the native enzyme was about 67% and for conjugated was about 44% in 28 days. The loading amount for native L-ASP was around 0.9%, while for PEG-ylated L-ASP of 0.8%, and encapsulation efficiency for native L-ASP was 78% (w/w) and for PEGylated, L-ASP was 65% (w/w). Authors suggested that the conjugated protein's increased molecular weight has led to a decrease in release rate.

Authors [43] compared the impact of biocompatible synthetic polymers, such as methoxy poly (ethylene glycol) (MPEG) and poly (lactic-co-glycolic acid) (PLGA), on the structure and dynamics of L-asparaginase (EcAII) using molecular dynamics (MD) simulations. The results showed that the protein encapsulated in PLGA was more stable

Table 1
The encapsulation performance, biochemical indicators and pharmacokinetics of polymer based the L-ASP encapsulation systems and their reduced side effects

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP L-ASP source *Acinetobacter glutaminasificans*	Acrylamide microparticles (Emulsion polymerization method)	L-ASP and L-glutaminase 1: 1	Acrylamide	~1 μm	–	–	–	At 4°C >10 weeks	Free L-ASP given intraperitoneally to rats didn't effect on the amino acids; Immobilized (1000 units/kg) –reduction of L-asparagine for up to 5 days.	–	–	–		Enhanced stability, reduced toxicity	[44]
L-ASP	Polymer nanoparticles (w/o/w double emulsion solvent evaporation method)	L-ASP aqueous solution	Poly (lactide-*co*-glycolide)	Adjustable between 196 and 226 nm	40.3 ± 2.9% (Resomer RG 503H (mol. Weight 34,000)), 21.4 ± 3.4% (Resomer RG 503 (mol. Weight 34,000)), 15.2 ± 0.9% Resomer RG 502 (mol. Weight 12,000)	4.86 ± 0.23% (Resomer RG 503H), 2.65 ± 0.09% (Resomer RG 503), 1.82 ± 0.05% (Resomer RG 502)	Up to 81,9% (Resomer RG 502) and 46.3% (Resomer RG 503) after14 days of incubation	Up to 7 days (released Resomer RG 502); the free enzyme was not stable longer than 3 days	–	–	–	–	–	Delivery of L-ASP for an extended period of time	[45]
L-ASP source *Escherichia coli*	PLG nanospheres (w/o/w double emulsion solvent evaporation technique)	L-ASP	PLGA, Pluronic F68	Adjustable 340–420 nm	Adjustable from 26.4 to 79.2%	Adjustable 0.13–0.45%	24% after 1 h of end-over-end rotation at 37°C; 19.5% after 20 h of incubation at 42°C.	In aqueous solution for 24 h at pH 8.0: in case of addition of PEG 400–70%, carboxymethylcellulose - 81%, Pluronic F68–100%	–	–	–	–	–	Improved stability of L-ASP	[54]
PEG-L-ASP	PLG nanoparticles (w/o/w double emulsion solvent evaporation technique)	L-ASP aqueous solution	PLG	150–300 nm	77.88 ± 2.01% (native L-ASP); 65.1 ± 1.47% (PEG-L-ASP) .	0.932 ± 0.04% (native L-ASP); 0.802 ± 0.02% (PEG-L-ASP)	66.66% (native L-ASP) and 44.45% (PEG-L-ASP) in 28 days	0.87 ± 0.1 units/mg (free PEG-L-ASP) and 0.72 ± 0.03 units/mg (after release of PEG-L-ASP); 1.4 ± 0.2 units/mg (free L-ASP) and 0.13 ± 0.01 units/mg (after release L-ASP)	–	–	–	–	–	The enhanced stability of the enzyme and protection against denaturation during encapsulation	[46]
L-ASP, PEG-L-ASP L-ASP source *Escherichia coli*	PHBV nanocapsules (w/o/w double emulsion-solvent	L-ASP aqueous solution	PHBV	Adjustable from 196 ± 12 nm to 509 ± 13 nm	L-ASP 23.7% Isoelectric ASP 28.0% PEG-ASP 27.9%	–	–	–	–	–	–	–	–	Improved the encapsulation efficiency	[49]

(*continued on next page*)

Table 1 (*continued*)

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
	evaporation technique)														
L-ASP, PEG-ASP, 2–4 bis(o-methoxy-plyethylene glycol) 6-chloro-*s*-triazine) (PEG_2-L-ASP) L-ASP source *Escherichia coli*	PHBV nanocapsules (w/o/w double emulsion-solvent evaporation technique)	L-ASP aqueous solution	PHBV conjugates with heparin	200 nm	28.0% (imm. L-ASP), 27.9% (imm. PEG_2-L-ASP)	–	–	–	4 h (L-ASP in PHBV); 4,5 h (PEG-L-ASP in PHBV); 6 h (L-ASP in heparin-PHBV)	–	0.276 U/mg (encapsulated L-asp) and 0.429 U/mg (encapsulated PEG_2-L-ASP)	–	Free L-ASP 2 of 3 mice had anaphylaxis and died within 30 min of injection; No adverse effect for L-ASP in PHBV	Improved EE, activity, decreased immunogenicity and toxicity, increased it's *in vivo* half-life in mice	[50]
L-ASP Source *Bacillus circulans*	Polyaniline nanofibers (stirring)	L-ASP	Polyaniline	–	–	48% at pH 7.0	–	–	–	1.809 mM L^{-1} (imm. ASP), 3.705 mM L^{-1} (free ASP)	90.57 μM/ min mg^{-1} (immobilzed L-ASP); 48.04 μM/ min mg^{-1} (free L-ASP)	–	–	Temperature and pH stability	[52]

"-" not provided.

Table 2
The encapsulation performance, biochemical indicators and pharmacokinetics of the L-ASP encapsulation systems based on polymersomes and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP Source *Escherichia coli*	Nanocapsules (Polymerization-Induced Self-Assembly)	L-ASP	PEG shell and PHPMA membrane	340 ± 3 nm	–	9%	–	Normalised activity after 18 h was >0.9 (1 max). Free ASP was 0.	–	–	–	Reduction in anti-proliferation activity for ASP-loaded vesicles (58%), and free ASP (78%),	–	Reduced antibody binding and proteolytic susceptibility	[55]
L-ASP	Asymmetric polymersomes (film hydration method)	L-ASP	A mixture of the copolymers PMPC-PDPA and PEO-PBO	104 to 120 nm	Adjustable from 6.6 to 10%	Adjustable from 252 to 845 molecules per particle	–	–	–	Adjustable from 3 to 16 mM	–	–	–	High loading capacity	[57]
L-ASP	Polymersomes (thin film hydration method)	L-ASP	PEG-PCL	200–400 nm	~20–25%	–	The release was not detected over 10 h at pH 7.4	–	–	–	–	–	–	The potential targeted delivery	[58]

"-" Not provided.

Table 3
The encapsulation performance, biochemical indicators and pharmacokinetics of the polyelectrolyte-based L-ASP encapsulation systems and their reduced side effects

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP Source *Saccharomyces cerevisiae*	Microcapsules (layer-by-layer technique)	$CaCO_3$	Two or three bi-layers of poly dextran/poly-L-arginine	1–2 μm	–	–	–	Storage stability – 50% of encapsulated L-ASP and 0% of free L-ASP remained after 12 weeks at 4°C	7 h	–	–	51% MOLT-4 cells and 30% SD1 cells survived for encapsulated L-ASP; for free enzyme 62% MOLT-4 and 32% SD1 cells survived	–	Resistance to proteolysis, thermal stability, storage stability	[62]
PEG-L-ASP L-ASP source *Escherichia Coli*	Nanocapsules	Polyphosphazene polyelectrolyte complex	PEG	20–100 nm	–	–	–	2.5-fold increase in thermal stability and a 2-fold increase in proteolytic resistance	~150 min compared to 10 min for native L-ASP	–	–	–		Resistance to proteolysis, thermal stability, reduced protein antigenicity	[63]

"-" not provided.

Table 4
The encapsulation performance, biochemical indicators and pharmacokinetics of the chitosan based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP	Nanoparticles (ionotropic gelation method)	L-ASP in the chitosan solution	Chitosan, Tripolyphosphate	340 ± 12 nm	76.2%	47.6%	At pH 7.0–70% after 360 h; at pH 7.4–82% after 168 h	Max. activity was at pH 8–9 for free L-ASP, pH 9.0 for imm. L-ASP (after 24-h incubation at 4°C); Both free and imm. ASP was active in temp. range of 37°C to 80°C	33 h (free L-ASP), 6.4 days (imm.L-ASP) in (PBS with 5% glycerol); 26 h (free ASP), 23 days (imm. ASP) (in double distil.water in the 5% glycerol)	–	–	–	–	Increased *in vitro* half-life, encapsulation efficiency, pH and thermal stability	[66]
L-ASP Source *Purpureocillium lilacinum*	Nanoparticles (ionic gelation method)	L-ASP in the chitosan solution	TPP/Chitosan in ratio 1:3		72%	53%	50% of drugs are releases within the first 24 h	The free enzyme retained 40%, imm. ASP retained 60% of the activity (after 60 min at 60°C)	–	–	–	The MICs of free L-ASP range from 21.06 to 84.25 mg/mL, and the MICs of imm.L-ASP reduces to 5.26–21.06 mg/mL	–	Improved stability and enhanced antimicrobial properties	[65]
L-ASP Source *Escherichia coli*	Nanovesicles (modified reverse-phase evaporation method)	L-ASP	Chitosan / soybean phospho- lipid and cholesterol	426 ± 36 nm	66,47 ± 2,81%	–	–	At 4°C after 28 days: 60% for imm.ASP, and 30% for free ASP; At 37°C: imm. ASP 60% after 5 days, and no activity for free ASP after 48 h; At 55°C 60% after 4 h for imm.ASP, and no activity after 3 h for free ASP	–	–	–	Imm.L-ASP 80%, free L-ASP 60% (inhibition rate of lung cancer H446 cells)	–	Enhanced catalytic activity, increased stability *in vitro* and anti-cancer effects on H446 cells	[67]
L-ASP	Nanocapsules (electrostatic complexation)	L-ASP	Carboxymethyl konjac glucomannan-Chitosan (CKGM-CS)	300–3000 nm with increase in either CKGM or CS concentration	68%	0,9%	–	25,8% for imm. ASP and 17,9% for free ASP (300 min incubation at pH = 6.0);	–	–	–	–	–	Immobilized enzyme system showed significantly higher thermostability and had preferable	[71]

(*continued on next page*)

Table 4 (*continued*)

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
								77% for imm. ASP, and 60% for free enzyme (90 min incubation at 60°C)						resistance to acid and alkaline environments	
L-ASP	Magnetic-propelled carriers	Iron oxide	Chitosan-L-ASP	>200 nm, 26.8 ± 0.87 mV	73.2%	–	–	The residual activities: for imm. ASP at pH 4.0–43.3%, at pH 10.0–76.14%, For free ASP At pH 4.0–12.3%, at 10.0–40.6%. Maximum activity: Imm.ASP at 60–70°C, free ASP at 45°C	–	Free 0.65 ± 0.078 Imm. L-ASP 0.14 ± 0.007	Free 578.3 ± 38.0 Imm. L-ASP 284.7 ± 12.5	–	–	Enhanced reusability, better pH tolerance in acidic or alkaline medium, long-term storage and incubation stability, thermal stability	[70]

"-" not provided.

Table 5
The encapsulation performance, biochemical indicators and pharmacokinetics of the alginate based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP source *Escherichia coli*	Hollow nanospheres (self-assembly)	L-ASP	Alg-g-PEG/ α-CD	467 nm	81.9%	Formulation: 1 mg of L-ASP per 2 mL of alginate (up to 6% (w/v)) and per 6 mL α-cyclodextrin (up to 6% (*w/v*)) solution	–	Maximum activity: Imm.ASP at pH 7.0–11.0 and temp. 35–65°C, and free enzyme at pH 8.6, temp.65°C. Remaining activity after incub. 270 min in the pH 5 solution: 83.05% (imm. ASP), 55.36% (free ASP)	–	$4.65 \cdot 10^{-3}$ M (imm. L-ASP), $7.17 \cdot 10^{-3}$ M (free L-ASP)	–	–	–	Prevented the enzyme from leaking (into the blood), improved enzyme activity at wider temperature and pH range, a greater loading of enzymes	[75]
L-ASP Source *Escherichia coli*	Hollow nanospheres (self-assembly)	L-ASP	Alg-g-PEG/ α-CD	600 nm at 50% of α -CD	Up to 81.9%	Formulation: 1 mL 0.25% Alg-g-PEG solution with 0.5–10 mg L-asparaginase was added into 3 mL a-CD (6%) solutions	–	Relative activity of encapsulated enzyme 110.7%	–	–	–	–	–	The encapsulation efficiency increased with the amount of enzyme increasing, prevented the enzyme from leaking (into the blood)	[76]

"-" not provided.

Table 6
The encapsulation performance, biochemical indicators and pharmacokinetics of the gel based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP Source *Escherichia coli*	Gel particles	L-ASP	Poly(2 hydroxyethyl methacrylate)	12 μm	–	–	–	–	Imm.ASP – >40 days at 37°C, *in vitro*. The clearance for the gel ASP activity in plasma had an initial half-life of 13 h in contrast to a half-life of 2 h for the free ASP	Gel Km was similar to that of the free enzyme	–	6C3HED murine lymphoma was inhibited by 35, 78 and 100% after single intraperitoneal injections of immobilized L-ASP of 2, 4 and 8 I.U., respectively, as compared to 36, 53 and 86% for the free ASP by the 14th day.	–	Significant plasma L-asparagine depression, antitumor activity without major toxicity	[80]
L-ASP	Gel nanoparticles (*In-situ* crosslinking of the precipitated nanoparticles by bioorthogonal copper catalyzed click chemistry)	L-ASP	Dendritic polyglycerol	Adjustable between 100 and 1000 nm	100%	–	Full release at pH 4 after 2 h	–	–	The activity of encapsulated and released at pH 5.0 asparaginase was of 86.2 U/mg and equal to the non-processed sample		–	–	pH triggered release	[78]
L-ASP	Nanogel (inverse electron demand Diels-Alder (iEDDA) crosslinking chemistry)	L-ASP	PG-BA-norbonene and PG-THP-norbonene	47–200 nm	81% (dPG-norbonene) and 93% (dPG-BA-norbonene)	5 wt%	9 h at pH 3.0 (dPG-BA-norbonene); 24 h at pH 3.0 (dPG-THP)	All gels were stable in PBS at pH 7.4 for 3 to 8 months	–	–	–	dPG-THP-norbonene at concentration 156 μg/mL showed 80% cell viability (McF7 cell line)	–	pH triggered release	[79]

"-" not provided.

Table 7
The encapsulation performance, biochemical indicators and pharmacokinetics of the oil-based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP	Solid lipid nanoparticle (w/o/w modified double emulsion method followed by solvent evaporation technique)	Glycerylmonostearate	Span 20 and Tween 20	<198 nm	76%	82%	–	–	–	–	–	–	–	Improved enzyme loading capacity	[41]
L-ASP Source from *Streptomyces griseoplanus*	Nanoemulsion (vortexing)	Oleic acid	Tween 20/ Tween 80 as surfactants and propylene glycol as co-surfactant	70.89 ± 15.37 nm (Tween 80), 85.45 ± 20.54 (Tween 20)	–	10–15% (v/v)	–	Encapsulated preserved 59.11% of its residual activity at 50°C; free L-ASP preserved about 33.84%. Imm.ASP was max.stable at pH 8 to 8.5	–	–	323 U/mL (Tween 80 NE), 197 U/mL (Tween 20 NE)	–	–	Enhance efficiency and thermal stability	[81]

"-" not provided.

Table 8
The encapsulation performance, biochemical indicators and pharmacokinetics of the solid particles based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP Source *Escherichia coli*	Dual porosity hollow nanospheres (Nanomasking method)	L-ASP	Silica	110 ± 5, 221 ± 8, 534 ± 13 nm	–	>1500 mg/g (using *B. cereus* penicillinase)	–	–	–	0.001838 mM (imm. ASP); 0.001989 mM (Free ASP)	0.3087 μM/min (imm. ASP); 0.3108 μM/min (Free ASP)	–	–	Protection from antibody access, neutralization and proteolysis	[24]
L-ASP Source *Escherichia coli*	(Ca-ALG/MWCNT-COOH/L-ASP hybrid beads (sonication)	L-ASP	Calcium-alginate/multi-walled carbon nanotube	2 mm	97.0%	–	–	>70% (imm.ASP) and 20% (free ASP) of residual activity after 4 weeks at ~30°C. Max. activity: 45°C pH 8.5 (imm. ASP), 35°C, pH 7.5 (free ASP)	–	0.33 mM (imm. ASP), 0.42 mM (free ASP)	60.2 μM/min (imm. ASP), 70.0 μM/min (free ASP)	–	–	Enhanced storage stability and reusability (36% of its original activity even after consecutive 14 reuse)	[83]

"-" not provided.

Table 9
The encapsulation performance, biochemical indicators and pharmacokinetics of the liposome-based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP	Liposomal formulations (simplified dehydration-rehydration vesicles (sDRV) or extruded vesicles (VET))	L-ASP	PC-Chol-SA	From 85 to 250 nm (VET), from 501 to 1249 nm (sDRV)	Adjustible from 24 to 100% in (sDRV), from 26 to 55% (VET)	–	–	95% (imm. ASP, sDRV) after 15 days at 4°C	At 48 h retention of activity in human serum was up to 65% (sDRV) and 80–90% (VET)	–	–	ID_{50} (against CHO cells) 14.0 ± 1.7 U/mL (imm.ASP) 3.3 ± 0.3 U/mL (free enzyme)	–	preservation of active enzyme, high entrapment efficiency, prolonged serum half-life	[85]
L-ASP	Liposomal formulations (simplified dehydration-rehydration vesicles (sDRV) or extruded vesicles (VET))	–	PC, PI, bovine brain monosialoganglioside (GM1), Chol, and SA	1249 ± 249 nm (sDRV) 158–180 nm (VET)	72% (sDRV), 34–36% (VET)	–	–	Retention of activity of 78–90% (VET) and 65% (sDRV) after 48 h of incubation in human serum.	0.11 h (PC-Chol-SA (sDRV) (7:2:0.25)), 9.35 h (PC-Chol-SA (VET) (7:2:0.25)); 11.04 h (PC-Chol-PI(VET) (10:5:1)); 28.75 h (PC-Chol-GM1 (VET) (10:5:1)), 1.96 h (free ASP)	–	–	Cured animals number at concentration of 800 U/kg: 9 of 10 (PC-Chol-GM1, VET), 7 of 10 (PC-Chol-PI, VET), 5 of 9 (PC-Chol-SA, VET) 0 of 10 (PC-Chol-SA, sDRV) 2 of 10 (Free ASP)	Imm.ASP – no death. Free ASP mortality of 17% and 83% of after the i.v., and 80% and 83% after 2nd i.v of 2000 and 4000 U/kg, respectively .	Prevented the induction of anti-asparaginase antibodies and mitigated the anaphylactic reaction	[86]
L-ASP Source *Pectobacterium carotovorum*	Nanoliposomes (hydrated thin-film method)	–	Soybean lecithin, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG-2000)	93.03 ± 0.49 nm	53.99 ± 5.44%	–	–	–	–	–	–	IC_{50} (UI/mL) values for LLC cells: 0.23 (imm.ASP) 0.079 (free ASP); for HEPG2 cells: 0.28 (imm.ASP) 0.044 (free ASP); for SK-LU-1 cells: 0.21 (imm.ASP) 0.037 (free ASP) for MCF-7 cells: 0.36 (imm.ASP) 0.12(free ASP); for NTERA-2: 0.23 (imm.ASP) 0.038 (free ASP).	–	Improved pharmacological properties	[87]

(*continued on next page*)

Table 9 (*continued*)

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
Palmitoyl-L-ASP	Liposomes (Extrusion)	L-ASP	PC-Chol-PI; PC-Chol-SA	192 ± 69.3 μm (PC-Chol-SA), 208.6 ± 32.6 μm (PC-Chol-PI)	66 ± 6% (PC-Chol-SA), 46 ± 6% (PC-Chol-PI)	–	–	–	24.3 h (PC-Chol-SA); 23.7 h (PC-Chol-PI); 2.88 h (free palmitoyl-L-ASP)	–	–	Antitumor activity (LLC cells) in 28 days tumor size ~6309 mm^3 (imm.ASP), ~9319 mm^3 Cured animals number at a concentration of 800 U/kg: 5 of 9 (PC-Chol-SA); 2 of 8 (PC-Chol-PI); 5 of 7 (free palmitoyl-L-ASP). At 2000 U/kg: 7 of 9 (PC-Chol-SA); 7 of 9 (PC-Chol-PI); 9 of 10 (free palmitoyl-L-ASP)	Anaphilactic shock of animals: 6 of 6 (PC-Chol-SA), 0 of 6 (PC-Chol-PI), 6 of 6, 2 of them died (free palmitoyl-L-ASP)	Reduced toxicity, prolonged half-life	[89]

"-" not provided.

Table 10
The encapsulation performance, biochemical indicators and pharmacokinetics of the red blood cells based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
LMWP-ASP	RBC (involved a membrane-translocating peptide)	LMWP-ASP	RBC membrane			8 IU ASP per 100 μL of packed erythrocytes	–	70% of the ASP activity were recovered after 14 days at 4°C.	*In vivo* half-life of enzyme activity of RBC/LMWP-ASP was 4.5 ± 0.5 days whereas that of RBCs loaded with ASP *via* a hypotonic method was 2.4 ± 0.7 days	–	–	The mean survival time of DBA/2 mice (mouse lymphoma cell L5178Y) was improved from 10.0 ± 1.4 to 14.4 ± 2.3 days compared to the saline control group	–	Increased mean survival time by almost 44%, increased hal-life of L-ASP	[92]
L-ASP	L-ASP-loaded RBCs product GRASPA (eryaspase)	L-ASP	RBC membrane							Selectivity: the glutaminase activity of GRASPA was 10-fold lower than that of L-ASP, with initial rates of approximately 0.8 μM/min and 8.0 μM/min	–	–	–	Increased selectivity for asparagine over glutamine	[90]

"-" not provided.

Table 11
The encapsulation performance, biochemical indicators and pharmacokinetics of the biophages based L-ASP encapsulation systems and their reduced side effects.

Type of encapsulated L-ASP and source	Type of encapsulation system and preparation method	Encapsulation system composition		Encapsulation system main characteristics				Biochemical indicators and pharmacokinetics						Improved properties compared to free L-ASP	Ref
		Core segment	Shell segment	Size	Encapsulation efficiency	Load capacity	Cumulative release	Stability compared to native L-ASP during storage at 4, 25, 37, 45°C	Half-life compared to native L-ASP at 37°C	Activity		Cytotoxicity against cancer cells	Acute toxicity		
										K_m, mM	V_{max}, μM/min				
L-ASP Source *Escherichia coli*	Virus-like particles of the bacteriophage P22	L-ASP	Bacteriophage P22 coat protein, isopropyl β- d-1-thiogalactopyranoside	71.61 ± 24.21 nm	–	41.6 ± 8.1%	–	**Aggregation:** 60% (imm. ASP); 100% (free ASP) at 37°C after 12 days. **Thermostability:** At 4°C,up to 168 h imm. and free ASP were similar. At 25°C, after at 60 h imm. ASP decreased 75% of the initial rate. At 37°C after 24 h and 48 h 50% less than free enzyme.	Imm.ASP was stable for up to 24 h at 37°C	0.227 mM (imm. ASP) and 0.015 mM (free ASP)	131.78 μM/min (imm. ASP), for free enzyme NR	ASP-P22 at 20 U/mL around 65% cell viability, and for free enzyme at 20 U/mL around 20%. (leukemic cell line MOLT-4)	–	Improved stability to aggregation and activity	[96]

"-" not provided.

than in MPEG, mainly due to electrostatic interactions between the enzyme and PLGA.

Baran and *co*-authors reported the use of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) for encapsulation of L-ASP [49,50]. In [49] the encapsulation was performed using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) through a double emulsion-solvent evaporation procedure (w/o/w). To improve the EE and activity of the encapsulated enzyme, numerous modifications like changing of medium pH, relative proportions of the ingredients and the molecular weight of the polymer were made. The enzyme activity increased when low molecular weight PHBV was used, even if the amount of protein encapsulated remained, suggesting that these nanocapsules had better solute permeability. The encapsulation yields of L-ASP were significantly improved by adjusting the second water phase to the proteins' isoelectric point [49].

Further authors [50] encapsulated L-ASP into PHBV nanocapsules to decrease the immunogenicity and toxicity of the enzyme and to increase L-ASP *in vivo* half-life in mice by water-in-oil-in-water approach. Authors reported [50] EE increase from 23.7% to 28.0% by changing the pH and the isoelectric point, suggesting that the encapsulation allowed protection of the enzyme against denaturation. In the case of unmodified PHBV nanocapsules, 4 h after injection, the enzyme activity in the blood decreased to 38% of its initial value; in the case of PHBV nanocapsules with heparin, enzyme activity had increased to almost 50%after 6 h [50].

Authors [53] reported that redox-responsive vesicles assembled from pillar[*n*]arenes for drug delivery could enhance the bioavailability of drugs for cancer cells and reduce the adverse side effects for normal cells; also these systems can also overcome the drug resistance of cancer cells [51], and these systems can be used for L-Asparaginase.

Polyaniline nanofibers were also used as an immobilization matrix of L-ASP to improve its stability and activity [52]. Polyaniline nanofibers possess a large specific area and higher porosity, allowing higher enzyme loading, higher conductivity, and more facile fabrication. The enzyme was characterized by XPS, XRD, TEM and FTIR, and the enhanced stability and activity of the enzyme after immobilization and the effect of temperature and pH on the stability were determined [52]. The calculated K_m values were 1.809 and 3.705 $mM \cdot L^{-1}$ for immobilized L-ASP, respectively, which are around twice lower in magnitude in comparison with K_m values for free L-ASP; the V_{max} values for immobilized and free L-ASP were 90.57 and 48.04 $\mu M \cdot min^{-1} \cdot mg^{-1}$, respectively [52].

Biodegradable, biocompatible polymeric capsules demonstrated the suitability to improve the stability and activity of L-ASP compared to its free form. Nevertheless, to date more alternative materials are being used to safely deliver L-ASP and improve its other properties, which will be discussed in the following sections.

The summary of the encapsulation performance, biochemical indicators and pharmacokinetics of the L-ASP encapsulation systems described in this review are shown in Tables in each section.

2.1.1. Polymersomes

As a potential substitute for PEGylation, using polymersomes for an L-ASP delivery system can provide size-selective permeability to the vesicle, structure versatility, better enzyme stability and decreased immunogenicity. Polymersomes are composed of a double layer amphiphilic block copolymer, similar to liposomes with two phospholipids layers. Encapsulation can be used to deliver a variety of therapeutic enzymes, regardless of their structure, as no chemical modification of the enzyme is needed [55,56].

Authors [55] reported inherently size-selectively permeable vesicles comprised of a PEG shell and a poly(2-hydroxypropyl methacrylate) (PHPMA) membrane (Fig. 3). L-ASP encapsulation was done through one-pot synthesis using polymerization-induced self-assembly (PISA). Encapsulation protects the enzyme against external proteases and antibody recognition, allowing permeability to small molecules, such as L-asparagine. The diameter of obtained polymersomes was around 340

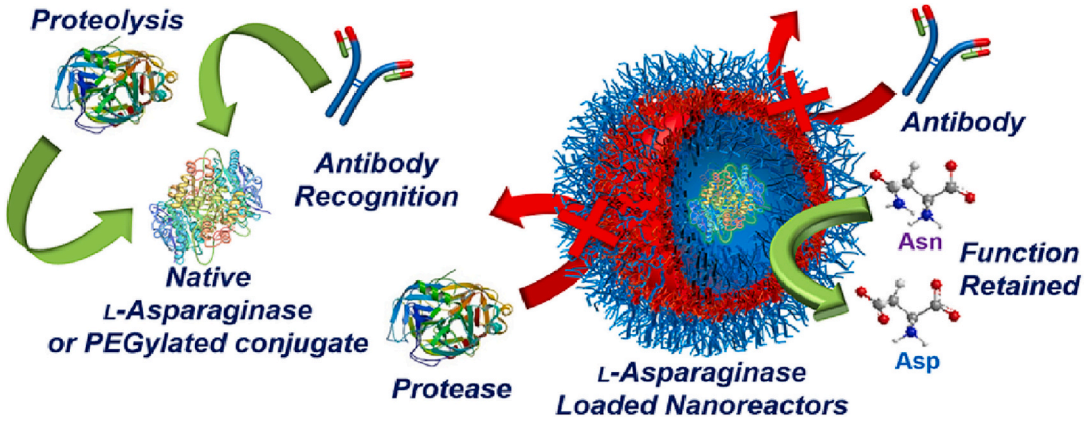


Fig. 3. Schematic representation of the size-selectively permeable polymersomes mechanism [55]. Reprinted with the permission of Ref [55] Copyright 2018 American Chemical Society.

nm. The activity of the ASP-loaded capsules was 52 ± 8% lower than of the free enzyme. Authors suggested that this is due to semipermeable vesicles' diffusive barrier. Following 24 h of incubation in fetal bovine serum solution, a little size increase for both blank and ASP-loaded capsules was detected, indicating gradual agglomeration with blood proteins, but a slight size decrease was found after incubation in cell growth medium. The authors assessed anti-ASP binding using a sandwich enzyme-linked immunosorbent assay. When compared to the native L-ASP, the binding affinity of ASP-loaded capsules was 2 orders of magnitude lower, and the binding affinity of PEG-ASP conjugation was 1 order of magnitude lower. The authors also studied the cytotoxicity *in vitro* on A549 cells (human lung cancer fibroblasts). A comparable reduction in proliferation of cells for ASP-loaded capsules was 58%, PEG-ASP of 61% and for free ASP was 78% [55].

Bueno et al. [57] reported homogenous polymersome formulations with a mean diameter of 104 to 120 nm were prepared by the film hydration method using the copolymers poly[(2-methacryloyl)ethyl-phosphorylcholine] – poly[2-(diisopropylamino) ethylmethacrylate] (PMPC–PDPA) and poly(ethylene glycol) – poly- (butylene oxide) (PEO – PBO). The L-ASP was incorporated through electroporation. The shell of asymmetric nanocapsules consisted of 90% PMPC–PDPA and 10% PEO – PBO with separated membrane domains, containing a small permeable bud formed by the minor PEO – PBO constituent (Fig. 4). Enzymatic activity studies with aspartate and ammonia production demonstrated that both ammonia and aspartate concentrations tend to be higher for asymmetric polymersomes. The authors explained their higher permeability due to the PEO – PBO bud and higher loading capacities.

Polymersomes based on semi-crystalline copolymers of PEG-b-poly (ε-caprolactone) (PEG-PCL) were investigated for the encapsulation of L-ASP [58]. Polymersomes with a 200–380 nm diameter was obtained using the film hydration method and the following post-film techniques: sonication, centrifugation, extrusion and filtration. It was suggested that the PEG chains might interact with one another during the centrifugation process to create an entangled polymeric network that could capture free enzyme molecules and drag them down. As a result, the amount of enzyme still present in the supernatant would be lower than its actual non-encapsulated content. Therefore, it would not accurately the initial and encapsulated variation enzyme mass. The authors reported no L-ASP release over 10 h at pH 7.4 [58].

2.1.2. *Polyelectrolyte multilayers and complexes*

Another approach to encapsulate enzymes is using several layers of oppositely charged biocompatible polyelectrolytes to form a shell of the capsule [59–61].

Authors reported L-ASP encapsulation through coprecipitation of calcium carbonate ($CaCO_3$) and an enzyme with the following coating with two or three poly dextran/poly-L-arginine-based bilayers (Fig. 5) [62]. After the polymer shell was assembled, the $CaCO_3$ core material was dissolved. While free enzyme was particularly vulnerable to trypsin treatment, as indicated by the fact that after 1 h of incubation, its remaining activity was below 5%, encapsulated L-ASP resisted trypsin exposure substantially better, keeping up to 20% activity even after 7 h. Encapsulation of L-ASP enhanced its thermostability only slightly. The storage stability of the free enzyme was 0% at the end of 12 weeks, whereas for the encapsulated enzyme was 50% at 4°C. Two leukemic

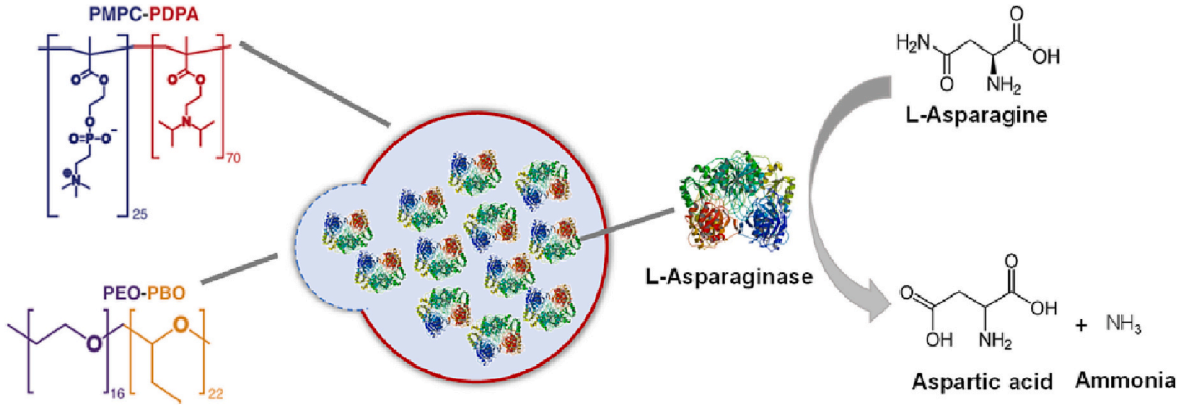


Fig. 4. Schematic illustration of an asymmetric polymersome [57].
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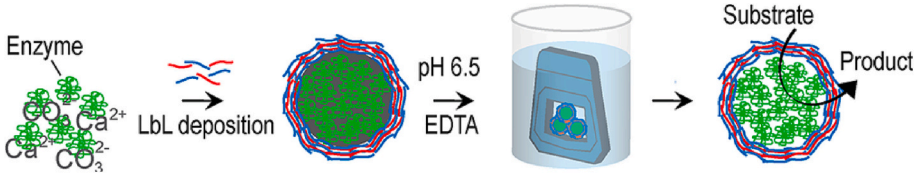


Fig. 5. The illustration of the formation of L-ASP-loaded polyelectrolyte capsules [62].
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cell lines SD1 and MOLT-4 were studied for antiproliferative activity. 51% MOLT-4 cells and 30% SD1 cells survived for encapsulated ASP compared to untreated cells. For the free L-ASP, 62% MOLT-4 and 32% SD1 cells survived [62].

In 2018, Andrianov et al. [63] reported the synthesis of oppositely charged polyphosphazene polyelectrolytes containing grafted PEG chains as modular components for the assembly of biodegradable PEGylated L-ASP delivery vehicles (Fig. 6). Here, poly[di(carboxylatoethylphenoxy)phosphazene]-*graft*-PEG (AP-PEG) was used as anionic graft copolymer, and poly[di(dimethylaminopropyloxy)phosphazene]-*graft*-PEG (CP-PEG) was utilized as cationic graft copolymer. Supramolecular assemblies were below 100 nm. The authors suggested the compact polyelectrolyte complex core and hydrophilic PEG shell structure with an electroneutral surface charge assessed by zeta potential measurements. Covalently bound PEG chains can block active sites of the enzyme, which was overcome by PEG bound electrostatically. Neutral polyelectrolyte complexes of both anionic and cationic PEG with L-ASP (NP-PEG-ASP) showed lesser antigenicity than CP-PEG-ASP (cationic PEG). Also, NP-PEG-ASP had the highest thermal stability and protection against proteolysis compared to CP-PEG-ASP and free L-ASP. All PEGylated polyacids degraded quickly at 65°C and slightly more slowly at 37°C, with the rate of hydrolysis increasing as the PEG group content in the polymer rose [63].

The studies suggest the use of biodegradable polyelectrolytes offers potentially lowered complexity of the synthesis and reduced expenses of reactions and purification processes compared to covalent PEGylation technology.

2.1.3. Chitosan

Due to its biocompatibility, biodegradability, low toxicity, and structural diversity, chitosan (CS), a natural polysaccharide primarily derived from marine crustaceans, is a viable drug delivery platform for medicines and many other ingredients [64]. In the case of L-ASP chitosan, besides improving stability, and protecting against the proteolytic enzyme, it is also known to enhance its pharmacokinetics and provide controlled release, biocompatibility and biodegradability. And on top of it, chitosan has antimicrobial properties, which can result in the synergetic effect of two materials [65].

Some authors [65–67] proposed the ionic gelation method for loading the enzyme L-ASP through chitosan nanoparticle formation. This technique is based on the electrostatic interaction of oppositely charged species, where at least one of them is a polymer [68]. However, there are other methods for chitosan particle preparation, such as the emulsion droplet coalescence method, reverse micellar method, *etc.* [69].

In work [66] to get optimal entrapment efficiency, chitosan nanoparticle (CSNP) preparation, chitosan/tripolyphosphate (CS/TPP) ratio, and L-ASP loading were investigated. The study revealed that entrapment effectiveness and loading capacity increased when CS and TPP concentrations rose. It was found that the size of the optimal ASP-CSNPs was 340 ± 12 nm, zeta potential of 21.2 ± 3 mV, entrapment efficiency of 76.2%, and loading capacity of 47.6%. *In vitro* half-life of the immobilized enzyme was longer than that of the free enzyme. The immobilized enzyme had similar pH and thermostability to the free enzyme. This research advances CSNPs preparation knowledge, increases encapsulation efficiency for high molecular weight proteins, and extends protein release from CSNPs [66].

Authors of the article [65] found that after encapsulation, the minimum inhibitory concentration of L-ASP reduces from 21.06 mg/mL to 5.26 mg/mL, which testifies to increasing efficiency 4 times compared with the free enzyme. Other properties, such as the thermal and pH stability of the L-ASP, also increased after immobilization. Other works also showed that conjugated L-ASP presented higher thermostability and a more comprehensive range of pH stability than free L-ASP [65,70]. Authors [65,70] showed that conjugated enzyme had higher residual activity than free form and was stable in the pH range of 4.0–10.0. If compared, an activity at pH 4.0 and 10.0 comprised 43.3% and 76.14% for the conjugated enzyme and 12.3% and 40.6% for free L-ASP,

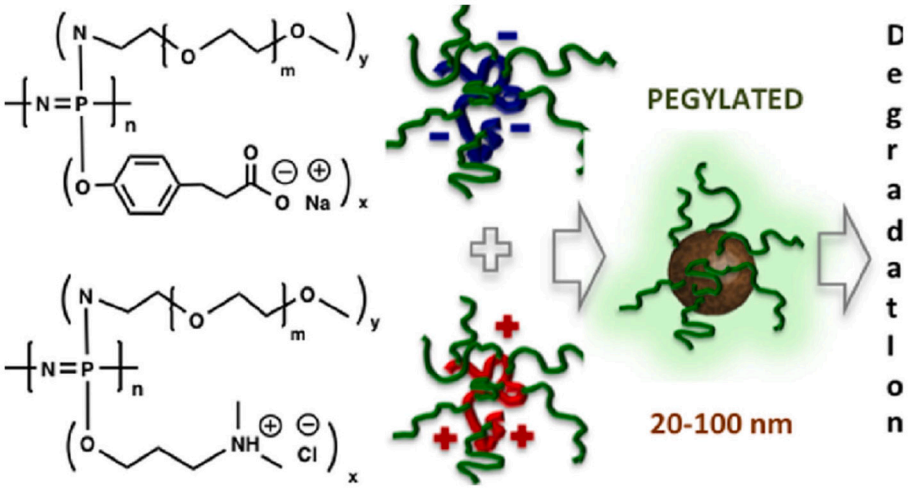


Fig. 6. Synthesis of CP-PEG and AP-PEG and schematic presentation of their spontaneous complexation in aqueous solution [63].
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respectively. This testified about good adaptability and better tolerance to acidic or alkaline environments owing to the shell surrounding it [65,70]. Also, while the optimum temperature for the free enzyme was 45°C, the conjugated enzyme showed maximum activity at 60–70°C. Authors found that enzyme nano-carriers have more significant therapeutic potential than free enzymes. Additionally, these studies examine yet another important use of this enzyme as an antimicrobial agent and encourage further research into its potential as an anti-biotherapy [65].

Authors [70] immobilized L-ASP onto magnetic-propelled carriers comprising magnetic Fe_3O_4-chitosan nanoparticles. It was found that L-ASP demonstrated enhanced catalytic activity in a weak magnetic field and thermal and pH stabilities. Conjugated L-ASP's reusability also considerably increased and, after 16 cycles retained 60.5% of its initial activity. After 4 weeks of storage at 4°C and room temperature, the conjugated L-ASP still had >50% and 48% of its initial activity, respectively. The authors also demonstrated that the activity of conjugated L-ASP onto magnetic Fe_3O_4-chitosan particles compared to free L-ASP increased by around 3-fold in the weak magnetic field at specific frequencies and flux densities. Thus, the report showed that this mechanism could transport and activate L-ASP and improve its catalytic activity, stability, and efficiency [70].

All of these works confirmed that the toxicity of encapsulated L-ASP is lower compared to the free enzyme, they have increased catalytic activity, stability *in-vitro* and anti-cancer effects, improved thermostability and a more comprehensive range of pH stability compared to free L-ASP; they also use of chitosan improves encapsulation efficiencies of L-ASP [65–67,70,71].

2.1.4. Alginate

One of the natural polymers widely employed in protein and drug delivery systems is sodium alginate. Alginate is a polysaccharide extracted from brown seaweeds [72]. Alginate provides several benefits, including simplicity in production, biocompatibility, biodegradability, and nontoxicity [73]. The release mechanism is based on the property of alginate carboxyl groups to protonate in acidic conditions, thus, eliminating drug release. The alginate-based delivery systems preparation is based on an ionotropic gelation process above pH 6, when crosslinking with Ca^{2+}, Ba^{2+} or Zn^{2+} ions occurs [74].

Authors [75] reported alginate-*graft*-poly(ethylene glycol) assembled with α-cyclodextrin (Alg-g-PEG/α-CD) hollow nanospheres for L-ASP encapsulation (Fig. 7). It was reported that the hollow spheres not only enable a high loading of an enzyme but also show semi-permeability which could prevent the enzyme from leaving the core while allowing substrates and products to pass through to maintain the enzyme activity. Additionally, the encapsulated L-ASP showed significantly higher stability in an acidic environment than the free enzyme. Authors [75] suggest that the encapsulation method presented in their paper represents an approach to preserving the biological activity of protein drugs for medical purposes.

The same authors in [76] reported that the encapsulation capacity of

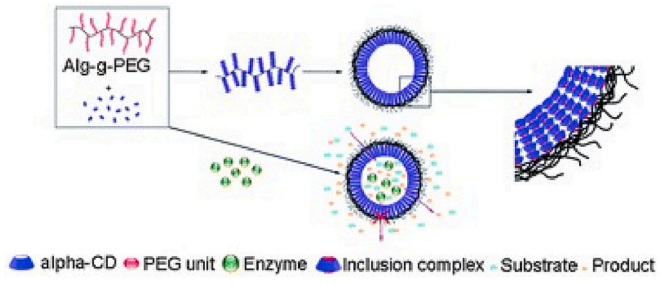


Fig. 7. Schematic illustration of alginate-based hollow spheres and the process of enzyme encapsulation [75].
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Alg-g-PEG/α-CD increased with the amount of enzyme increasing, The maximum encapsulation capability for L-was estimated to be approximately 4 mg per 4 mL Alg-g-PEG/α-CD (0.25%/6%) solution. Additionally, high PEG graft density increased EE but did not impact leakage efficiency. The possibility of using hollow nanospheres as L-ASP carriers and as controlled release systems are made possible by specific properties of alginate [76].

2.1.5. Gels

Gels are widely used in medicine due to their biocompatibility, biodegradability, stability, rheological properties, ease of preparation, and good adherence [77]. Gels are systems of liquids dispersed within a solid medium. Thus, they can be used to encapsulate hydrophilic ingredients. Examples of natural polymers used for gel formation are chitosan, alginate, dextran and also various synthetic polymers can be used for gel fabrication.

Surfactant-free dendritic polyglycerol (dPG) gels were fabricated by the authors [78] for pH-controlled release of L-ASP. The diameters of gels could be adjusted between 100 and 1000 nm, and enzyme encapsulation efficacy was 100%. At acidic pH values, the polyglycerol nanogels quickly degraded allowing the release of the enzyme (Fig. 8). The acidic pH is typical for inflamed and tumor tissues as well as intracellular organelles. However, the gel remained stable at physiological pH values for a long time. After the release, the total enzyme activity of 86.2 U/mg and the initial structure was retained.

In 2020, Oehrl et al. [79] improved the pH-degradable polyglycerol-based nanogels by incorporating of benzacetal (BA) and tetrahydropyran (THP)-based acetals into the macromonomers which cleave at pH values of 5 and 3, respectively. pH-degradable nanogels degraded at their acetal linking points. The EE were up to 93%. Gels displayed high stability in phosphate-buffered saline at pH 7.4 for extended periods. Furthermore, the macromonomers did not show cell toxic effects up to 2.5 $mg{\cdot}mL^{-1}$, except for dPG-THP-norbonene.

Later, Maryam Monajati *etc.* [25] developed the nanogels prepared by adding bisCD to P(HPMA-MPEGA)-L-ASP. Using this nanogel improved the kinetic behaviour and antitumor activity of the enzyme. The K_m parameter, a measure of an enzyme affinity to its substrate, was reduced compared to free L-ASP. Heat stability of immobilized L-ASP was higher than free enzyme; for example, at 70°C, almost the entire enzyme activity of free ASP was lost, P(HPMA-MPEGA)-L-ASP and L-ASP bisCD nanogel retained 34% and 47% of their initial activity, respectively. After 1 h incubation with trypsin, the remaining activity of free L-ASP was only 17%, and immobilized L-ASP maintained up to 80% of activity.

2.2. Solid lipid nanoparticles and emulsions

The emulsion is one of the encapsulation strategies allowing to improve the stability of the enzyme and increasing its resistance to proteases. An emulsion is a liquid droplet dispersed in another liquid medium (oil and water) and surface-active materials, such as surfactants, solid particles, among others stabilize the interface here. The main advantages of emulsions are the ability to control such parameters as core/shell size, release rate and release triggers, interfacial properties, surface zeta-potential, and hydrophobic-hydrophilic balance, among others.

Sharma et al. [41] used a statistical experimental design to optimize the relative amount of lipid/oil, surfactant/s, the ratio of lipid: drugs, temperature, homogenization speed *etc.* This allowed controlling the physicochemical properties like mean particle size, zeta potential, PDI and drug-release profiles of L-ASP loaded solid lipid nanoparticles (SLN). Glyceryl monostearate was used as a solid lipid and Span 20 and Tween 20 were chosen as emulsifying agents. The authors reported spherical in shape nanoparticles with a mean size of <198 nm. The enzyme drug loading was equal to 10.11% and the enzyme encapsulation efficiency of 76.19%.

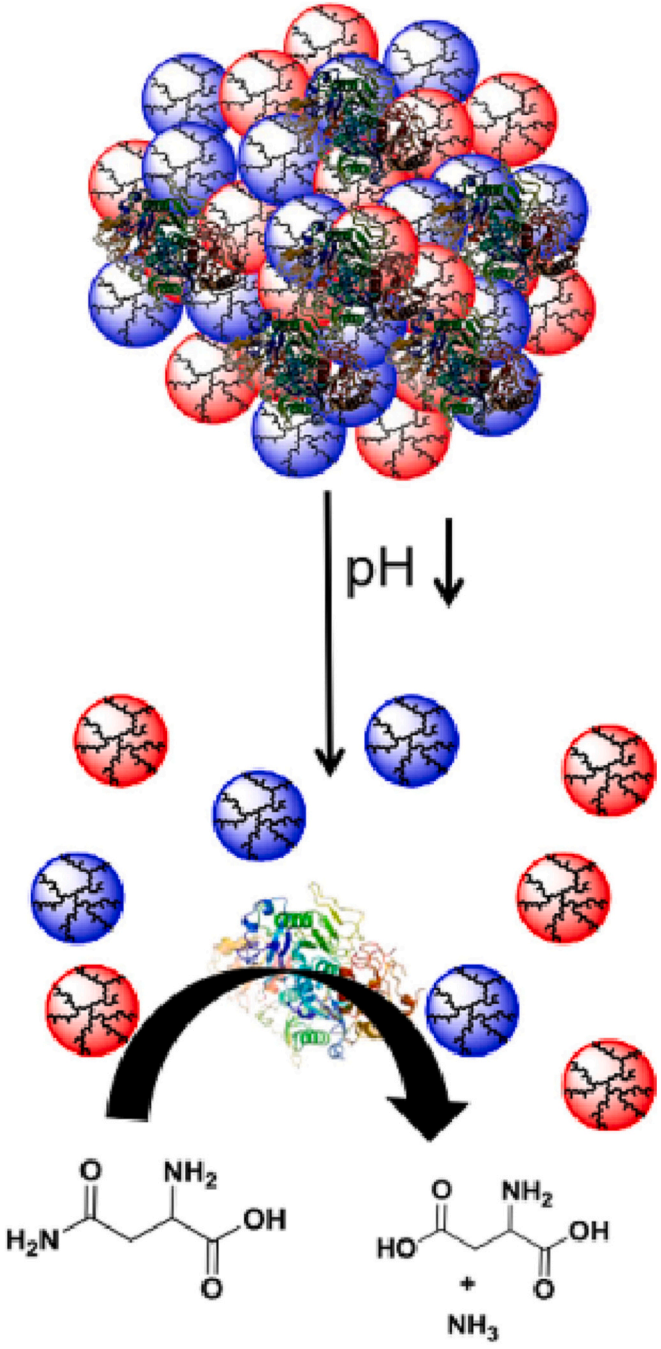


Fig. 8. The illustration of degradation triggered release of L-ASP and its catalytic conversion of asparagine to aspartic acid under ammonia generation [78]. Reprinted with the permission of Ref [78] Copyright 2012 Elsevier Ltd.

El-Hadi et al. [81] developed an L-ASP-loaded nanoemulsion (NE) to enhance its efficiency and thermal stability. Oleic acid was used as an oil phase core, Tween 20/Tween 80 as surfactants and propylene glycol as a co-surfactant. Authors reported that immobilized L-ASP was more stable at a pH of 8–8.5 compared to free L-ASP. In addition, the immobilized L-ASP maintained about 59.11% of its original activity at 50°C, while the free enzyme showed only 33.84%.

2.3. Solid particles

Colloidal solid particles can be used as a promising approach for drug delivery. Due to their high specific surface area, they can be used as a carrier of the drug attached to their surface [82], used for emulsion stabilization, and in case they have a hollow core that can be considered for drug loading and controlled release. In addition, this allows immune isolation and protection of active ingredients.

The authors [24] developed hollow silica nanoparticles with dual-porosity (nanoporous of <2 nm and mesoporous of 5–50 nm), allowing substrate to access the enzyme. After loading the enzyme the mesopores were sealed with the same nanoporous material. This allowed to trap the enzyme inside the particles and protect it from neutralization, antibody access, and proteolysis without losing functionality in serum immunized against the load for at least two weeks. In addition, nanopores provided smaller substrates, such as asparagine, easy access inside, which allowed them to interact with encapsulated enzyme and diffuse out (Fig. 9). *In vivo,* localized activity was reported, as well as the in-tissue-residence time of about two months, which can contribute to achieving systemic effects.

Inorganic materials can be combined with organic ones to form hybrid materials possessing beneficial properties of each component, including flexibility, processability, and mechanical, chemical and thermal stability, to name a few.

This approach was used to fabricate L-ASP immobilized calcium-alginate/multi-walled carbon nanotube hybrid beads (Ca-ALG/MWCNT-COOH/L-ASP) with a size of 2 mm [83]. The beads showed high resistance against temperature and pH changes. Above 55°C free enzymes underwent thermal denaturation, while Ca-ALG/MWCNT-COOH/L-ASP maintained 11.13% of initial activity at 65°Cdue to the limited conformational flexibility caused by immobilization. The enzyme-loaded beads showed maximum activity at 45°C, pH 8.5, while free enzyme – at 35°C, pH 7.5. Also, storage stability was improved and retained >70% of the initial activity after 4 weeks at 30°C, while free enzyme demonstrated only 20% of residual activity. Furthermore, immobilized enzymes possessed >36% of their initial activity after 14 reuses. It is worth noting that the original activity did not change significantly after eight consecutive uses. Thus, hybrid materials with storage stability and reusability can be promising carriers for enzyme delivery.

2.4. Liposomes

L-ASP encapsulation in liposomes is a promising approach due to the increased therapeutic index of L-ASP. This is achieved *via* the prolongation of the half-life of the enzyme and the ability of liposomes to overcome the immune response by masking the antigen determinants.

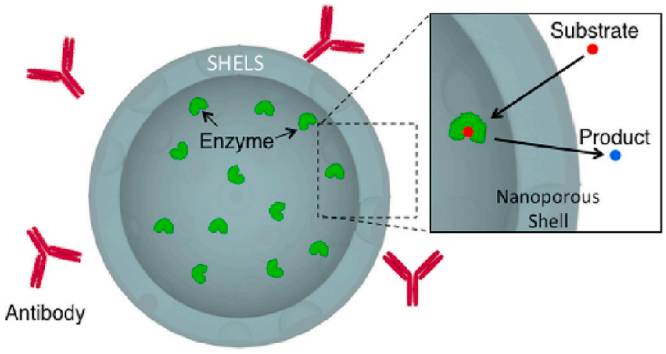


Fig. 9. Illustration of silica nanoparticle loaded with L-ASP. Encapsulated L-ASP (green dots) cannot escape through the nanopores of the shell. The blow-up of a section of the particle demonstrates the small molecule substrate (red dots) diffusion inside through the nanopores surface, interaction with L-ASP and the reaction product diffusion out (blue dots) [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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Liposome systems are widely used in medicine and attract the researcher's interest due to their safety and biocompatibility [84].

Cruz and colleagues [85] reported preserving active enzyme, high entrapment efficiency, prolonged serum half-life and reduced toxicity compared with the free enzyme loading L-ASP in liposomal formulations. Liposome formulations were prepared using simplified dehydration-rehydration vesicles (sDRV) or extruded vesicles (VET). The sizes of extruded vesicles were 85 to 250 nm in diameter and for sDRV, from 501 to 1249 nm. The authors of this work reported that encapsulation efficiency and other parameters depend on various concentration ratios. It was found that the most optimal molar ratio of lipid combination (egg phosphatidylcholine (PC) – cholesterol (Chol) – stearyl amine (SA)) was 7:2:0.5, at which EE reached up to 100%, and enzyme activity of 99.5 ± 0.7%. Extruded vesicles had a more extended circulation period in the blood while having inferior encapsulation parameters to those of sDRV. The studies of L-ASP loaded liposomes in human serum showed that at 48 h the retention of activity for sDRV formulations was up to 65% for PC-Chol-SA, and for VET formulations composed of PC-Chol-SA were more stable, with initial enzyme activity retention of 80–90%. In addition, authors demonstrated that the liposomal formulations were less cytotoxic than the free enzyme against Chinese hamster ovarium cells. This was explained by a slower release of L-ASP and slower substrate depletion, allowing cells to adapt and synthesize asparagine [85].

In the later report [86] authors found that incorporating L-ASP into the liposomes increases factors for immunogenically safe and efficient use in the practice. The encapsulation of L-ASP reduced acute toxicity drastically. When liposomal L-ASP was used as the sensitizing and challenging agent, the survival was 100% for VET and sDRV liposomes, for both 2000 and 4000 U/kg doses, and for first- and second-challenge administration. At the same time, the mortality of free enzyme was 17–67%. After a second challenge dose of VET L-ASP of 80% of the animal survived, while in the case of free enzyme the survival rate was 20%. Also, it was demonstrated that L-ASP VET formulation increased antitumor activity 2-fold at intravenous doses of 400 and 800 U/kg and increased the number of cured animals compared with a free enzyme.

Authors [87] prepared L-ASP-loaded liposome formulation using the dehydrate-rehydrate thin film method. The average size of the formulation was about 93 nm and EE of around 54%. They also found that PEGylated nano liposomal L-ASP significantly suppressed tumor growth. However, similar to results in [85] due to slow uptake liposomal formulations showed 3–5 times lower cytotoxic activity against cancer cells compared to those of free L-ASP.

Unfortunately, liposomes have a relatively short half-life, which limits the drug delivery time relatively short half-life, which limits the drug delivery periods. But new recent researchers show that modification of liposomes can solve these problems for optimum affinity, with the use of chemical functionalization strategies [88].

2.5. Red blood cells

Red blood cells (RBCs) can deliver enzymes [90–93]. RBCs are about ~7 μm in diameter and ~ 2.5 μm in thickness, and the internal volume is 185–191 μm^3 [94]. Also, RBCs are biocompatible, biodegradable, and in blood circulation have a life span of 120 days and thus can carry active ingredients for sustained drug release [95]. L-ASP-loaded RBCs ard a new approach to treating asparagine auxotrophic malignancies [90].

The majority of techniques of protein encapsulation into RBCs are based on a partial breach of the cell membrane, which permanently alters the physical and chemical characteristics of RBCs (Fig. 10). A novel technique for encapsulating therapeutically active L-ASP into intact RBCs was described by the authors [92]. It involved a membrane-translocating peptide – low molecular weight protamine (LMWP). The results showed that the structural and functional integrity of both the

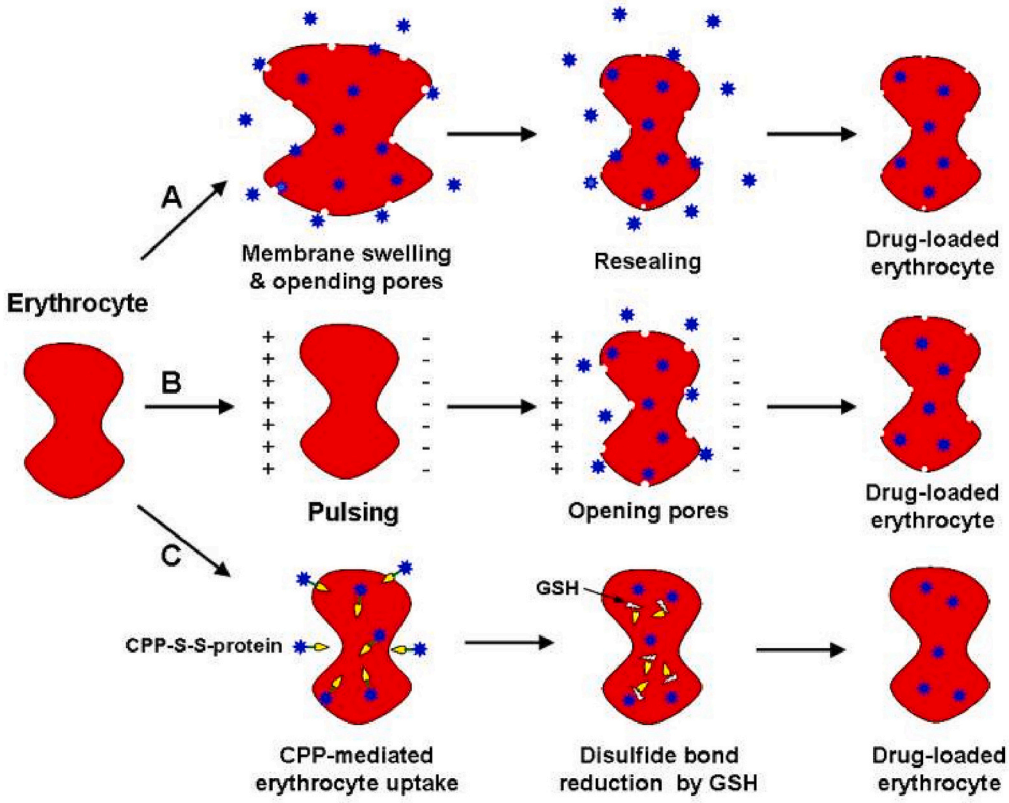


Fig. 10. Schematic illustration of methods for loading protein drug into erythrocyte: (A) Osmotic dialysis, (B) electroporation, and (C) CPP-mediation method [91]. Reprinted with the permission of Ref [91] Copyright 2013 Elsevier B.V.

loaded L-ASP and processed erythrocytes were utterly reserved. In addition to significantly longer plasma half-lives that were almost twice as long as those of untreated RBCs, erythrocytes treated with the LMWP-mediated method showed improved therapeutic effects of the entrapped protein drug. This is likely because the erythrocytes protected the drug from potential proteolytic degradation and phagocytic clearance.

Authors [81] suggested using L-ASP-loaded RBCs product GRASPA (eryaspase) to reduce L-ASP side effects. They demonstrated an improved toxicity profile. When treated with GRASPA none of the patients exhibited any signs of allergic responses during induction, in contrast to 46% of patients treated with free L-ASP. Also, encapsulated L-L-ASP has led to less pancreatic or hepatic toxicities compared to free L-ASP. Furthermore, L-ASP-loaded demonstrated 10-fold increase in selectivity for asparagine over glutamine due to a significant decrease in glutaminase activity relative to L-ASP. The authors concluded that in the presence of GRASPA, asparagine was rapidly and extensively converted to aspartic acid inside the RBCs of GRASPA, whereas no aspartic acid accumulated in the RBC following treatment of whole blood with L-ASP. So, the RBC membrane of GRASPA imbues L-ASP with improved target selectivity, which may explain the better toxicity profile of GRASPA. Altered target selectivity has been added to a growing list of beneficial properties of the RBC membrane, including improved half-life and decreased immunogenicity.

Using biomaterials, such as red blood cells for L-ASP encapsulation might be limited due to the threat caused by the specific features of blood.

2.6. Biophages

Virus-like particles (VLPs) are monodisperse nanostructures consisting of self-assembled proteins, identical to the original virus capsid. However, instead of genetic material, they contain the active ingredient molecules, thus unable to cause infections. VLPs can be used as delivery systems because they possess a longer half-life, are biocompatible, can be chemically functionalized and can be produced in large amounts.

L-ASP from *E. coli* was encapsulated in bacteriophage P22-based virus-like particles (ASP-P22 nanoreactors) to obtain a minimal immunological response upon application to patients [96]. ASP-P22 nanoreactors were composed of nearly 60% coat proteins (58.4 ± 7.9%) and 40% ASP-scaffold proteins tetramers (41.6 ± 8.1%), with average sizes of 71.61 ± 24.21 nm (Fig. 11). The K_m value of ASP-P22 was 15-fold higher than that obtained for the free enzyme (0.227 mM for ASP-P22 and 0.015 mM for the free enzyme). In addition, the authors reported improved stability against aggregation at 37°C for ASP-P22, which showed an aggregation of 60% of the volume sample compared to commercial L-ASP aggregated entirely under the same conditions. However, thermostability studies showed that at 37°C the initial rate of ASP-P22 was 2-fold lower compared to commercial L-ASP. But the use of glycerol at 10% *v*/v as a nanoreactor-stabilizer allowed to keep the enzymatic profile up to 24 h at the same level as for commercial ASP, which is almost half the life of the commercial L-ASP Leunase®. After 48 h of incubation, the reaction efficiency of ASP-P22 remained 50% less compared to Leunase®, and in the presence of glycerol allowed decreasing only by 25%. On the other hand, L-ASP nanoreactors showed concentration-dependent cytotoxicity activity against the leukemic cell line MOLT-4, even though 3-fold less compared to Leunase®. Authors suggested that the lowered functionality is attributed to lower stability at 37°C, restricted conformational changes during catalysis, lower substrate diffusion rate into VLPs, or a non-observable population of the encapsulated inactive enzyme. Therefore, further research is needed to extend the lifetime of ASP nanoreactors to be competitive with commercial formulations.

The encapsulation in such particles as VLP can be a promising approach for L-ASP due to essential advantages attributed to biophages.

3. Bio-characteristics of L-ASP encapsulation systems

L-ASP shows cytotoxic effects on both cancerous and non-cancerous cells, which is why it is used as a chemotherapy drug. The cytotoxicity of L-ASP is due to its ability to deplete the level of asparagine, an amino acid essential for the survival and growth of cancerous cells. The enzyme has been extensively characterized biochemically, and here are some of its key properties:

- Molecular weight: L-asparaginase is a homotetramer protein consisting of four identical subunits, with a molecular weight ranging from 130 to 160 kDa depending on the source of the enzyme.
- Optimal pH: L-ASP has an optimal pH range of 7.5–8.5. The enzyme is active in slightly alkaline conditions.

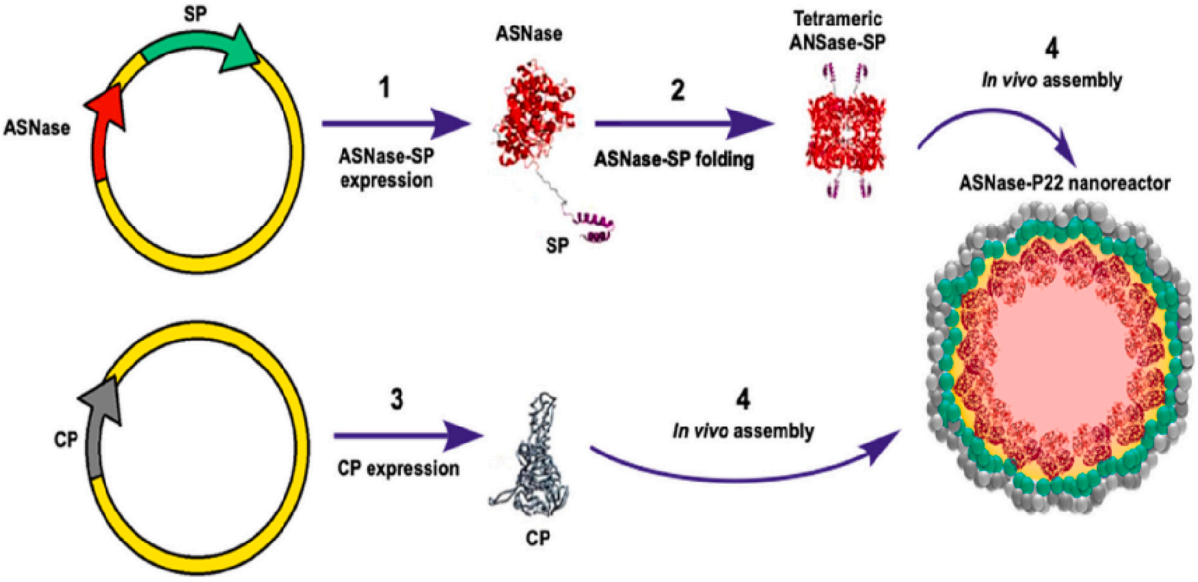


Fig. 11. Illustration of ASP-P22 virus-like particle consisting of L-ASP (red)-scaffold proteins (green) tetramers and coat protein (grey) [96]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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- Substrate specificity: L-ASP hydrolyses L-asparagine, not D-asparagine or other amino acids.
- Inhibition: L-ASP can be inhibited by various compounds, including L-glutamine and L-aspartic acid.
- Stability: L-ASP is relatively stable at low temperatures and can be stored at −20°C for several months. However, the enzyme is sensitive to heat and can be inactivated at temperatures above 50°C.
- Glycosylation: L-ASP from some sources, such as *E. coli*, is not glycosylated, while others, such as *Erwinia chrysanthemi*, are glycosylated.
- Immunogenicity: L-ASP can be immunogenic and may cause allergic reactions in some patients.

Overall, these biochemical characteristics of L-ASP have important implications for its use in cancer therapy, including the need for careful dosing and administration, as well as the potential for allergic reactions or other adverse effects. Each carrier system of L-ASP exhibits different biochemical properties. For example, PEGylation involves the attachment of polyethylene glycol to L-ASP to increase its half-life and reduce its immunogenicity. PEGylation can also reduce the cytotoxicity of L-ASP to healthy cells, while maintaining its efficacy against cancer cells [25,26].

Liposomes have been shown to reduce the cytotoxicity of L-ASP to healthy cells while maintaining its efficacy against cancer cells. This is because liposomes can help to target L-asparaginase specifically to cancer cells and protect the enzyme from degradation, while minimizing its exposure to healthy cells [76,77].

L-ASP has been extensively studied for its *in vitro* and *in vivo* cytotoxicity against cancer cells with different carrier systems. *In-vitro* system, L-ASP has been shown to induce cytotoxicity in a wide range of cancer cell lines, including acute lymphoblastic leukemia, acute myeloid leukemia (AML), and various solid tumors. The cytotoxicity of L-ASP is thought to be due to its ability to deplete extracellular L-asparagine, which is essential for the growth and survival of cancer cells that cannot synthesize their L-asparagine. However, L-ASP can also induce cytotoxicity in normal cells that rely on extracellular L-asparagine for their growth, such as lymphocytes, hepatocytes, and pancreatic acinar cells.

In *in-vivo* studies, L-ASP has been used as a standard of care in treating ALL for several decades and has been shown to improve the survival rate of patients. However, using L-ASP is associated with various adverse effects, such as hypersensitivity reactions, coagulopathy and liver dysfunction. In addition, some studies have reported that L-ASP can induce cytotoxicity in normal tissues, such as the pancreas, liver, and kidney, leading to long-term complications. Overall, the *in-vitro* and *in-vivo* cytotoxicity of L-ASP is a double-edged sword. It is essential for its therapeutic efficacy against cancer cells but can also lead to adverse effects and toxicity to normal tissues.

3.1. Bioavailability of L-ASP

The bioavailability of L-ASP refers to the fraction of the administered dose that reaches the systemic circulation and is available for distribution to target tissues. Various factors, including the route of administration, the formulation, and the pharmacokinetic properties of the enzyme can influence the bioavailability of L-ASP. Some key factors that affect the bioavailability of L-ASP:

- Route of administration: L-asparaginase can be administered intravenously (IV) or intramuscularly (IM). IV administration typically results in higher bioavailability than IM administration due to the more rapid absorption and higher plasma concentrations.
- Formulation: The formulation of L-asparaginase can influence its bioavailability. For example, encapsulation of L-ASP in liposomes or other carriers can improve its bioavailability by protecting it from degradation and improving its pharmacokinetic properties.
- Pharmacokinetics: The pharmacokinetic properties of L-ASP, such as its half-life and clearance rate, can also affect its bioavailability. L-ASP with a longer half-life or slower clearance rate can have higher bioavailability due to the more prolonged exposure to target tissues.
- Interactions with other drugs: L-ASP can interact with other drugs that affect its absorption, distribution, metabolism, or excretion, leading to changes in its bioavailability.

Overall, the bioavailability of L-ASP is a crucial factor that determines its efficacy and toxicity in clinical settings. The optimization of the formulation and administration route of L-ASP, as well as the management of drug interactions, can improve its bioavailability and enhance its therapeutic benefits for cancer patients.

Depending on the L-ASP source, the significant challenges of its pharmaceutical application are the hypersensitivity that it causes in ALL patients and its short half-life and fast plasma clearance in the blood system by native proteases. Today most commercial L-ASP is producing from bacterial source mainly *Erwinia* and *E.coli*. Due to its bacterial nature, immune responses to L-ASP during treatment can occur in between 30 and 70% of patients and have been associated with the generation of potentially neutralizing antibodies [30].

4. Conclusion

L-ASP is one of the key enzymes known for anti-tumor activity used in cancer treatment. However, it possesses some weak points such as pH and temperature sensitivity, short half-life and side effects, such as immune response, and temporary blood clearance, complicating the treatment procedure. The immobilization of the enzyme can solve these side effects. Compared to enzyme conjugation and cross-linking approaches, where an enzyme is partly exposed to the environment, the encapsulation technique avoids the abovementioned side-effects and improves performance properties, such as selectivity, stability against degradation, shelf-life, affinity, as well as acute toxicity.

Choosing of type of encapsulation system is crucial in therapy due to its effect on the performance parameters of the L-ASP. The analysis of the reports showed that almost all encapsulation systems allow for reducing immune response due to screening the enzyme from antibodies, as well as prolonging its half-life. However, the enzyme's activity and stability depend on the encapsulation system type. Therefore, while designing an encapsulation system, attention must be paid to the disposition of enzyme inside the core matrix, which directly affects enzyme activity.

Considering the research results on encapsulating L-ASP into different encapsulation systems and comparing their resulting performance, it can be suggested that the promising systems attracting the researcher's interest are biodegradable and biocompatible materials, such as chitosan, alginate, liposomes, among others. For example, all reports on chitosan encapsulation systems confirmed that toxicity of encapsulated L-ASP is lower than that of free enzyme, they have enhanced catalytic activity, increased stability *in vitro* and anti-cancer effects, higher thermostability and a wider range of pH stability in comparison with those of free L-ASP, also chitosan improves encapsulation efficiencies of L-ASP nanoparticles. It is worth noting that each encapsulation system has its advantageous feature, which results in the improvement of one or more properties of the enzyme.

In conclusion, the use of carriers for the delivery of L-ASP has shown promising results in reducing its toxicity to healthy cells while maintaining its efficacy against cancer cells. Various carrier systems, such as liposomes, nanoparticles, and hydrogels, have been investigated for the delivery of L-ASP, and have shown improved therapeutic outcomes in preclinical and clinical studies. However, the development of carrier systems for L-ASP delivery is still a field of active research, and further studies are needed to optimize the carrier design, improve their safety and efficacy profiles, and evaluate their long-term effects in clinical settings. Nevertheless, with continued progress in carrier-based delivery

systems, L-ASP has the potential to become a more effective and safer treatment option for cancer patients.

CRediT authorship contribution statement

V.S.S.L. Prasad Talluri: Conceptualization, Investigation, Visualization, Writing – original draft. **B. Mutaliyeva:** Supervision, Validation, Writing – original draft, Writing – review & editing. **A. Sharipova:** Investigation, Funding acquisition, Writing – original draft. **V. Ulaganathan:** Investigation, Visualization, Writing – original draft. **S.S. Lanka:** Investigation, Writing – original draft. **S. Aidarova:** Validation, Writing – review & editing. **A. Suigenbayeva:** Investigation, Writing – original draft. **A. Tleuova:** Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data availability

No data was used for the research described in the article.

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