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Research Article

Conjugation and encapsulation of L-asparaginase: an effective strategy for augmenting enzyme potential for anticancer activity

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Abstract
Extensive research is being conducted for cancer treatment but eradicating cancer cells is a great challenge till date due to lack of understanding regarding cancer development and manifestation in the systemic and local environment. L-asparaginase (ASNase), a well-known drug since decades for treatment of paediatric Acute Lymphoblastic Leukemia (ALL) is an enzyme that restricts the ability of the non-essential amino acid asparagine for tumor progression by breaking it down to aspartic acid and ammonia thereby depriving tumor cells from their key nutrient for survival. Though the enzyme has gained immense interest in clinical and scientific community the major challenge posed by the use of this specific enzyme in antilymphoproliferative studies is the induction of several immunogenic responses. In this review, we have highlighted the different encapsulation techniques using emerging carrier molecules to increase enzyme efficacy with a minimum negative impact on the living cells. Further we have highlighted the recent approach in which encapsulation of the enzyme using diverse nanoparticles which have proved its effectiveness in rendering target-specific action along with reduced immunogenicity, improved half-life, thermal stability and better enzyme activity.

Keywords
asparaginase, lymphoproliferative, immunogenicity, encapsulation, nano-particles

Abbreviations
AAR – amino acid response; ASNase – L-asparaginase; ALL – acute lymphoblastic leukemia; ASNS gene – asparagine synthetase; HMP – hexametaphosphate; mTOR – mammaliantarget of rapamycin; mTORC1 – mammaliantarget of rapamycincomplex 1; NMR spectra – nuclear magnetic resonance spectroscopy; PBS – phosphate buffered saline; PEG – polyethylene glycol; P-gp – P-glycoprotein; PHBV – poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PIC – polyion complex vesicles; PISA – polymerization-induced self-assembly; Poly(HEMA-GMA) – poly(2-hydroxyethyl methacrylate-glycidylmethacrylate); TPP – tripolyphosphate; UPR – unfolded protein response

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Introduction

The ethiology of cancer arises from aberrations in the DNA. Point mutations, inversion and deletions of chromosomes, base substitutions, dimer formation, and various other changes contribute to the different abnormalities that arise in the DNA. The information derived from the DNA thus drives the biosynthesis of different proteins that are required by an organism to perform its intrinsic activities. There are several factors like hormones and biomolecules (lipid, protein, carbohydrate, vitamins, etc.) that control the gene expression in a system. When this interconnected network is disturbed by any of the factors involved, abnormalities occur. Owing to the abnormalities and subsequent mutations caused by different agents, there is an up-regulation of protein biosynthesis. This phenomenon thus controls cellular division, differentiation, and uncontrolled proliferation, leading to malignancies.

Asparagine is a non-essential amino acid. Therefore, it can be synthesized by normal cells. The enzyme asparagine synthetase catalyzes the ATP - dependent conversion of aspartic acid and glutamine to asparagine (Asselin et al. 2015). The compound effect of asparagine exhaustion in the plasma level and the asparagine synthetase gene silencing in vivo indicated a deterrence in malignant cell growth (Hettmer et al. 2015). This suggested the role of asparagine in tumor growth. Previous studies pointed out that the hyper-activation of the mammalian target of rapamycin (mTOR) pathway promotes cell and tissue division (Sarbassov et al. 2005). Later, it was found that asparagine acts as an exchanger of amino acids thus, regulating the mammalian target of rapamycin complex 1 (mTORC1) mediated protein synthesis (Krall et al. 2016). In a healthy individual, the concentration of asparagine in the circulation is regulated between 40 and 80 μM (Boos et al. 1996; Vieira Pinheiro et al. 1999). Therefore, if the availability of asparagine is scarce within the system, hindrance is observed in protein synthesis and consequent cell division.
Lymphoblastic leukemic cells lack the Amino Acid Response (AAR) and the Unfolded Protein Response (UPR) pathway mediated ASNS gene (Asparagine synthetase) expression. This makes the lymphoblast auxotrophic for asparagine (Chiu et al. 2019), thus depending upon exogenous asparagine. If the externally available asparagine level is curbed down, the malignant cells are starved. This can cause a flattening of the cell proliferation curve. This is the junction where asparaginase finds its role as an anti-cancer drug. L-asparagine amidohydrolase, (E.C.3.5.1.1) is an enzyme that takes part in the hydrolysis of asparagine. The reaction consecutively yields aspartic acid and ammonia. Owing to its functional abilities, the enzyme is used for treating patients diseased with ALL and Non-Hodgkin Lymphoma. It was first reported by Kidd (Kidd et al. 1953). Broome went on ahead to establish its effectiveness as an anti-cancer drug (Broome et al. 1963).

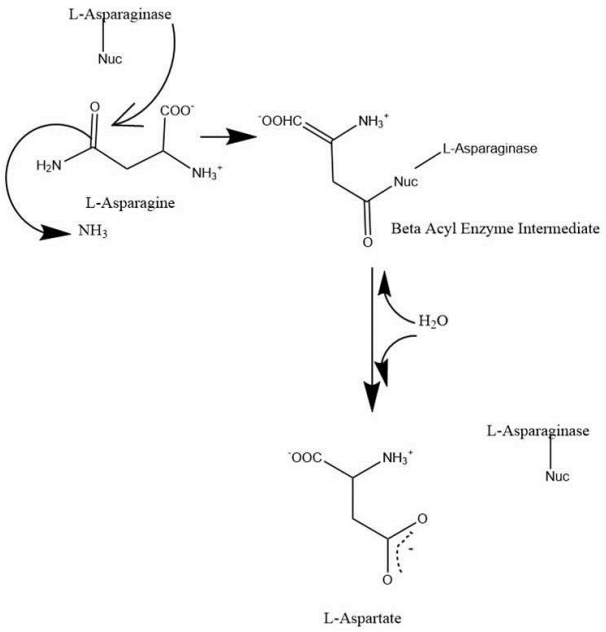


Figure 1. Mechanistic pathway of ASNase-mediated L-asparagine hydrolysis

Asparaginase has been extracted from several sources ranging from diverse bacterial strains to endophytes (Chow et al. 2015). In general, the enzyme ASNase is a homotetramer with 222-symmetry and belongs to the class of alpha/beta proteins. The enzyme is composed of four identical subunits namely A, B, C, and D. The active site of the enzyme is located between N- and C- terminal domains of adjacent monomers and hence can be treated as dimers of dimer (Kotzia et al. 2007). However, few formulations of the enzyme are used as per health standard protocols. They are asparaginase extracted from *Escherichia coli*

(commercially known as ELSPAR) which was approved in 1978, asparaginase extracted from *Dickeya dadantii* and the PEGylated form monomethoxy polyethylene glycol ASNase, (PEGASNase, commercially known as ONCASPAR) approved in 1994 and ASNase from *Dickeya dadantii* (ERWINASE), approved in 2011 (Heo et al. 2019). The degree of effectiveness of the drug ASNase against tumor will depend upon the affinity of the enzyme to its substrate. Thus by varying the substrate concentration the Km value for *E. coli* ASNase was determined to be 18±3 μM ASN and on the other hand, *D. dadantii* asparaginase reported K_m of - 33±6 μM ASN (Lanvers-Kaminsky 2017).

One of the major problems surrounding the use of asparaginase as an anti-cancer drug is the immune responses triggered by its administration. The modes of administration of the drug vary. It can be intramuscular or intravenous. Since, the source of asparaginase corresponds to bacterial origin, the immune cells (mainly the T-cells and dendritic cells) (Duval et al. 2002) trigger host response upon interaction with the antigen. Gene expression of the asparagine synthetase enzyme is observed in organs like the pancreas, liver, testes, brain, and thyroid. Therefore, the circulation of asparagine is prevalent at these sites, regulating various metabolic activities. Apart from hypersensitive reactions, the decrease in the level of asparagine in the body also causes various complications since it regulates enzymes like amylase and lipase (Piatkowska-Jakubas et al. 2008; Raja et al. 2012). The clinical symptoms of hypersensitivity caused by the drug administration are diverse. They are hemorrhage, puritis, hepatic dysfunction, erythema, fever, pancreatitis edema, urticaria, Quincke's edema, skin rashes, leukopenia, neurological seizures, and anaphylaxis (Duval et al. 2002; Nowak-Göttl et al. 2001; Panosyan et al. 2004).Hypersensitive reactions also include bronchospasm ketoacidosis, hypofibrinogenemia, hypercoagulable state-coagulopathies, hypoalbuminemia (Avramis et al. 2006). Reportedly 60% of the patients under this treatment show such complications (Shrivastava et al. 2016). Other than these complications, hepatic toxicities constitute the onset of oxidative stress, glutamine deficiency, and decreased hepatic protein synthesis (Bodmer et al. 2006; Fromenty et al. 1995; Fromenty et al. 1997).

Cancer is singularly responsible for killing millions of people worldwide. The commonly used treatment to address this disease has been limited to chemotherapy. There are several drawbacks related to this method including cytotoxicity and chemo resistance among the most widely studied ones. Besides, the chemotherapeutic agents often target non-specifically which leads to several other complications. Therefore, the need for agents that specifically target cancer cells and eliminate chemoresistance has arisen. To address these problems, nanoparticle-based therapy has emerged as a new trend since it can accumulate in solid tumors. This activity is facilitated by increased permeability and retention. Moreover, the use of nanoparticles can bypass the P-glycoprotein (P-gp) activity and reverse the multiple drug resistance. The pioneer application of PEGylation of chemotherapeutic agents marked the beginning of encapsulation to avoid side effects. To enhance the clinical efficacy of the molecule in recent years primary focus has been given on isolating novel enzyme from microbial sources, protein engineering, chemical modifications like covalent bonding, crosslinking and enzyme confinement. The present review deals with various encapsulation and immobilization techniques of ASNase by different synthetic and non-synthetic components to maximize the effectiveness of the drug and minimize the complications. The *in vivo* performance of the drug can be effectively increased by nano-encapsulation techniques. The carrier molecule will prevent the enzyme from the local environment hence protecting the enzyme from protease degradation, increasing half-life and in large extent reducing immunogenicity (Villanueva-Flores et al. 2021). Indeed, the encapsulation technique prevents the enzyme from getting identified by the immune system thus reducing clinical hypersensitivity. In spite of being an old drug molecule, intensive research and development of treatment strategies have given this enzyme a new dimension in the field of cancer research.

PEGylation of L-asparaginase

Polyethylene Glycol. Polyethylene glycols (PEG) are water soluble polymers having a wide range of biomedical application. The molecular weight of the polymer ranges from 200 to several million

depending upon repeating ethylene oxide units (Leung 2014). It has a neutral pH and insoluble in water. It consists of two terminal hydroxyl groups and repeating ethylene oxide subunits. The structure of the compound varies between linear and branched (Bailon et al. 1998). The PEGylation of substances generally includes easily alterable molecules. The PEGylation of protein-like structures involves nucleophiles as reactive groups. This category includes thiol, an α-amino group, epsilon amino group, carboxylate, hydroxylate (Veronese 2001). The polyethylene glycol involved in the PEGylation of asparaginase is the branched type. Two linear chains joined by a trifunctional space mark the structure of a branched PEG. The carboxylic group in the spacer is activated as - OSu (**hydroxysuccinimidyl esters of carboxylated PEGs**). This ensures protein binding (Veronese 2001). This structural attribute of PEG is advantageous since it accounts for lower inactivation of the enzyme.

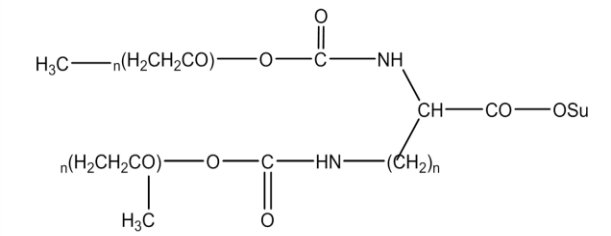


Figure 2. Structure of branched PEG

Facilitated by its structure, PEG can become hydrated in an aqueous solution. Owing to its hydrophilic nature, it is in constant motion while in solution. This helps create a barrier around the enzyme which protects it from proteasome degradation and being identified by antibodies (Kozlowski and Harris 2001). The steric hindrance induced by the branched-chain polymer and its umbrella-like conformation provides for better results (Veronese et al. 1996;Veronese et al. 1997). The pharmacokinetic properties of PEG depend upon factors like the molecular weight and site of administration (Mehvar et al. 2000). The binding affinity of the PEG to the enzyme depends on several physico-chemical parameters as well. They include hydrophobicity, electrostatic affinity, steric hindrance, and conformations (Molineux 2002). Initially, PEGylation was done using unbranched polyethylene glycol.

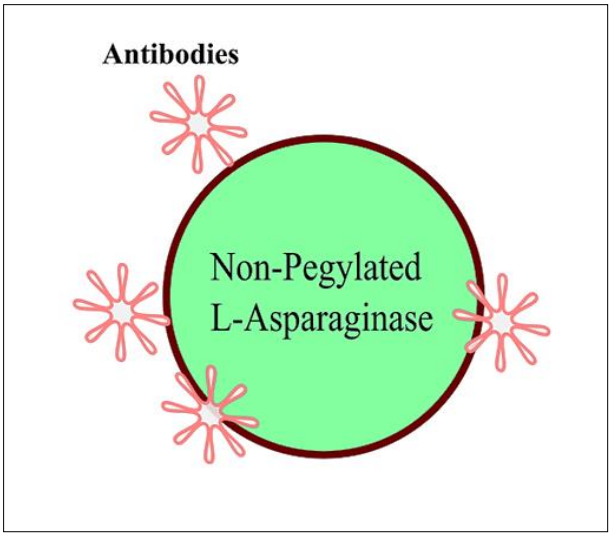


Figure 3a. The absence of PEG allows degradative enzymes and antibodies to come in contact with the enzyme surface

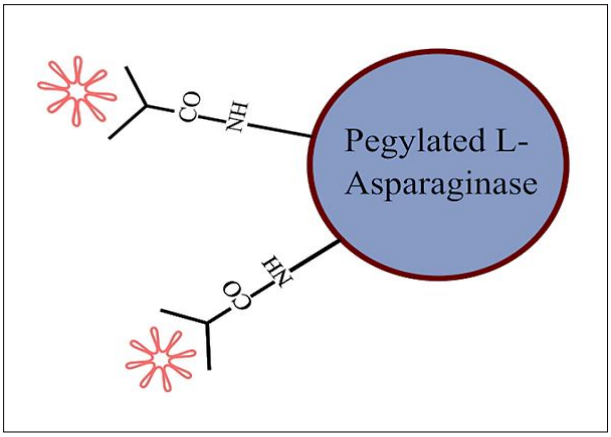


Figure 3b. The structure of the PEG prevents degradation of enzymes and antibodies from coming in contact with the enzyme surface (*Adapted by Veronese et al. 1997*)

The PEGylation of proteins or enzymes hinders the approach of antibodies. However, the same character of PEG also deters the incoming substrates. This acts as a shortcoming of the PEGylation technique. Nonetheless, it was later found out that the use of branched PEG curbed the problem and increased the effectiveness of the compound. A conformational analysis using NMR spectra exhibited that the PEGylation of a protein does not distort its structure (Ravera et al. 2016).

PEGylated L-asparaginase and its anticancer activities. The treatment regime adopted for cancer patients incorporate the use of ASNase as an anti-cancer drug. In this specific regime, the first line of treatment is done using *E. coli* derived ASNase and PEGASNase (ONCOSPAR). PEGylation of asparaginase reduces immunogenic potential and extends the half-life of the enzyme from 1-3 d to 5-7 d and decreases the number of dosages.

During the 1970s, various experimental observations established *E. coli*-ASNase as an anti-cancer drug. Currently, the limelight has shifted towards it again due to its effective activity in the therapy of non- Hodgkin lymphoma. It has also shown efficacious results against extra nodal natural killer/T cell patients (Yong et al. 2003; Yamaguchi et al. 2008; Jaccard et al. 2009). The PEGylation of *E. coli* derived asparaginase is done using monomethoxy polyethylene glycol. This confers various advantages to the clinical use of the enzyme as a drug. It increases the half-life and prevents identification by degradative enzymes or antibodies. However, these escalated changes do not change the antineoplastic action of the enzyme (Avramis et al. 2009). The clinically suggested dosage of *E. coli* derived asparaginase amounts to 2500 IU/m^2 (Shinnick et al. 2013). Clinical studies showed the administration of different doses of the PEGylated asparaginase to patients newly diagnosed with ALL. The dosage range corresponded to the values of 500 $U.m^{-2}$, 1000 $U.m^{-2}$, and 2000 $U.m^{-2}$. Two doses were given each week. It was observed that the disappearance of PEG-ASNase was low, almost 5% (Lanvers-Kaminsky et al. 2020). It can be administered via intravenous or intramuscular routes. An analysis of several patients receiving PEGylated asparaginase via intravenous and intramuscular route exhibited a 32.2% hypersensitive response in the case of IV against 13.3% of IM. Another clinical trial demonstrated reduced anxiety in patients upon IV administration of the drug (Place et al. 2015).

The treatment of cancer with ASNase faces certain drawbacks owing to its natural origin. Since they are mostly derived from bacterial sources, they have a reduced half-life in the blood serum. The degradation of the enzyme by proteolytic enzymes and its identification by antibodies contribute to the conflicting phenomenon (Lanvers-Kaminsky 2017; Avramis et al. 2006; Shrivastava et al. 2016; Narta et al. 2007; Patel et al. 2009; Liu et al. 2009). PEGylation, on the other hand, addresses these shortcomings and increases the stability of the enzyme in the serum by securing the epitopes (Mishra et al. 2016; Veronese and Mero 2008; Milla et al. 2012).

PEGylation causes an inverse relationship between pharmacokineticsm and pharmacodynamics. The increase in the degree of PEGylation causes a decrease in pharmacodynamic properties. It includes catalytic activity and substrate affinity (Rodríguez Martínez et al. 2008). An 8% decrease in the catalytic activity has been recorded. Clinical trials also substantiate the observation (Avramis et al. 2002). Other disadvantages include the generation of anti-PEG antibodies. Clinical trials confirmed the role of anti-PEG antibody in the clearance of PEGylated asparaginase from the blood serum if patients suffering from ALL (Garay et al. 2005). A study was conducted to observe if pre-sensitization of animal models can improve the activity of the bacteria-derived enzyme. The observation indicated the development of anti-PEG antibodies in 88% of animals. Therefore, it was concluded that pre-sensitization causes no change to the pharmacokinetic properties of the enzyme or prevents hypersensitive response (Poppenborg et al. 2016).

Since a patient is subjected to the PEGylated enzyme for a prolonged period, there is a higher chance of development of antibodies against the enzyme molecule (Garay et al. 2005; Armstrong et al. 2007; Hashimoto et al. 2014). These problems can be addressed by the conjugation of PEG at pre-determined sites on the enzyme interface (Shaunak et al. 2006; Jevševar et al. 2010; Zhang et al. 2012). This helps in keeping the active site free from obstruction. This ensures the sustenance of the pharmacodynamics properties.

To improve the efficacy of the ASNase drug, a study was conducted where site-directed mutagenesis was executed. The cysteine residue was targeted in this experiment. The experimental method involved the conjugation of the enzyme subunits with site-specific conjugation. This helps avert the loss of activity of the enzyme caused due to dissociation. The conjugated entity exhibited a similar efficiency of activity to the native form of the drug. The mechanism behind this phenomenon might influence a rise in the hydrodynamic size.

This consequently reduces the glomerular filtration and contributes to an improved half-life along with complete retention of the effectivity (Ramirez-Paz et al. 2018).

The appearance of hypersensitive reaction upon the use of *E. coli* asparaginase or PEG-asparaginase prompts the use of *Pectobacterium carotovorum* derived asparaginase as the second line of treatment. However, it shows hypersensitive reactions too. The disadvantage consists sofa reduced half-life in the bloodstream. Moreover, 17% of patients undergoing this treatment exhibited hypersensitive reactions (Van den Berg 2011; Vrooman et al. 2010). This gave rise to the need for an improved form of the enzyme. When inspected in a mouse model, the PEGylated form of the enzyme recorded an increased half-life and a relatively lower immunogenic response. It showed improved results in an *in vivo* study where it increased the viability by 7 to 12 d with a dose of 2-20 $U.kg^{-1}$. Therefore, the PEGylation of *P. carotovorum* derived asparaginase can suffice for a potential cancer treatment drug. Reports also suggest a 65% retention of V_{max} in comparison to that of the original (Chien et al. 2014).

In a study, asparaginase was PEGylated using methoxy polyethylene glycol carboxymethyl N-hydroxysuccinimyl ester. It recorded an extended half-life, improved stability, and was impervious to the action of asparaginyl endopeptidase and similar degradative enzymes. It also showed potency against leukaemia cell lines in an *in vitro* environment. Therefore, PEGylated asparaginase established itself as a potential anticancer drug. However, since PEG generally targets lysine residues, the conjugation of the compound to the adequately available lysine residues caused polydispersity. An approximation of 62-82 molecules of monoethoxylated PEG can be conjugated to ASNase (Meneguetti et al. 2019).

Another report suggested the use of N-hydroxy succinimide ester of monoethoxy polyethylene glycol hemisuccinate as a PEGylating agent. It was used to conjugate asparaginase derived from *P. Carotovorum* (Melik-Nubarov et al. 2017).

A different study demonstrated the use of PEG-chitosan and glycol-chitosan as conjugative agents. The ASNase was derived from *P. carotovorum*. It showed an improved catalytic efficiency by a factor of 3 in comparison to the original enzyme. The conjugate exhibits an improved thermostability as well (Sukhoverkov and Kudryashova 2015).

Several clinical trials have been conducted over time to establish the mechanism and nature of the PEGylated form of asparaginase in cancer patients. It was reported that the use of PEG-ASNase caused hyperammonemia in children suffering from ALL. Eight samples were used for the study. It was found out that seven out of eight people showed an ammonia level greater than 100 µmol/L. The incident might have been a result of excessive generation of ammonia caused by the hydrolysis of asparagine and glutamine (Heitink-Pollé et al. 2012).

There are several implications of the use of PEGylated asparaginase. A patient reported acute functional liver failure upon being treated with the modified form of the enzyme. The condition was recovered by the application of plasmapheresis treatment (Göpel et al. 2016).

However, the potential advantages of PEGylating the enzyme includes improved biological activity, the improved half-life of the biological agent, lowered degradation by enzymes, reduced toxicity profile, lowered frequency of administration (Bowen et al. 1999; Gabizon et al. 1997; Harrington et al. 2001; Molineux et al. 1999).

Nano-encapsulation of L-asparaginase

L-asparaginase has a wide application in the therapy of diseases associated with malignancy. Such diseases include Hodgkin's disease, acute lymphoblastic leukaemia, retinosarcoma, lymphosarcoma, melanosarcoma, and several others (Nadumane et al. 2016). To incorporate ASNase in the treatment protocol of these diseases, the enzyme properties need to be optimized. The affinity of the enzyme for its substrate should be high with increased thermal stability and an extended half-life. To achieve these criteria, the immobilization or encapsulation technique is applied. The process includes the attachment of an enzyme to a support material via different forms of interaction to increase the stability of the enzyme. Over a decade, researchers have been invested in finding novel methods to encapsulate ASNase to make it suitable for extensive use in the cancer treatment regimen.

A potential approach strategizing an improved activity of ASNase uses Fe_3O_4-chitosan carriers. This combined entity works under the influence of a weak magnetic field. The conjugate maintained high thermal stability along with an efficacious behaviour across a wide pH range. The reusability was examined as well, by using it through 16 cycles. The outcome suggested 60.5% retention of activity. It also maintained 50% of its activity after 4-week storage at 4°C. This value was obtained as a result of implementing specific frequency control in a weak magnetic field (Ates et al. 2018).

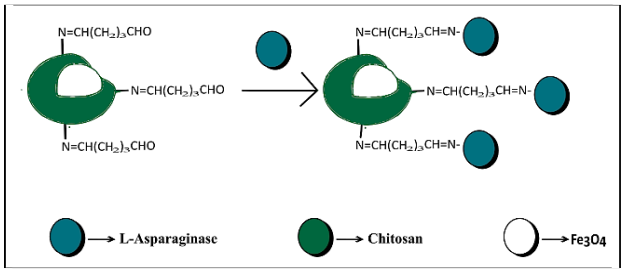


Figure 4. Encapsulation of L-asparaginase by Fe_3O_4-chitosan *(Adapted by Ates et al. 2018)*

ASNase derived from *E. coli* was modified using Poly - (N-vinylpyrrolidone-co-maleic anhydride). The improved form of the enzyme showed a reduced immunogenic response towards the anti-ASNase antibodies. It also corresponded to a higher stability against proteolytic enzymes. The incubation of the conjugate in the presence of trypsin retained 80% of its original activity in comparison to 14% for the native enzyme. The modification of the enzyme using poly-(N-vinylpyrrolidone-co-maleic anhydride) caused a pH shift towards the alkaline scale by a unit of 0.8. It also demonstrated a 10°C higher optimum temperature than that of the native form (Karsakevich et al. 1988; Qian et al. 1997).

Similar results were obtained using polyaniline nanofiber. It is widely being used as a carrier owing to its porous character and high specific area. It is a nanoparticle by nature and works by binding covalently to the enzyme. The immobilized enzyme shows a 2.05-fold reduction in the K_m value and an increased V_{max} value of 90.57 $U.min^{-1}.mg^{-1}$. Besides, it also demonstrated the retention of 70% of its activity at the optimum pH 8.5, whereas the native enzyme accounts for only 30% (Ghosh et al. 2012). Starch is a preferred carrier for immobilization because of its porosity. A study was conducted using poly (methyl methacrylate)-starch conjugate. The K_m yielded a value 8-fold less than that of the native enzyme. It also retained its functionality by 60% upon a storage duration of 1 month (Ulu et al. 2016).

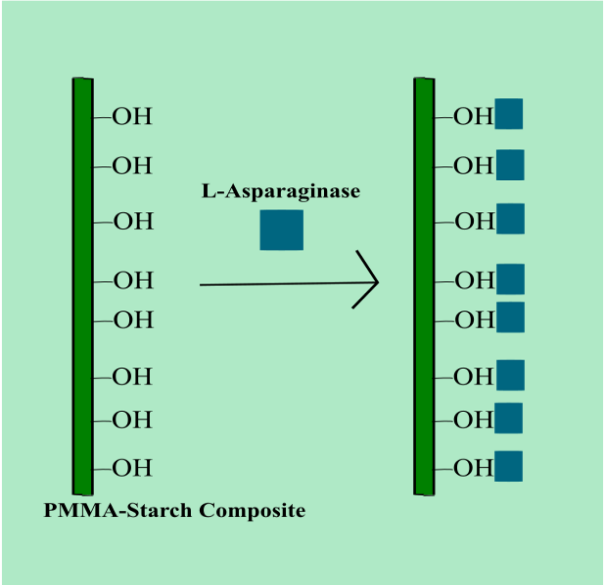


Figure 5. Encapsulation of ASNase by poly (methyl methacrylate)-starch conjugate *(Adapted by Ulu et al. 2016)*

The encapsulation of ASNase was also done using phosphorylcholine-based polymer. It was achieved by implementing a two-step procedure. The remodelled enzyme demonstrated an increased half-life along with an improved plasma circulation time (Zhang et al. 2016).

Magnetic nanoparticles are widely being used these days because of their increased surface area. It is also facilitated by low-cost production. Owing to the biocompatible nature of the nanoparticles, the improvement caused by using magnetic core-shell structure Fe_3O_4@MCM-41 as an encapsulating agent was accounted for a lower K_m value. This consequently meant a greater affinity of the enzyme for the substrate. It also has a 1.15 fold reduction in the K_m value and an improved effectivity over an extended range of temperature and pH (Ulu et al.2018). Poly (2-hydroxyethyl methacrylate-glycidyl methacrylate), Poly (HEMA-GMA) is a nanoparticle of size 117.5 nm. The ASNase enzyme exhibited an attachment to it at an amount of 66.42 $mg.g^{-1}$ nanoparticle. A pH shift was observed

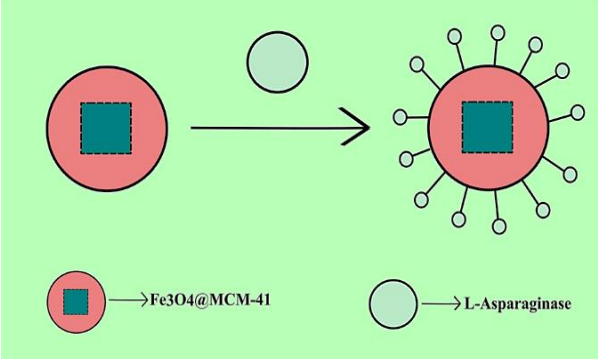


Figure 6. Encapsulation of L-asparaginase by Fe_3O_4@MCM-41 *(Adapted by Ulu et al. 2018)*

inclined towards the acidic scale. The optimum temperature accounted for a 10°C rise in comparison to the native form and was determined to be 55°C. After a 10-hour study implemented for the determination of the thermal stability, the derived results remained in favor of the modified enzyme by 50%. The immobilized conjugate not only corresponded to a lower K_m value but also retained 74.74% of its activity in an artificial serum medium (Orhan and Uygun 2020).

Chitosan nanoparticle is an effective vehicle for entrapment and delivery of the enzyme ASNase. Chitosan, a natural polymer, has gained immense importance in current research due to its safe, biocompatible, and biodegradable nature. To construct a stable chitosan nanoparticle having pharmaceutical utility ionotropic gelation method is reported (Debnath et al. 2011; Saeed et al. 2020). The polyanion cross-linkers like hexametaphosphate (HMP), tripolyphosphate (TPP), dextran sulphate interacts with the amino sugar monomeric unit by electrostatic interaction imparting mechanical strength to the nanoparticle. The studies focus on the entrapment efficiency of the drug and the mode of release of the enzyme. The enzyme ASNase showed burst release from the nanoparticle followed by a controlled release pattern. The release pattern was influenced by hydrogen bonding of glycerol in the release medium, pH, and ionic strength of phosphate buffered saline (PBS). The hygroscopic nature of glycerol facilitated the inflow of medium inside the nanoparticle and leads to diffusion of the entrapped enzyme. There is an increased half-life of immobilized enzyme of 64 days compared to that of free ASNase (only 33 h). The research depicts that at pH 5.7 the negatively charged ASNaseis linked with the chitosan chains by electrostatic interaction before the addition of TPP. Thus, the enzyme not only acts as a drug but also as a cross-linker that strengthened the nanoparticle. The research work also led to a finding that compared to free ASNase, immobilized enzyme was more resistant to high temperature and alkaline pH (8 to 9), showed increased half-life as well as high mechanical strength owing to slow degradation (Katas et al. 2013).

Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV nanocapsules were targeted on increasing *in vivo* half-life of modified ASNase with conjugation of polyethylene glycol (PEG). The modification is expected to protect the enzyme from denaturation during nanocapsule preparation. The research work reported that the combination of chemically modified ASNase encapsulated in lower molecular weight PHBV resulted in increased enzyme activity compared to unmodified ASNase. The activity increased from 0.074 to 0.429 $U.mg^{-1}$ nanocapsule. The increased activity reported is due to the higher permeability of lower molecular weight PHBV nanocapsule and higher stability of modified PEG conjugation. Now to determine the *in vivo* half-life of the enzyme, the pharmacokinetics of the drug was evaluated. The study showed that there was a sharp drop of enzyme activity of unmodified PHBV nanocapsule in the blood. So, to overcome the problem, heparin-coated PHBV nanocapsules were synthesized. Heparin being a hydrophilic molecule it extended outward in the aqueous phase creating a steric barrier and hence the nanocapsule showed a longer duration of circulation in blood. The experimental analysis reported 50% of the enzyme activity after 6 hours. Radioactivity study also confirmed that at 8 hours heparin-coated nanocapsule was detectable in the blood whereas only 30% radioactivity remained in circulation after 3 hours in case of unmodified nanocapsule.

The study also confirmed no adverse effect of the encapsulated ASNase PHBV nanocapsule when injected on mice compared to the unmodified enzyme having hypersensitivity reaction. Thus, the modified nanocapsule can be used as a potential therapeutic tool for cancer treatment (Baran and Hasirci 2002).

Liposomal encapsulation is an effective approach for targeted drug delivery without inhibiting the activity of the drug. Phospholipids, the main components of liposome, form a bilayer membrane that helps the drug to get encapsulated and then delivered to the targeted site imparting maximum release onsite. In the study, ASNase isolated from *Pectobacter iumcarotovorum* was loaded into liposome by the hydrated thin-film method. The enzyme encapsulation efficiency into liposome carrier was reported to be 53.99 ± 5.44%. This nanoencapsulation method enhanced potency, reduced toxicity as well as increased the half-life of the enzyme. However, one limitation of liposomal delivery is the size of the particles. Larger liposomes are opsonized and removed by the phagocytic system from blood. Liposomes less than 200nm are best suited for this purpose and help in prolonged retention time in the bloodstream and showed significant improvement in antitumor activities in lung carcinoma tumor-induced BALB/c mice (Afrin et al. 2002).

In a separate study, researchers determined the enhanced enzymatic properties of ASNase by encapsulating it using silica gel. The conjugate reported optimum activity in a pH range of 7.0 - 7.4, which corresponds to the pH of the body. The modified form also showed 80% retention of its activity after being incubated in 50 units of trypsin for 1 h. The K_m was estimated using the Lineweaver Burk plot to yield a value of 2.29±0.10 mM, while that of the native enzyme remained at 3.2 ±0.07 mM (Golestaneh and Varshosaz 2018).

Another experimental approach included the use of a hydrogel matrix developed from polyethylene glycol albumin. The improved enzyme showed retention of 90% of its activity even upon being stored for 50 days in comparison to 43% of activity retention by the native enzyme. This conjugation also helped in improving biocompatibility (Jean and Fortier 1996).

Several methods have been adapted to curb the issues concerning the efficacious activity of ASNase. A comparatively modern approach involves the production of enzymes loaded in the vesicle. It is based on size-selective permeability. The technique involved is referred to as polymerization-induced self-assembly (PISA). This capacitates the enzyme to execute its therapeutic activities, while remaining confined within a domain. The vesicle-loaded enzyme demonstrated colloidal stability coupled with proteolytic stability. It retained its activity upon 18 hours of incubation in chymotrypsin. Antibodies exhibited a two-fold less affinity towards the improved form in comparison to that of the native form. Despite such improved characters, the vesicle-loaded enzyme exhibited a similar metabolic pathway and tumor cell growth inhibition to the native enzyme (Blackman et al. 2018).

Immobilization of L-asparaginase

The different carriers that are used to immobilize or encapsulate the enzyme are organic, inorganic, or composite by nature.

Recent advancements in improved formulation of L-asparaginase. Since L-asparaginase is used extensively in the treatment regime of a wide variety of diseases associated with malignancy, numerous researchers are highly involved in the improvement of the enzyme. Apart from those that have already been highlighted previously in this review paper, there are several other emerging trends in this field. Calaspargasepegol is a form of PEGylated asparaginase where the SS linker present in polyethylene glycol has been substituted by succinimidyl carbamate. This attaches more stability to the polymer (Brumano et al. 2019). Another innovative approach is the PASylation technique. This technology has been developed by an Irish biopharmaceutical company that currently holds a license for it. The peptide residues of proline, alanine, and serine form a structural conformation similar to that of polyethylene glycol. Moreover, since alanine and proline are devoid of side chains, they do not interact using the side chains (Ahmadpour and Hosseinimehr 2018). The use of solid lipid nanoparticles (Sharma et al. 2019), calcium-alginate beads (Bahraman and Alemzadeh 2017) as masking agents, have also gained momentum in this genre. Another potential approach invested in the development of an effective ASNase formulation is the recombinant gene technology and bioengineering of metabolic pathway. In a particular study, the ASNase gene isolated from a halo-thermotolerant *Bacillus* strain was incorporated within the genetic material of *E. coli* strains (Safary et al. 2019).

Table 1. Immobilization of ASNase using different carriers

Immobilizing carrier	Optimum pH	Optimum temperature	Remarks	Reference
1. Organic carriers				
i. Albumin	6.8	55	The immobilization of ASNase with albumin was investigated in a mouse model having lymphosarcoma. Besides, the pharmacokinetic properties of the improved enzyme were also examined in a pancreatic tumor cell line. The results corresponded to increased resistance towards degradation and effective anti-tumor activity. It also contributed to the inhibition of malignant cell growth in a pancreatic tumor cell line	(Poznansky et al. 1982)
ii. Dextran	8.5	-	Dextran can be used as an immobilizing agent as well. It conjugates with the enzyme via covalent bonding. It showed less degradation by proteolytic enzymes. On the contrary, a reduction in activity was observed as well. In *in vivo* studies, the immobilized conjugate showed reduced immunogenic reactions	(Wileman et al. 1986)
iii. Colominic acid	-	37	Colominic acid can be classified as a polysialic acid with a low molecular weight. ASNase derived from the bacterial source of *P. carotovorum* was immobilized using colominic acid. The immobilized form retained 82-86% of the original activity. It was coupled with the maintenance of a similar Km value to that of the native enzyme. During an *in vivo* study conducted in a mouse model, it showed resistance towards degradation. It also sustained 65-83% of its initial activity and an increased half-life was reported	(Fernandes et al. 1997)
iv. Polyacrylamide gel	7	-	It also resisted degradation and brought about the complete decomposition of L-asparagine in the blood	(Mori et al. 1974)

2. Inorganic carriers				
i. Activated carbon	7	60	It binds covalently to the enzyme with a 100% retention of its activity even at 80°C. Further, the IC_{50} value obtained via using HCT-116 cell line was reported to be 218.7 µg/ml	(Maysa et al. 2010)
ii. Aluminium oxide	7.5	37	The improved version of the enzyme had a reduced K_m value.	(Agrawal et al. 2018)
3. Composite carrier				
i. Ca-alginate	8.5	50	Such a group of substances includes calcium alginate. The enzyme derived from *E. coli* upon undergoing immobilization showed optimal activity over an extended range of temperature and pH.	(Youssef and Al-Omair 2008)
4. Carbongels				
i. p(HEMA-GMA) cryogels	8.5-10	60	The enzyme in cryogel carrier matrix exhibited improved stability at extreme conditions (pH and temperature) and also showed high catalytic efficiency.	(Noma et al. 2021)
ii. Xerogel	It can adapt o both acidic and alkaline environment.	37	The enzyme retained 71% of original activity upon immobilization and there was a 3.9-fold increase in enzyme affinity for the substrate,	(Barros et al. 2022)

The initiative yielded an improved outcome that showed that recombinant strain from *Bacillus* sp.showed higher specificity for L-asparagine than L-glutamine. Also the enzyme proved to be highly thermostable and pH resistant (4.5-10.0) Several other studies concerning this particular niche have been discussed in greater detail (Vidya et al. 2017). Polyion complex vesicles (PICs), made of pairs of oppositely charged diblock co-polyelectrolytes, have emerged as novel nanocarriers and have proved to preserve enzyme folding and activity. When administered intravenously to mice it was observed that the blood circulation time of PICs-ASNase was prolonged compared to free ASNase (Villanueva-Flores et al. 2021). In a very recent study, it was observed that co-encapsulation showed a promising result in terms of drug efficacy and reduced toxicity. Co-encapsulation process enables multiple drugs into a single nanocarrier molecule. A dextran-based nanocarrier molecule was developed where co-encapsulation of ASNase and drug etoposide showed an excellent cellular uptake in chronic myeloid leukemia (Konhäuser et al, 2022). Potent antioxidant obtained from most common natural extracts like herbs, spices, vegetables, fruits can also be co-encapsulated with ASNase to improve its therapeutic efficacy (Kolev 2022).

L-asparaginase since its discovery and development as an anti-cancerous drug has become one of the major molecules for chemotherapeutic treatment especially for ALL. Apart from curing leukemia, the enzyme has also proved its potential in treating lung cancer, ovarian cancer, and breast cancer. Despite being a successful therapeutic agent for decades it has been observed that the enzyme has several shortcomings including reduced toxicity, immunogenicity, thermostability as well as reduced half-life. Though the drug is been successfully used to treat patients, it is still one of the most widely researched molecules across the globe. The availability of the molecule from various microbial sources and molecular analysis provided the researcher to unlock several information regarding structural and pharmacological properties of the enzyme. These properties of the enzyme further helped in better understanding of different techniques of encapsulation, immobilization, and chemical modification. The current review thus gives an overview of all possible new as well as modified techniques of immobilization and encapsulation of the enzyme to come up with some positive results in terms of biopharmaceuticals. The enzyme can also be modified by implementation of recent recombinant technologies and understanding the molecular pathway for development of bio betters in terms of drug safety and efficacy. Thus, ASNase has a wide array of application in the field of cancer research and a lot of studies and researches are still going on in order to achieve novel strategies for successful therapeutic application.

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