PTD-Mediated Red Blood Cell Encapsulation of L-Asparaginase for Potential Treatment of Acute Lymphoblastic Leukemia

by

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To my parents Shin Kwan and Young Soon Chung

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ALL	Acute lymphoblastic leukemia
ASN	L-Asparagine
ASNase	L-Asparaginase
AS	Asparagine synthetase
ASP	L-Aspartic acid
FPLC	Fast protein liquid chromatography
GOT	L-Glutamate oxaloacetate transaminase
HBSS	Hank's balanced salt solution
HMDS	Hexamethyldisilazane
LMWP	Low molecular weight protamine
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin content
MDH	L-Malate dehydrogenase
NADH	Nicotinamide adenine dinucleotide, reduced
PBS	Phosphate buffered saline
PCV	Packed cell volume
PTD	Protein transduction domain peptide
R-HBSS	Reversed Hanks balanced salt solution
RBCs	Red blood cells
RDW	Relative distribution width
RES	Reticuloendothelial system
SEM	Scanning electron microscopy
SPDP	N-Succinimidyl 3-(2-pyridyldithio) propionate
TR	Traut's Reagent

Abstract

One of the primary drugs used in treatment of acute lymphoblastic leukemia is L-asparaginase (ASNase), which despite its therapeutic efficacy, faces challenges often faced in protein drug treatment: the immunogenicity and short half-life of the drug. In order to overcome these major challenges, the red blood cell (RBC) encapsulation method has been employed to prevent degradation by serum proteases and elimination by reticuloendothelial system, thereby increasing circulation half-life of the protein drug. However, the encapsulation methods used thus far, such as hypotonic dialysis/resealing and electroporation, showed compromise in the oxygen transport function and structural integrity of RBCs.

Herein we introduce a novel RBC encapsulation method that involves low molecular weight protamine (LMWP), a protein transduction domain (PTD) peptide. Protein drug was first conjugated to LMWP via disulfide bond and the cell penetrating property of LMWP allowed encapsulation of protein drug into RBCs as verified by the confocal microscopy images. The optimal encapsulation condition was determined to be 37 °C for 30 minutes. Hematological parameters as well as SEM and osmotic fragility curve of LMWP-ASNase encapsulated RBCs showed that the structural integrity was maintained while Hill coefficients and pO_{50} values indicated oxygen transport functionality was maintained. LMWP-mediated encapsulation method considerably increased the circulating half-life of ASNase compared to that of the hypo-osmotic

rupture/resealing method and improved the survival time of tumor-bearing mice by 44% compared to the saline control group.

Based on these data, novel method for encapsulating protein drugs into intact and fully functional RBCs was established for potential treatment of acute lymphoblastic leukemia.

Chapter 1

Background

Acute lymphoblastic leukemia (ALL)

Acute lymphoblastic leukemia (ALL) is cancer of the white blood cells, the cells that normally fight infections. As the number of lymphocytes increase in the blood and bone marrow, there is also less room for healthy white blood cells, red blood cells, and platelets. As a consequence ALL patients often suffer infections, anemia, and easy bleeding. Almost 4000 cases of ALL are diagnosed annually in the United States alone, approximately two thirds of which are in adolescent children, making ALL the most common cancer in this age group. Indeed, ALL represents 23% of the cancer diagnoses among children younger than 15 years of age, occurring at an annual rate of 30 to 40 per million[1]. While a cure rate of ~80% was estimated for childhood ALL, the experience with adult ALL was far less rewarding, as the reported cure rate seldom exceeded 40%[2].

L-Asparaginase

One of the primary drugs used in the treatment of ALL is L-asparaginase (ASNase). ASNase was first discovered in 1953 by Kidd in guinea pig serum[3] and has been in clinical use since 1967[4]. ASNase is an enzyme that hydrolyzes amino acid L-asparagine (ASN) to L-aspartic acid (ASP) and ammonia. Most human tissues can self-synthesize ASN from L-glutamine by the action of asparagine synthetase (AS). Certain neoplastic tissues, including ALL cells, however, express significantly lower levels of AS and thus

have to rely solely on extracellular source of ASN to maintain protein synthesis[5]. Systemic depletion of ASN by ASNase would therefore impair protein biosynthesis in these cells, leading to their deaths through cellular dysfunction[6].

However, ASNase therapy is not without problems. Aside from hypersensitivity, which will be addressed in the next section, the use of ASNase can result in liver dysfunction. The elevated transaminase, abnormal bilirubin and alkaline phosphatase levels, and depression in albumin and lipoprotein levels have been observed.[7] Other toxicities of ASNase include coagulation abnormalities, pancreatitis, cerebral dysfunction, parotitis, and immune suppression.

ASNase formulations currently in use originate from two bacterial sources: *Escherichia coli* and *Erwinia chrysanthemi*. The active form of enzyme is tetrameric with each monomer containing an active site and has an overall molecular weight of 133-140 kDa. The specific activity of purified ASNase ranges between 300-400 umole of substrate/min/mg of protein. The isoelectric point lies between pH 4.5-5.5 for the *E.coli* enzyme and 8.6 for *Erwinia* enzyme.[8] The Km is approximately 1 x 10^{-5} M.[9] ASNase is not adsorbed from the GI track and thus, in clinical use, is normally administered intravenously or intramuscularly.

Problems with Protein Drug Delivery and Remedies

As with most protein drugs, the clinical application of ASNase faces two major obstacles. First, due to the bacterial origin of ASNase its clinical use is associated with a high incidence of hypersensitivity. ASNase can trigger significant immunological consequences that include activation of B-lymphocytes and production of antibodies

causing severe anaphylactic reactions. When a patient has a reaction, the route of administration, form or source of drug is changed. The reaction rate with intravenous (iv) administration of the free form is very high. For this reason the free enzyme is almost always given by an intramuscular (im) or subcutaneous (sc) route. Most reactions occur within one to several hours after administration and include signs and symptoms typical of anaphylaxis, sometimes including death.

Secondly, as with most protein drugs, ASNase is susceptible to degradation by serum proteases and elimination by the reticuloendothelial system (RES). The plasma half-life of ASNase is estimated to be in the range of 8-30 hrs.[10] This rapid clearance necessitates frequent injection of large doses, further elevating the possibility of inducing immunological responses.

To overcome such problems of short circulating half-life and immunogenicity, various remedies have been attempted. The most successful or commonly employed methods to-date includes: attaching hydrophilic polyethylene glycol (PEG) to enzyme drug, or encapsulating drug into soluble, synthetic (e.g. polymers) or natural (e.g. liposomes, cells) carriers. Attachment of PEG chains to enzyme drug increases its mass as well as shields the enzyme from proteolytic degradation, improving pharmacokinetics of the drug.[11]

Indeed, the PEG-modified ASNase, with a trademark name of pegaspargase, has been successfully developed during the 1970s, with its first clinical trial in the 1980s. Clinical results showed that attachment of PEG molecule increased the half-life of ASNase from 26 hrs to 12 -15 days[13]. In addition, because of the assistance of PEG in alleviating detection by the host immune system, this new form of ASNase was better

tolerated than the free form, especially when given intramuscularly. Hence, pegaspargase has been specifically indicated for treating ALL patients who are sensitive to native ASNase. According to a review of clinical data, in re-induction therapy for patients who were hypersensitive to E. coli-derived ASNase, pegaspargase was able to reduce the frequency of drug administration from 6-9 times to 1-2 times per therapy. Nevertheless, pegaspargase has not yet been proven to be superior to E. coli ASNase for the first remission of ALL. Most critically, pegaspargase failed to completely abort the anaphylactic responses in patients who are hypersensitive to ASNase therapy.

Red Blood Cells (RBCs) as Drug Carrier

Among all carriers employed for ASNase encapsulation, the use of RBCs (red blood cells; RBCs) as the drug carrier appears to be most appealing, simply because the RBCs would not only protect the loaded protein drug from proteolytic degradation but also prevent detection of the drug by the host immune system. Furthermore, RBCs are completely biodegradable without generation of toxic products, and they are also biocompatible, particularly when autologous RBCs are used. In addition, RBCs are the most abundant cells of the human body ($5.4x10^6$ and $4.8x10^6$ RBCs/mL in men and women, respectively), therefore giving an affordable source of supply for use in drug encapsulation. Moreover, the biconcave disk shape of RBCs endows them with the highest surface to volume ratio ($1.9x10^4$cm/g) usable for drug encapsulation.[14] Most critically, RBCs possess a lifespan in circulation of approximately 120 days, which is significantly longer than any of the currently existing drug carriers. A detailed discussion

of the benefits of utilizing RBCs as the drug carrier can be found in a review article authored by Hamidi and Tajerzadeh.[15]

RBC Loading Methods

A variety of methods have already been developed to entrap protein drugs into RBCs. The most adapted techniques thus far include drug (e.g. primaquine, hydrocortisone, etc.)-induced endocytosis[16], electroporation[17], and hypo-osmotic-based pre-swelling[18], rupture/resealing[19, 20] or dialysis[21]. Using these methods to create sufficiently large pores or perturbations on the cell membrane, a number of the impermeable protein drugs including ASNase, erythropoietin, acetaldyhyde dehydrogenase, and alcohol dehydrogenase have been successfully loaded into RBCs.

Despite reasonable success, all of these methods are still beset by a host of shortcomings. The most crucial drawbacks come from two aspects following RBC processing. First, these techniques all require the application of a chemical (drug-induced endocytosis), electrical (electroporation), or mechanical (osmotic dialysis) force to the RBC membrane to create sufficiently large pores for the protein drug to diffuse through. Such disruption of the cell membrane often leads to partial, but irreversible, deterioration of the structural integrity and morphology of the RBC. A significant alteration of the RBC morphology from the native discocyte form (i.e. normal RBCs with a small area of central pallor and biconcave disc shape) to stomatocyte (i.e. abnormal RBCs with oval or rectangular area of central pallor) following treatment by electroporation[22] and hypo-osmosis[23] was observed (see Figure 1). Consequently, these processed RBCs will be

recognized by the phagocytic system as foreign entities, rendering their rapid destruction and clearance by the host immune system.

The second issue is that RBCs processed by any of the existing encapsulation methods, regardless of whether it is electroporation or hypotonic dialysis, would inevitably result in a loss of important cellular constituents, such as hemoglobin and cytoskeleton, from the cells. This is because all of these methods rely on a pore-opening and a resealing step, both of which involve a dialysis procedure.[20, 24] Thus far, the largest protein encapsulated in RBCs by using such methods was alcohol oxidase from *Pichea pastoria*,[25] which had a molecular weight (675 kDa) that was 10-fold larger than that of hemoglobin (65 KDa); the major constituent of an RBC. Since dialysis is an equilibrium process and with such large pores being created on the cell membrane, in theory and practice, it is inevitable that a certain portion of the cytosolic constituents including hemoglobin, glutathione, and cytoskeleton would be leaked out of the RBC. Indeed, loss of hemoglobin was clearly observed in RBCs treated with the hypotonic dialysis, and electroporation method, as evidenced by a decrease in mean corpuscular hemoglobin (MCH) (see Table 1) and presence of a pinkish color after resealing.[20]

It should be noted that aside from the principal activity of oxygen transport, RBCs also carry out other important biological functions, such as energy (ATP)-involved metabolic processes and scavenging of oxidative stressors.[26] Hence, a loss of hemoglobin would not only impair the oxygen transport function of RBCs, but also affect their ability to manage oxidative stress. Similarly, a loss of cytoskeleton from the RBCs would compromise it with a much weakened structural integrity, rendering it prone to destruction or recognition by the phagocytic system. Structural integrity of RBCs can be

evaluated by examining osmotic fragility of RBCs. As evidenced by osmotic fragility test, the RBCs that underwent hypo-osmotic dialysis procedure showed hemolysis at all osmotic pressures indicating existence of various cell populations, with the majority of hemolysis occurring at lower values of the osmotic fragility curve. On the other hand, normal RBCs displayed "sudden" hemolysis resulting in an osmotic fragility curve with a sigmoidal profile.[27]

It is important to point out that in order to inherit the benefits of RBCs as a natural and long-lasting drug carrier, it is absolutely essential to retain both the structural and functional integrity of the cell. Yet, all of the existing RBC encapsulation methods fail to recognize this critical point. Therefore, there is a need of a method that would permit encapsulation of therapeutically active protein drugs into fully functional RBCs.

PTD-Mediated Drug Loading

Recently, a family of small but extraordinarily potent membrane permeable peptides, classified as "PTD" (protein transduction domain) peptides that include TAT[28], ANTP[29], VP22[30], poly (arginine) peptides[31], and the non-toxic, naturally occurring low molecular weight protamine (LMWP) developed in our laboratory[32, 33] have been discovered. Both in vitro and in vivo studies revealed that, by covalently linking PTD to almost any type of molecular species including proteins (MW > 150 kDa; more than 60 different proteins have already been tested[34]), nano-carriers[35, 36], and liposomes[37], PTD was able to ferry the attached species across cell membranes.

More specifically, when a fusion protein of TAT, the most widely studied PTD, and β-galactosidase was injected into mice by intraperitoneal injection, β-galactosidase

activity was observed in every organ and tissue, including kidney, heart, and even brain[28]. This finding suggests that intracellular protein uptake mediated by the PTD peptide is not receptor- or transporter-dependent as different types of cells cannot possess the same types of receptors and transporters. In addition, PTD-mediated cell internalization did not induce perturbation or alteration of the cell membrane.[38] Based on this conclusion, in theory all cell types, including RBCs, should be transducible and PTD peptides could potentially be applied as a powerful tool to achieve non-invasive encapsulation of biologically active protein therapeutics into intact and fully functional cells.

LMWP

In order to encapsulate the therapeutic protein ASNase into RBCs, we propose the use of LMWP, one of the PTD peptides. LMWP is an arginine-rich peptide created by enzymatic digestion of protamine with thermolysin which results in 5 different fractions designated TDSP (thermolysin-digested segmented protamine). The fraction of the choice, TDSP-5, and now termed LMWP, has the sequence of VSRRRRRRGGRRRR and has molecular weight of 1880. Initially developed as a non-toxic antagonist to heparin and low molecular weight heparin (LMWH), the LMWP sequence bears close similarity to many of the arginine-rich PTD peptides, such as TAT, which has amino acid sequence of GRKKRRQRRRPPQ. When HeLa cells were incubated with LMWP labeled with FITC (fluorescein isothiocyanate), a commonly used fluorescence tag, LMWP displayed cellular uptake comparable to that of TAT[44].

LMWP has several unmatched advantages over other existing PTDS. First, unlike other PTDs that rely solely on chemical synthesis for their production, LMWP can be

manufactured in mass quantities using enzymatic hydrolysis and a single step purification system. Second, unlike most other PTDs that are derived from viral sources and, thus, present health concerns, LMWP is obtained from digestion of native protamine, a FDA approved clinical drug. Third, unlike all existing PTDs, the toxicology profile of LMWP has already been thoroughly established; the LMWP peptide is neither toxic nor immunogenic[33, 39, 40, 41]. Last but not least, since LMWP possesses only one single -NH2 group at the N-terminus, its conjugation to a protein drug can be precisely regulated and easily carried out using our established N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) activation method.[42]

Research Objectives

In this thesis dissertation, PTD-mediated RBC encapsulation of ASNase for potential treatment of acute lymphoblastic leukemia is investigated. The main goals of the project were to: 1) successfully produce LMWP-conjugated ASNase with sufficient retention of enzyme activity; 2) verify encapsulation of LMWP-ASNase conjugate in RBCs, optimize the RBC loading process, and determine whether PTD-mediated loading procedure alters functionality and/or physical properties of RBCs; and finally 3) determine whether encapsulation of ASNase in RBCs will extend circulating half-life of ASNase as well as extend survival time of tumor-bearing mice.

Loading Method	Electroporation		Hypo-osmotic Dialysis	
Species	Mouse		Human	
Parameters	Native RBCs	Loaded RBCs	Native RBCs	Loaded RBCs
MCV (fl)	52.1 ± 0.5	71.9 ± 0.9	94 ± 2	79 ± 1
MCH (pg)	17.5 ± 0.4	15.1 ± 0.9	32.0 ± 0.8	25.6 ± 0.9
MCHC (g/dl)	33.6 ± 0.7	21.1 ± 1.4	34.2 ± 1.1	33.1 ± 0.7
n	11	4	16	16

Table 1. (a) Hematological parameters for mouse RBCs encapsulated with alcohol dehydrogenase loaded by electroporation under optimal experimental conditions (420 V, four pulses of 1ms every 15 min. at 37°C; resealing for 1 hr at 37°C; n = number of experiments (means ± S.E.M.)) and (b) human RBCs encapsulated with xxx by dialysis bag method (45 min). The RBC suspensions were adjusted to 70% haematocrit and 4 mg/mL of L-asparaginase (115 ± 4 units/mL) (means ± S.E.M.).

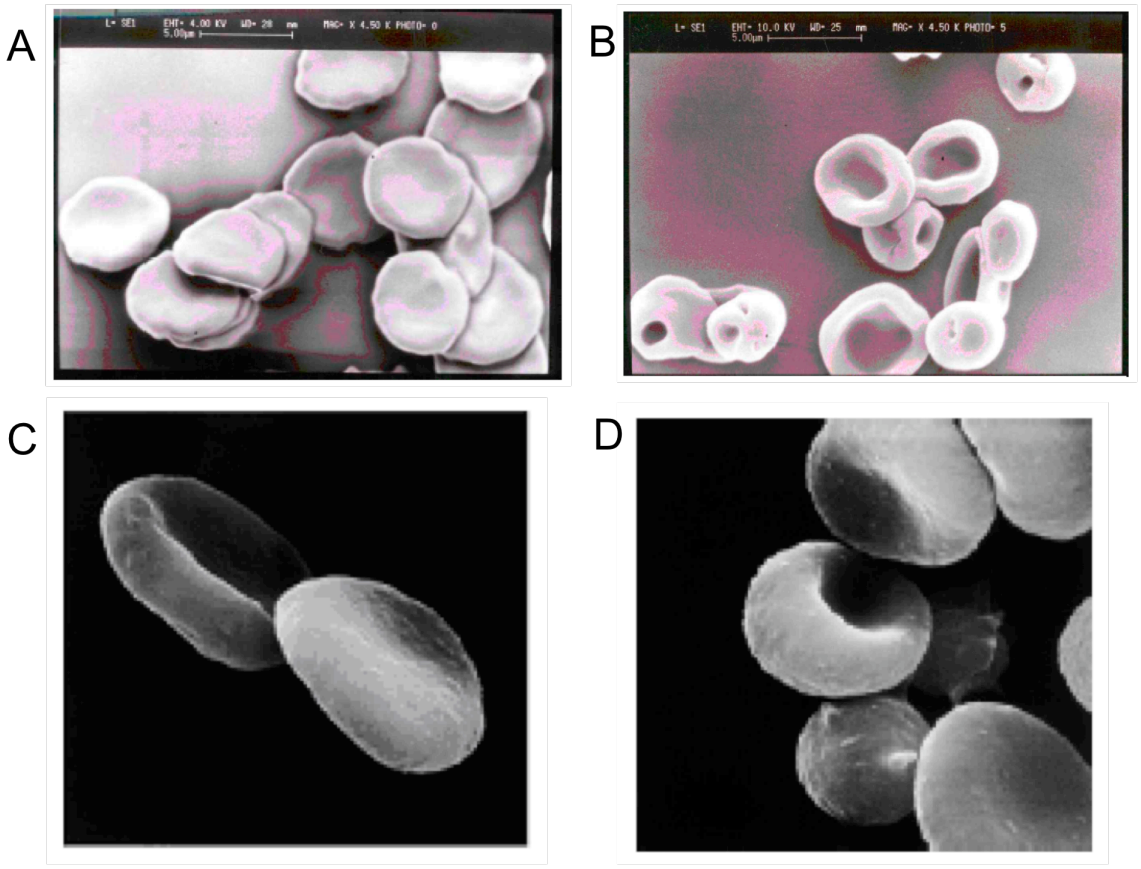


Figure 1. Scanning electron micrographs of human RBCs before (A) and after (B) undergoing hypotonic preswelling method; mouse RBCs before (C) and after (D) loading by electroporation method (magnification 35000).

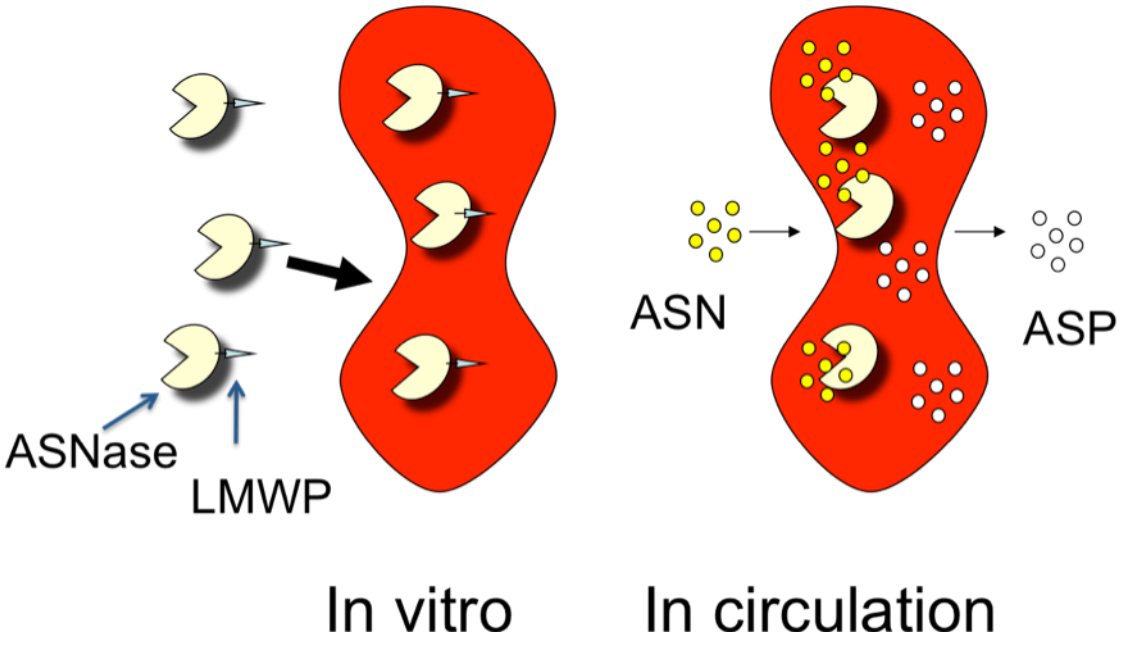


Figure 2. Schematic of LMWP-mediated ASNase loading into intact RBCs.

Chapter 2

LMWP-ASNase: Conjugation and Characterization

Introduction

Despite various methods used to encapsulate protein drugs into RBCs the circulating half-life as well as the structural and morphological integrity of RBCs show room for improvement. To this regard we proposed use of PTD-, more specifically LMWP-mediated RBC encapsulation to resolve these issues. The bioconjugation of LMWP to ASNase was then necessary. Another consideration was the type of linkage used between LMWP and ASNase. In order to avoid the possibility of LMWP-mediated leaching of ASNase out of the RBCs use of a disulfide bond was proposed. These bonds have been known to break under reducing environment, in this case, cytosol of RBCs, and will allow ASNase to remain inside upon encapsulation.

Conjugation of LMWP to ASNase via a disulfide bridge can be accomplished in three steps (see Figure 3) by use of two key reagents: Traut's reagent (also known as 2-iminiothiolane) and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP).

Traut's reagent, also known as 2-iminiothiolane, is a thiolating reagent used in the preparation of disulfide and thioether linked conjugates. It has a preference for primary amino groups and reacts at pH 7 - 10 to give amidine compounds that contain free sulhydryl groups. The amidine linkage preserves the original primary amine positive charge.[43] SPDP is a heterobifunctional cleavable cross-linking agent. It contains one amine reactive N-hydroxysuccinimide (NHS) residue and one sulfhydryl reactive pyridyl

disulfide residue. The NHS-ester cross-linking reactions should be performed in buffers such as phosphate, bicarbonate/carbonate and borate buffers that do not contain primary amines. The optimum pH for this reaction is between 7 and 8.

In the first step, ASNase is reacted with Traut's reagent to create a free thiol group. In the second reaction, a 2-pyridyl-disulfide group is introduced into LMWP by the reaction between the primary amine group of the peptide and the N-hydroxysuccinimide ester side of SPDP. Finally sulfhydryl exchange occurs between the thiol group of ASNase and the 2-pyridyl-disulfide group of LMWP. Pyridine-2-thione is released and its concentration can be determined by measuring the absorbance at 343 nm.

One of the important aspects of chemical modification of ASNase to keep in mind is its enzyme activity. Chemical modification of an enzyme drug has been known to alter its activity depending on the site of modification. When conjugating LMWP to ASNase it is necessary to control for the degree of conjugation while retaining sufficient specific enzyme activity to preserve its therapeutic effect.

For the RBC-encapsulated ASNase to function properly in vivo, it is crucial for the enzyme to maintain its therapeutic activity during the encapsulation process as well as in circulation. In this chapter we describe: first, the chemical reaction and purification steps undertaken to conjugate LMWP to ASNase by a disulfide bond; second, the characterization of the final product MALDI-TOF MS method, followed by effect of the conjugation process on specific enzyme activity of ASNase; and last, the storage stability of the final product.

Materials and Methods

LMWP-ASNase Preparation

Low molecular weight protamine (LMWP) was produced in our lab according to the method previously described[44]. To activate LMWP, a five molar excess of SPDP (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO and added drop-wise to LMWP (5 mg/mL) dissolved in phosphate buffer (0.1 M, with 1mM EDTA, pH 8.0), and the mixture reacted at room temperature for 2 hours. Activated LMWP detectable at 214 nm was purified by HPLC on a heparin affinity column (Buffer A: 50 mM phosphate, 1 mM EDTA, pH 7.5; Buffer B: 50 mM phosphate, 1 mM EDTA, 2 M NaCl, pH 7.5) followed by concentration using an ultrafiltration cell with a 500 MWCO membrane (Spectrum Laboratories, Inc. Rancho Dominguez, CA). Degree of activation was determined by monitoring pyridine-2-thione generation, detectable at 343 nm, upon addition of DTT (dithiothreitol) to purified LMWP-PDP.

ASNase (Elspar, Ovation Pharmaceuticals, Inc., Deerfield, IL) was dissolved in 0.1 M HEPES buffer containing 5mM EDTA and reacted with a 10 fold excess (per monomer) Traut's reagent (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 hour and purified by HPLC using two desalting columns in series (HiTrap, GE Healthcare, Piscataway, NJ; isocratic, Buffer A: 50 mM phosphate, 1 mM EDTA, pH 7.5). Monitoring the fractions at wavelength of 254 nm allowed detection of both ASNase-SH and unreacted Traut's reagent. Degree of thiolation was determined using Ellman's Reagent (Sigma-Aldrich, St. Louis, MO), which reacts with free sulfhydryl groups. Finally, to the thiolated ASNase, 5 fold excess activated LMWP (relative to thiol)

was added to react at room temperature for 2 hours and the final product was purified by HPLC on a heparin affinity column (Buffer A: 50 mM phosphate, 1 mM EDTA, pH 7.5; Buffer B: 50 mM phosphate, 1 mM EDTA, 2 M NaCl, pH 7.5) and concentrated using an ultrafiltration cell with a 10,000 MWCO membrane (Spectrum Laboratories, Inc.).

MALDI-TOF Analysis

MALDI-TOF-MS method was used to determine actual degree of LMWP conjugation to each ASNase monomer. MALDI-TOF-MS analysis was run in linear mode on Waters Tofspec-2E, calibrated with bovine serum albumin. The data was acquired and processed using Masslynx 3.5 software. Sinnapinic acid was used as matrix.

ASNase Activity Measurement

ASNase activity was determined by direct nesslerization of produced ammonia. In this reaction, ASNase is incubated with its substrate ASN at 37 °C, pH 8.6, for a specified time and generated ammonia is captured by Nessler's reagent. More specifically, for each measurement, a microcentrifuge tube containing 850 uL of 60 mM ASN in Tris-HCl (50mM, pH 8.6) and 100 uL Tris-HCl (50mM, pH 8.6) was pre-incubated at 37 °C for 5 minutes to achieve temperature equilibration. To this solution, 50 uL of LMWP-ASNase or native ASNase solution was added and incubated for 10 minutes before quenching the reaction with 100 uL of trichloroacetic acid (1.5 N, Sigma-Aldrich, St. Louis, MO). The reaction mixture was centrifuged at 15000 rpm for 3 minutes and 50 uL of the supernatant was added to a microcentrifuge tube containing 700 uL of DI H_2O and 100 uL of Nessler's Reagent (Sigma-Aldrich, St. Louis, MO). The resulting solution was incubated for 15 minutes at room temperature before measuring

absorbance at 480 nm. Enzymatic activity unit is defined as μmol ammonia produced per minute. Specific activity of native ASNase ranged from 206 to 259 units/mg of protein.

LMWP-ASNase Thermal Stability

Solution of LMWP-ASNase at a concentration of 50 ug/mL was prepared. Three aliquots of this solution was stored at 4°C and another three at 37°C. At specific time points, 50 μL from each vial were collected and measured for enzyme activity by direct nesslerization.

LMWP-ASNase Storage Stability

Two hundred microliter aliquots of LMWP conjugated- or native- ASNase solutions (~1 mg/mL each) were prepared in reversed Hank's balanced salt solution (R-HBSS; KCl 10.18 g/L, KH_2PO_4 0.1 g/L, $NaHCO_3$ 1.273 g/L, NaCl 0.316 g/L, Na_2HPO_4 0.08 g/L, glucose 2.0 g/L; pH adjusted to 7.4 with H_3PO_4, 85 % w/v)[45] and stored at 4, -20, and -80 °C. At specified time points, one vial was removed from each storage location and diluted to 50 ug/mL before analysis by direct nesslerization.

Results and Discussion

LMWP-ASNase Conjugation and Characterization

With the use of heterobifunctional linker SPDP and Traut's reagent, LMWP-ASNase linked by disulfide bond was synthesized. The SPDP activated LMWP is easily separated from the biproducts and excess SPDP with use of a heparin affinity column, as seen by FPLC chromatogram in Figure 4. With Traut's reagent and ASNase both absorbing at 254 nm, the ASNase and excess Traut's reagent fractions were sufficiently separated

when two desalting columns were used in series with the thiolated ASNase eluting at approximately 2.6 minutes (Figure 5).

LMWP-ASNase conjugation products were purified by FPLC on a heparin affinity column. The elution profile shown in chromatogram (Figure 6) in conjunction with MALDI-TOF analysis of separated fractions verified that ASNase fractions eluted in order of increasing degree of LMWP conjugation, positive charge from LMWP contributing to increased affinity to heparin.

The initial RBC loading experiment indicated that the amount of ASNase encapsulated was higher for the fraction with greater degree of LMWP-modification. When the ASNase fractions eluting at 0.6 M and 1.2 M NaCl were compared for RBC encapsulation, the fraction at 1.2 M showed significantly higher loading as seen in Figure 7. The MALDI-TOF MS analysis also verified that the fraction at 1.2 M had a higher degree of LMWP conjugation per ASNase tetramer than the fraction at 0.6 M (data not shown).

With the base peaks at *m/z* 34526, *m/z* 36639, *m/z* 38761, and *m/z* 40828 corresponding to ASNase monomer, monomer with one LMWP, two LMWPs, and three LMWPs, respectively, MALDI-TOF-MS results indicated that up to 3 LMWP peptides could be conjugated to each ASNase monomer, or up to 12 LMWP peptides per ASNase tetramer, the active form. At the end of conjugation and purification process, 55 - 60 % of the original specific enzyme activity was retained.

LMWP-ASNase was found to have V_{max} and K_M values of 627.7 and 0.031, respectively, while corresponding values for native ASNase were 339.8 and 0.016. Although these Michael-Menton kinetic parameters differed for two ASNase forms, their

catalytic efficiency, the ratio between V_{max} and K_M, was comparable at 2.12 x 10^4 and 2.02 x 10^4, indicating that in terms of depleting systemic ASN level the two forms should be on par with each other.

In order to verify that the LMWP-ASNase conjugate will remain stable/active during storage before the loading process, storage stability test was performed at 4, -20, and -80 °C. Results indicated that while storage at 4 °C results in loss of slightly over 10 % of original activity over 25 days, the enzyme activity is well preserved when stored frozen at -20 and -80 °C shown in Figure 9.

One of the requirements for the RBC loading process is that the protein drug of interest remains active during the loading procedure and also during circulation in vivo. To test this issue, the conjugate stability at 4 and 37 °C was tested. While a slight decrease in enzyme activity was observed for LMWP-ASNase stored at 4 °C, the enzyme activity remained more or less constant when incubated at 37 °C for up to 72 hrs (Figure 10).

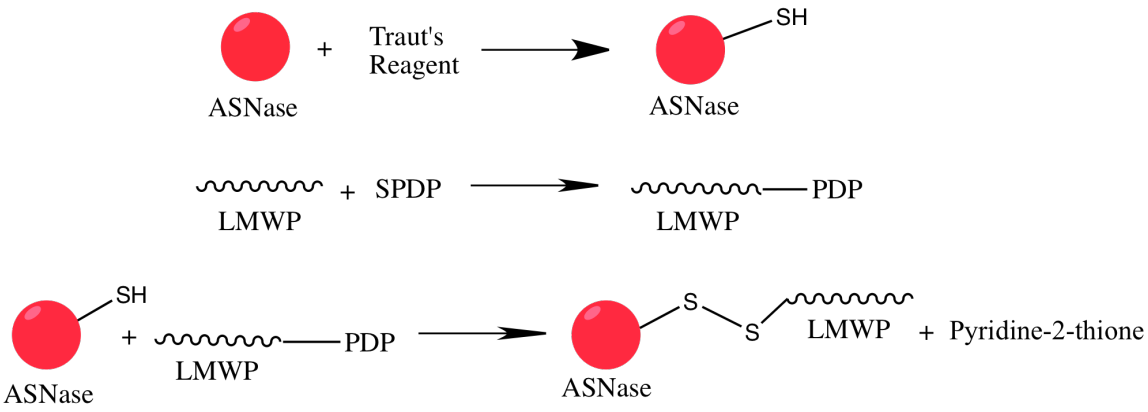


Figure 3. Schematic of LMWP to ASNase conjugation procedure. A disulfide bridge between ASNase and LMWP is created by activation of LMWP with SPDP and thiolation of ASNase with Traut's reagent.

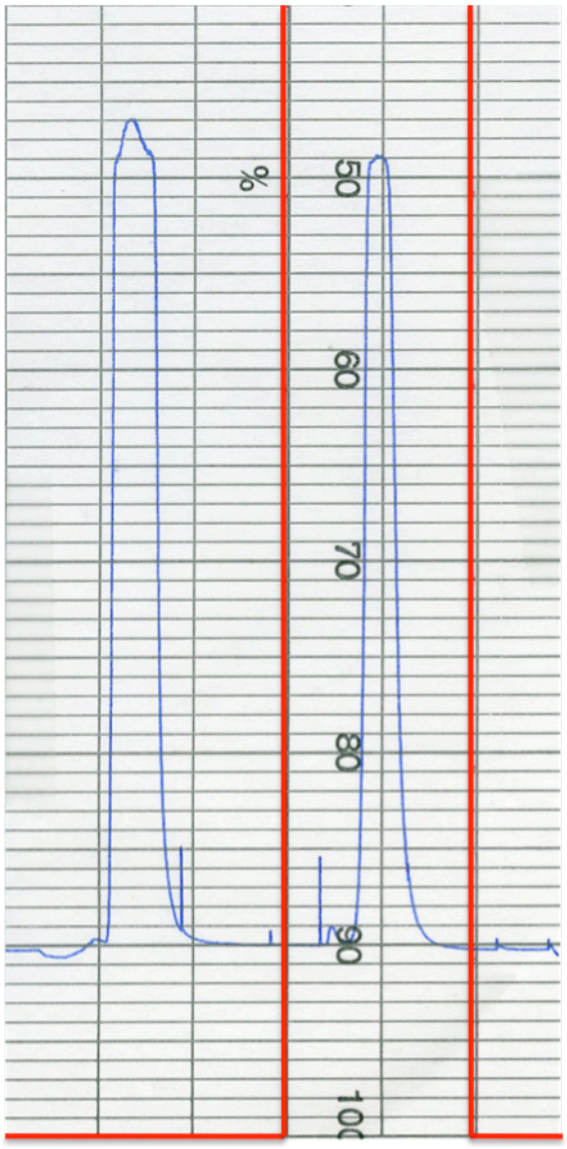


Figure 4. FPLC chromatogram of purification of SPDP activation of LMWP reaction product.

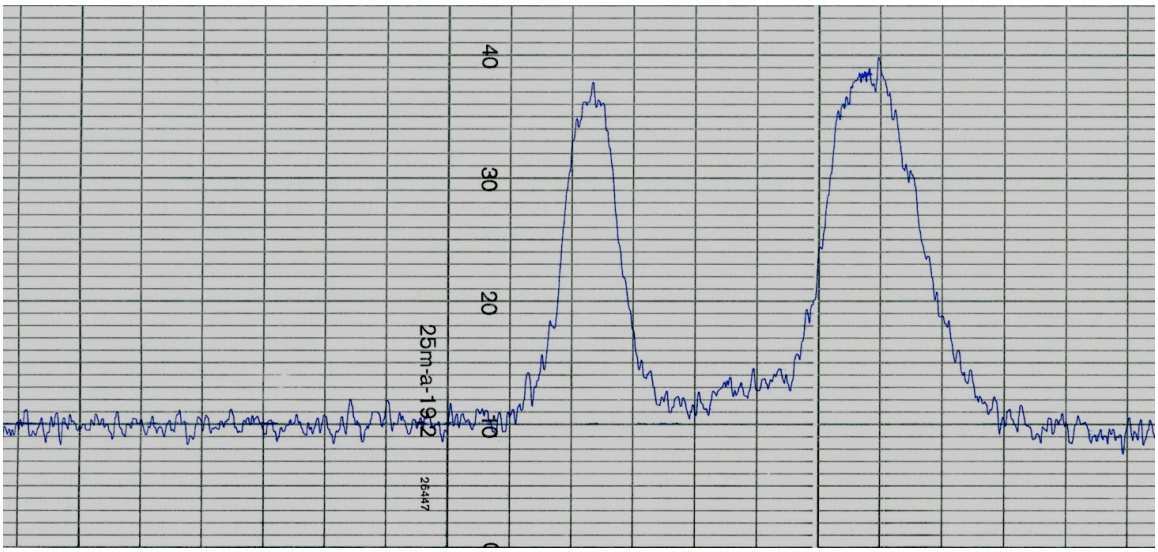


Figure 5. Purification of ASNase thiolation with Traut's reagent reaction by FPLC with two desalting columns in series. (Isocratic; Buffer A: 50 mM phosphate, 1mM EDTA, pH 7.5)

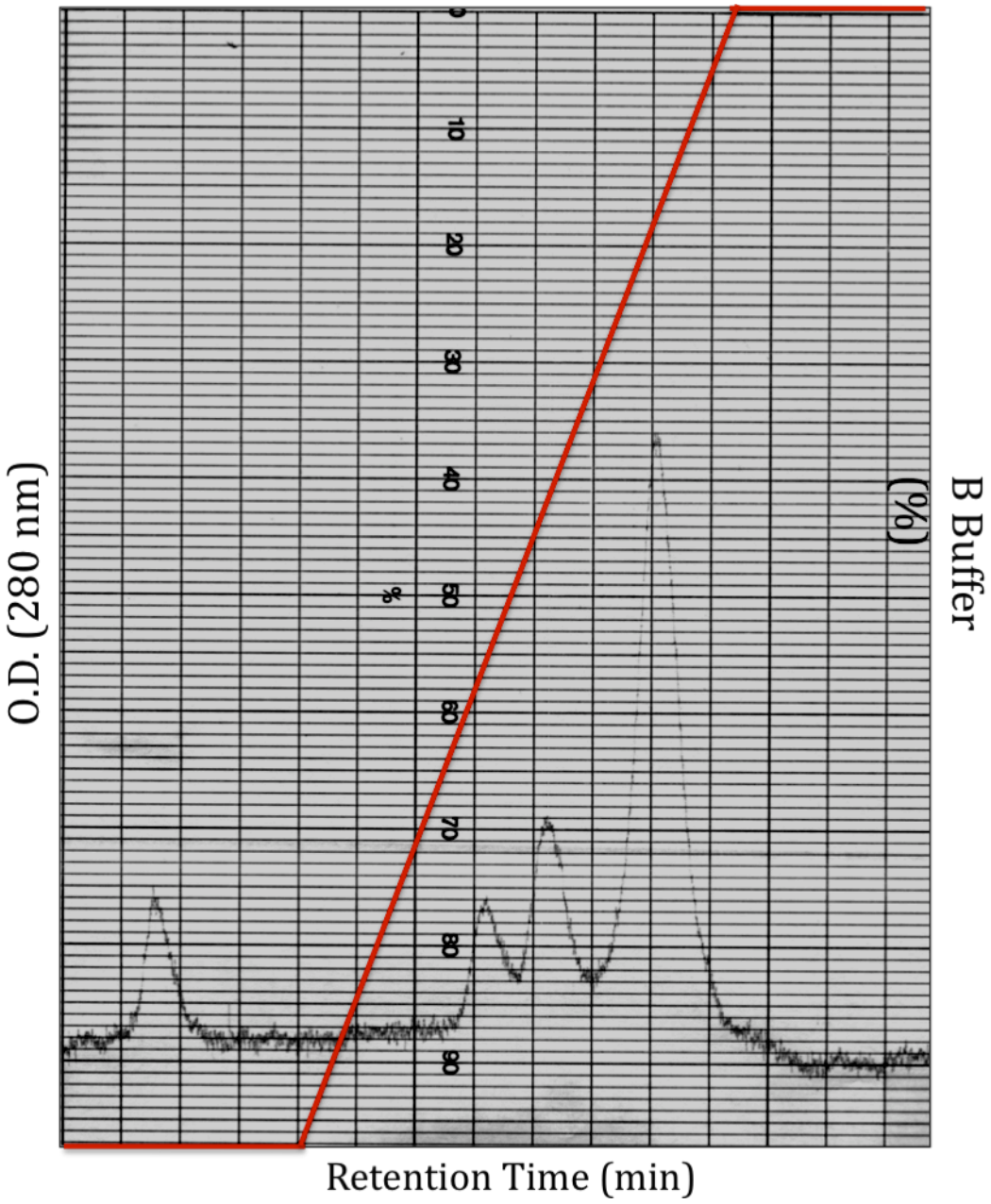


Figure 6. FPLC chromatogram of LMWP-ASNase conjugation mixture. (Buffer A: 50 mM phosphate, 1 mM EDTA, pH 7.5; Buffer B: 50 mM phosphate, 1 mM EDTA, 2 M NaCl, pH 7.5)

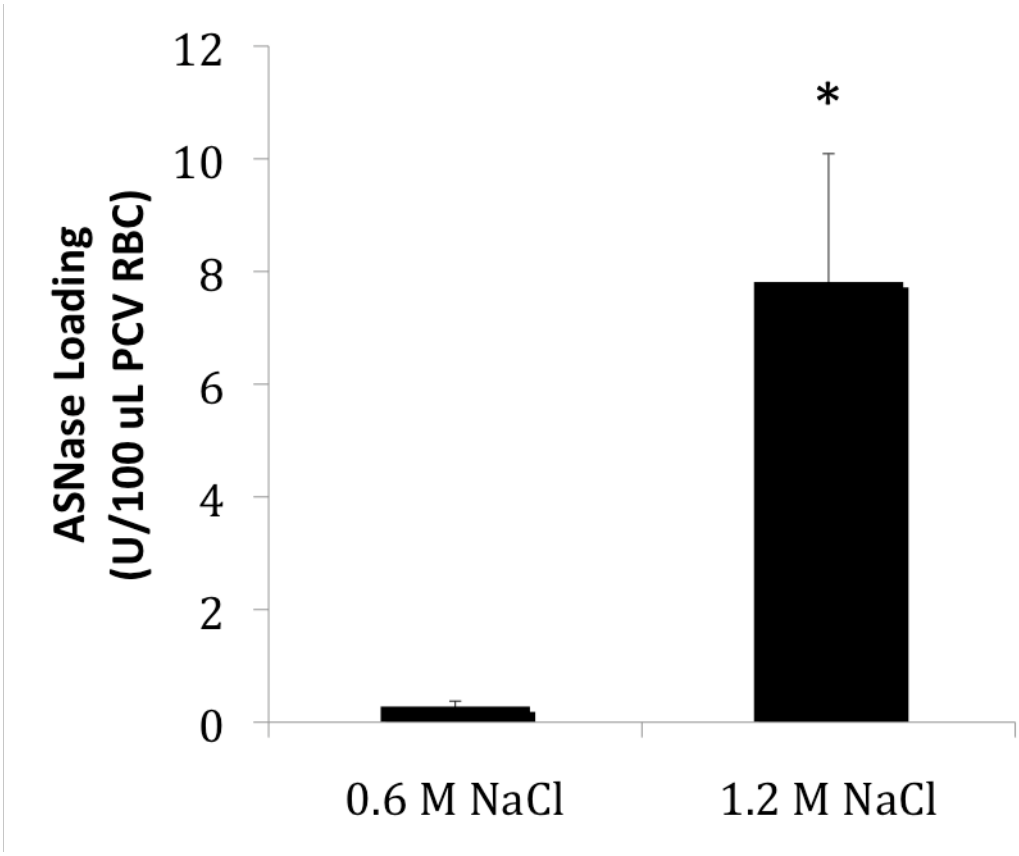


Figure 7. Fraction eluting at higher salt concentration shows higher loading efficiency compared to fraction eluting at lower salt concentration.

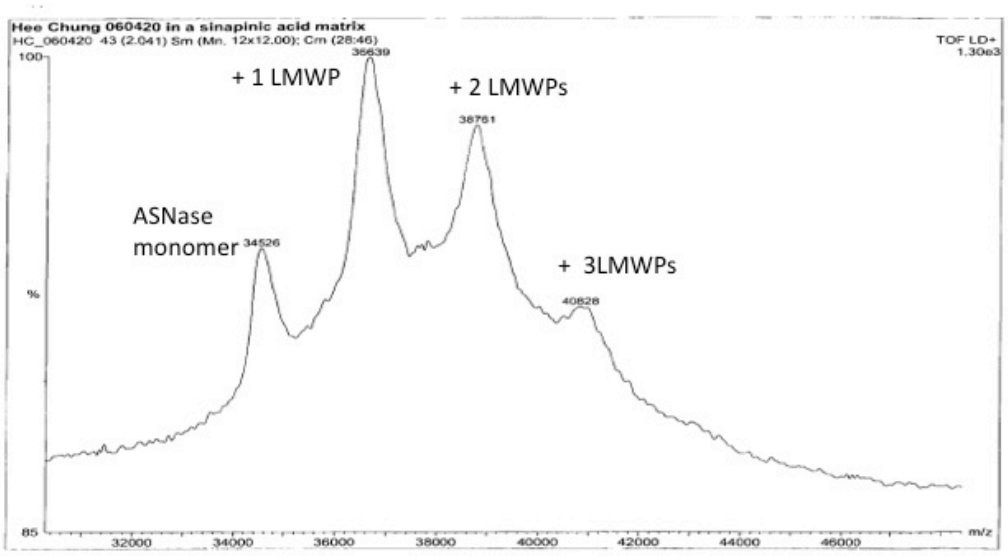


Figure 8. MALDI-TOF mass spectra of LMWP-ASNase conjugates prepared by coupling SPDP activated LMWP to the thiolated ASNase. Up to three LMWP peptides were found to be conjugated to each ASNase monomer.

Paramter	Native ASNase	LMWP-ASNase
V_{max}	339.8	627.7
K_M	0.016	0.031
V_{max}/K_M (catalytic efficiency)	2.12×10^4	2.02×10^4

Table 2. Michaelis-Menten kinetics parameters for native and LMWP-conjugated ASNase.

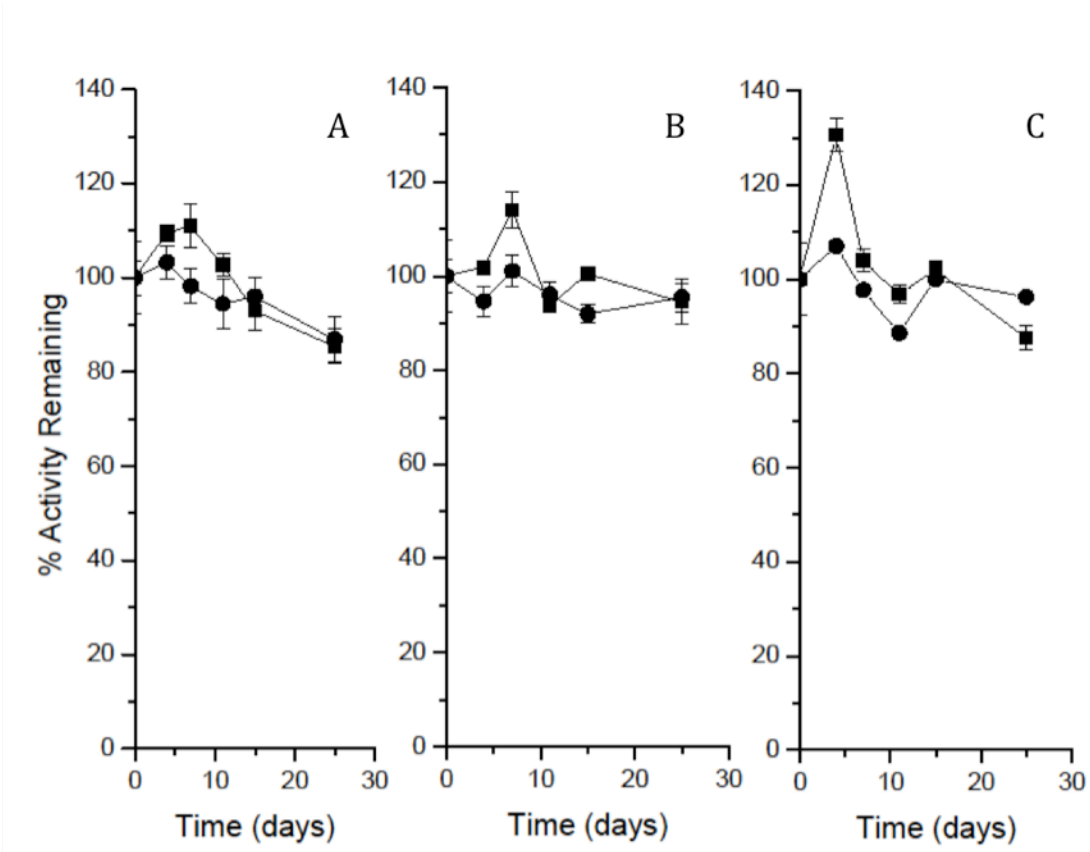


Figure 9. Storage stability comparison of LMWP-ASNase to native ASNase. Two forms of ASNase were stored at 4 (A), -20 (B), and -80 (C) °C and enzyme activity was measured at specified time points using direct nesslerization method (■: LMWP-ASNase, ●: ASNase).

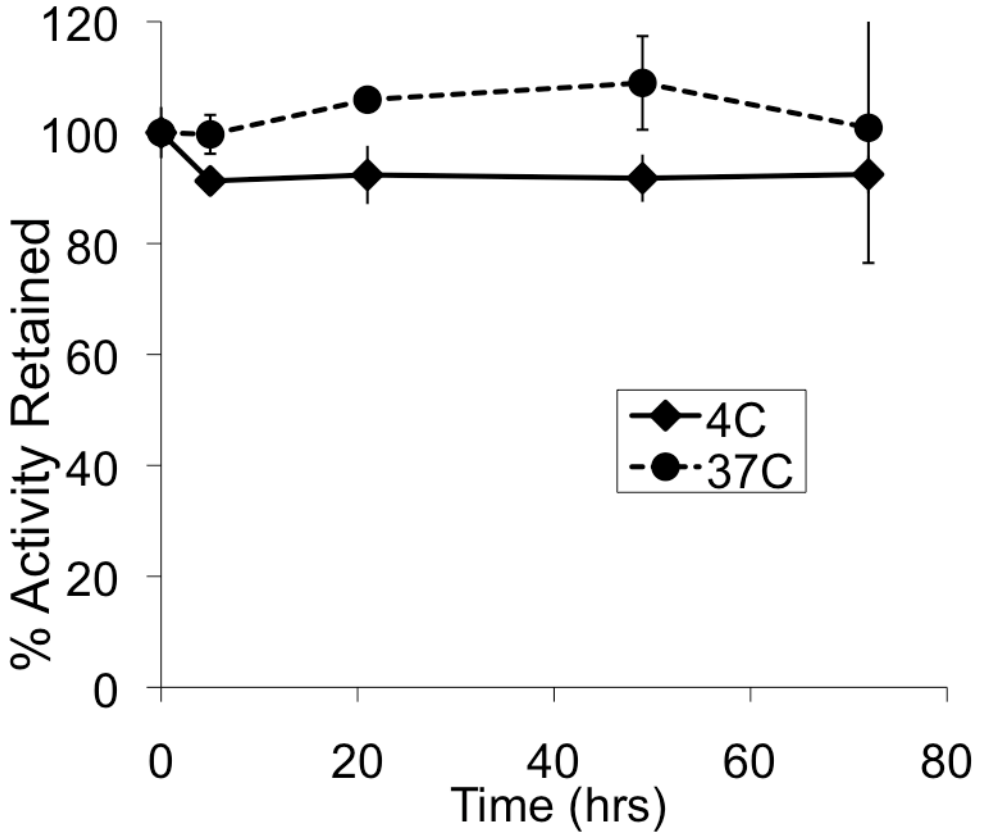


Figure 10. LMWP-ASNase stability at 4 and 37 °C. LMWP-ASNase conjugate was incubated at specified temperatures and an aliquot was removed at each time point to determine enzyme activity by direct nesslerization.

Chapter 3

PTD-Mediated RBC Encapsulation of L-Asparaginase

Introduction

Active research on use of RBC as a drug carrier has been going on since the 1980s. However, the encapsulation methods used with these therapeutic protein drugs was shown to compromise structural and functional integrity of RBCs. In order to take most advantage of the long life span of RBCs, it is necessary to preserve its major function as an oxygen carrier as well as its morphology and structural integrity. With the use of LMWP as a PTD peptide, these issues can be overcome, extending the circulating life span of RBCs, which in, turn will extend the circulating half-life of encapsulated protein drug.

In this chapter we will show LMWP-mediated cell uptake of protein into RBCs followed by steps taken to optimize LMWP-mediated RBC loading process. In addition, we demonstrate that these enzyme-encapsulated RBCs retain their natural morphology and their major function as oxygen carriers.

Materials and Methods

Blood Collection

Mice were anesthetized with ketamine/xylazine and blood collected by cardiac puncture was immediately transferred into microcentrifuge tube containing EDTA as an

anticoagulant. Blood was centrifuged to remove serum and separated RBCs were washed three times with R-HBSS before use.

RBC Uptake of Fluorescence-Labeled LMWP-Ovalbumin Conjugates

Commercial Alexa Fluor-488-labeled ovalbumin (Invitrogen, Carlsbad, CA) was activated using SPDP and then conjugated to LMWP. For uptake experiments, fresh sheep RBCs (MP Biomedicals, Solon, OH) were suspended in Hank's balanced salt solution (HBSS) at a density of 5 x 10^8 cells/ml, and were then incubated with a 0.5 mg/ml solution of the LMWP-ovalbumin conjugates for 30 min at room temperature under gentle shaking. RBCs were then washed with HBSS, fixed with 2% paraformaldehyde for 20 min, mounted on glass chamber slides, and uptake was examined using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany).

Effect of Temperature on Loading

RBCs were incubated in LMWP-ASNase in R-HBSS (20 uL PCV RBC/ 75 IU ASNase/ mL) for 1 hour at 4°C or 37°C. RBCs were then washed and lysed to measure for enzyme activity.

Loading Kinetics Experiment

Washed RBCs were added to R-HBSS containing LMWP conjugated-, free-, or no ASNase and incubated in shaking water bath at 37 °C. An aliquot of 1 mL was removed from each vial at previously specified time points, centrifuged, washed 3 times with R-HBSS. Washed RBCs were lysed with DI H_2O and analyzed for enzyme activity.

Twenty microliters of packed cell volume (PCV) RBCs were added to vials containing 1 mL of ASNase-LMWP at 20 IU/mL and incubated in a shaking water bath

at 37°C. At specified time points, vials were removed and centrifuged to collect RBCs, which were subsequently washed three times with R-HBSS before lysing to analyze for enzyme activity. For each time point, RBCs incubated in R-HBSS only were used as control.

RBC Encapsulation

Washed RBCs, 100 uL PCV (packed cell volume), were added to pre-warmed vial containing ASNase in R-HBSS (100 U/mL). The RBC-suspended ASNase solutions were then incubated in a shaking water bath at 37°C for 30 minutes. The loaded RBCs were then washed three times with R-HBSS and measured for ASNase activity.

Hematological Parameters

To determine the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin content (MCHC), and relative distribution width (% RDW), RBCs resuspended in R-HBSS at 50% hematocrit were analyzed using a commercially available veterinary hematology system (Drew Scientific, Dallas, TX).

Oxygen Dissociation Measurement

A 10% solution of ASNase-loaded RBCs in Hank's Buffered Salt Solution (HBSS) was washed three times and lysed with distilled water. The resulting hemolysate was centrifuged and the supernatant was diluted to 5 ml with a 1:1 mixture of HBSS and distilled water. Dissolved oxygen was measured using a Clark electrode (World Precision Instruments, Sarasota, FL) connected to a data acquisition system described by Frost et al.[46] Oxyhemoglobin was measured using a 37°C thermostated spectrophotometer (UV 2501PC, Shimadzu, Columbia, MD) at 540 nm, and change in

oxyhemoglobin content of the hemolysate due to decreases in oxygen concentration was monitored.

Scanning Electron Microscopy

LMWP-ASNase-loaded and sham-loaded (control) RBCs were fixed in 2.5% glutaraldehyde in R-HBSS for 1 hour at 4°C and washed three times with R-HBSS. Cells underwent dehydration in graded ethanol starting from 50% and finally in absolute ethanol. Dehydrated RBCs were washed four times with HMDS (hexamethyldisilazane) and air dried over night. After gold sputtering (Polaron E5100) cells were examined by scanning electron microscope (1910 Field Emission Scanning Electron Microscope, Amray).

Osmotic Fragility

LMWP-ASNase- and sham-loaded RBCs were resuspended to 50% hematocrit, and 20 µl of the RBC suspension was added to 1.0 ml NaCl solutions with osmolality ranging from 0 to 300 mOsm/Kg. The solutions were incubated at 37°C for 30 min, centrifuged, and the absorbance of each supernatant measured at 540 nm. The absorbance for 0 mOsm/Kg solution was taken as 100% hemolysis. The osmolality of each solution was measured using a vapor pressure osmometer (Wescor, Logan, UT).

Statistical Analysis

All data are expressed as mean ± S.D. A two-tailed *t*-test was used in assessing the hematological parameters. A *p*-value of less than 0.05 was considered significant.

Results

LMWP-Mediated Encapsulation of Proteins into RBC Carriers

In order to provide physical evidence of the ability of LMWP to transduce proteins into RBCs, LMWP was first activated by introducing a thiol moiety to its N-terminus, followed by conjugation to a commercial ovalbumin already labeled with the fluorescent dye, Alexa Fluor 488. While control native RBCs showed weak autofluorescence from hemoglobin excitation (Figure 11A), RBCs incubated with the Alexa Fluor 488-labeled ovalbumin also displayed only weak fluorescence on the cell surface, with no observable uptake of the labeled protein within the interior of the RBCs (Figure 11B). However, after conjugation of labeled ovalbumin with LMWP, significant intracellular fluorescence was detected in the cytosol of RBC carriers Figure 11C).

Optimization of PTD-Mediated ASNase Loading into RBCs

Results in Figure 11 already provided physical evidence that LMWP was able to transport protein molecules into RBCs. By following the similar protocol, we conjugated ASNase with LMWP via disulfide linkages as described in Chapter 1. When RBCs were incubated with LMWP-ASNase solutions for varying times to determine the kinetics of RBC loading, no statistically significant difference was noted within a 10 to 90 minute period (see Figure 12). The age of RBCs did not significantly affect ASNase loading as long as RBCs were kept in original serum (see Figure 13). When RBCs were incubated with LMWP-ASNase conjugates at a total enzyme concentration of 100 IU/mL, a loading efficiency of 19.7 ± 3.63 % was observed, with a loading capacity of 19.7 IU of ASNase per 100 uL packed cell volume (PCV) of RBCs.

RBC Functionality after Encapsulation of LMWP-ASNase

Since preservation of morphology and physical properties of the RBC through drug loading are important, we utilized the SEM method to examine the morphology of the ASNase-loaded RBCs obtained by LMWP-mediated encapsulation process. While the ASNase-loaded RBCs created using the conventional osmotic rupture-resealing technique showed distinctive changes in morphology, RBCs treated with LMWP-ASNase displayed morphology identical to that of the control RBCs, with full preservation of the customary biconcave shape and no observable deformities (data not shown). In addition when the SEM image of ASNase-loaded mouse RBCs prepared by the LMWP-mediated method was compared to that of control RBCs, retention of their original biconcave shape made them almost indistinguishable from each other (see Figure 14).

Also of great significance was that when RBCs loaded with LMWP-ASNase were incubated in solutions of decreasing osmolality, the displayed osmotic fragility curve was virtually identical to the result of control RBCs. As shown in Figure 15, these RBCs displayed "sudden" hemolysis, or a sigmoidal curve, characteristic of normal RBCs. On the other hand, had their structure been compromised the resulting osmotic fragility curve would have indicated hemolysis of RBCs at all osmolality values, an indicator for a heterogeneous population of RBCs as mentioned with glutamate dehydrogease and alcohol dehydrogenase encapsulated human (and sheep) RBCs by hypotonic dialysis/isotonic resealing method.[47]

In addition to confirming the structural integrity of the LMWP-ASNase loaded RBCs, we also examined their oxygen transport functionality. As noted with conventional methods for protein encapsulation, the loss of intracellular components during pore formation or osmotic swelling could result in changes to the normal oxygen-

binding capabilities of the drug-loaded RBCs. When subjected to varying oxygen concentrations, we found that the hemoglobin of the LMWP-ASNase loaded RBCs exhibited the characteristic sigmoidal profile indicative of cooperative binding. The Hill coefficient and pO_{50} values for the LMWP-ASNase loaded RBCs were equivalent to those of the control RBCs or the previously established values for sheep RBCs (Table 4), confirming the presence of the same level of fully functional hemoglobin.

Additionally, MCV, MCH, MCHC, and RDW showed no statistically significant difference between normal and LMWP-ASNase loaded RBCs (Table 3). Comparable MCV values further supported results from SEM indicating preservation of RBC morphology while MCH values indicated retention of hemoglobin inside RBCs since no large pores were created, as observed with electroporation and hypotonic RBC loading methods. Furthermore the distribution width (RDW), which is a measure for distribution of RBCs with varying sizes, was comparable between two RBC groups suggesting homogeneous population of RBCs in terms of size. The onset of hemolysis for majority of RBCs at 150 mOsm in osmotic fragility curve also verifies this result. In comparison the RDW is wider for hypo-osmotically loaded RBCs. These data demonstrated that cells loaded with ASNase using LMWP were virtually identical to normal RBCs with regard to both structural and functional properties.

Discussion

Specific substrate selectivity and unparalleled reaction efficiency bestow proteins with the promise of being potent therapeutic agents. Yet, clinical applications of protein drugs face two major hurdles; one is their premature degradation and inactivation by

endogenous proteases as well as elimination by the reticuloendothelial system (RES), and the other is the manifestation of immunological responses and toxic side effects by the host immune system towards foreign protein compounds. To overcome such problems, the most widely employed strategy is encapsulation of the protein into a soluble and biocompatible drug carrier; which can either be a synthetic polymer (e.g. PLGA) or a multi-component particulate structure such as liposomes or cells. Among all of these carrier systems, RBCs feature a number of distinctive advantages. Not only are RBCs the most abundant cells in human body and thus affordable for use in drug encapsulation, but they are also completely biocompatible and biodegradable, particularly when autologus RBCs are used. In addition, the biconcave disk shape of RBCs endows them with the highest surface to volume ratio (1.9×10^4 cm/g) usable for drug encapsulation. Most importantly, RBCs possess a lifespan in circulation of approximately 120 days, which is significantly longer than any of the currently existing carriers.

ASNase represents a typical example of these protein therapeutics. Despite its wide use in treating ALL, clinical application of ASNase is hindered by the short plasma half-life and high potential for inducing allergic responses. Hence, ASNase has been attempted in almost every method involved in RBC encapsulation. We, therefore, selected ASNase to examine the PTD-mediated RBC encapsulation technology, not only because of the clinical significance of ASNase, but also a direct comparison of the benefits of the new method over existing techniques could be readily attained and justified. We also chose the LMWP peptide developed in our laboratory as the representative PTD, simply because it possessed similar membrane-translocating activity to the most widely studied TAT and yet was proven in animal studies to be neither toxic

nor immunogenic.[44] Furthermore, to provide direct physical evidence of the utility of the new method, we decided to study the LMWP-mediated cell uptake examined via confocal fluorescence microscopy, utilizing the Alexa 488 fluorescent dye-labeled ovalbumin as the protein marker. As demonstrated in Figure 11, despite a significant overlap between the emission wavelengths of Alexa 488 and absorption wavelengths of hemoglobin, the green fluorescence from the LMWP-ovalbumin conjugates inside the cells were nevertheless quite evident. The horizontal sections along the z-axis (z sections) were also taken to ensure that the conjugates were indeed inside the RBCs (data not shown). In contrast, without the aid of LMWP, the dye-labeled ovalbumin could not enter the cells and only weak fluorescence was observed on the surfaces of RBCs. Based on these findings, we were convinced that LMWP would be able to translocate other attached proteins such as ASNase into living RBCs. Indeed, by utilizing an optimized encapsulation protocol, our results showed that a reasonable ASNase loading efficiency (~4%) a RBC loading capacity (8 IU of ASNase per 100 μL of packed RBCs) was achieved. Clinically, the dose of ASNase as a sole induction agent in the treatment of ALL is about 200 IU/kg body weight.[48] Hence, even based on our currently established loading protocol, this clinical dosing regimen, which can be translated into a dose of 2.5 mL of ASNase-loaded RBCs per kg of body weight, is obviously quite achievable.

In vitro characterization showed there was no leaching or activity decay of the RBC-encapsulated ASNase over a 3-day incubation period; under which the RBCs remained visibly intact. Since LMWP was linked to ASNase via disulfide linkages, it was speculated that detachment of LMWP from ASNase via degradation of such bonds by the elevated glutathione activity in the cytosol caused the membrane-impermeable ASNase to

be trapped inside of the RBC. In addition, it has so far not been established that the PTD-mediated cell entry is a reversible process. Hence, the permanent entrapment of the protein drug inside RBCs could also result from this irreversible translocation mechanism. Overall, the absence of leaching and activity decay of the entrapped ASNase fulfills one of the essential requirements for the ASNase-loaded RBCs to eventually be useful clinically.

Evidence gathered from our experimental results all point towards the same direction; i.e. RBCs after processing by the new encapsulation method remain both structurally and functionally intact. The morphology of treated RBCs were indistinctive from the untreated cells (see Figure 14) whereas all of the important cellular parameters including MCV, MCH, MCHC, and RDW were all statistically indistinguishable from that of untreated RBCs (see Table 3). The most convincing evidence came from the oxygen transport capability of the treated RBCs, as ASNase-loaded RBCs displayed virtually statistically indistinguishable oxygen transport characteristics, such as the measured Hill coefficients and pO_{50} values, from the untreated, normal RBCs (see Table 4).

It should be noted that RBCs treated by any of the existing encapsulation methods, either by electroporation or hypotonic dilution, may result in a loss of cellular components such as hemoglobin. Several investigators reported that under a carefully managed process of hypotonic dialysis and with the aid of a rejuvenating agent during the resealing procedure, drug-loaded RBCs could preserve an intact structure and initial chemical balance similar to those of native RBCs.[49, 50] Nevertheless, in this hypo-osmotic drug-loading method, a pore-opening and a resealing step, both involving dialysis, were

required. Thus far, the largest protein being encapsulated in RBCs by using this method was alcohol oxidase from Pichea pastoria[51], which has a molecular weight (675 kDa) 10-fold larger than that of hemoglobin (65 kDa), the major component of an RBC. Since dialysis is an equilibrium process and with such large pores being created on the cell membrane, in theory and practice, it is inevitable that some constituents in the cytosol such as hemoglobin, glutathione, or cytoskeleton would be leaked out of the RBC. Indeed, loss of hemoglobin was observed in RBCs treated with the hypo-osmotic dialysis method, as evidenced by a decrease in MCH after resealing.**Error! Bookmark not defined.** As known, aside from the principal activity in oxygen transport, RBCs also carry out other important biological functions including energy (ATP)-involved metabolic processes as well as scavenging of oxidative stress[52]. Hence, a loss of hemoglobin would not only impair the oxygen transport function of RBCs, but also affect their ability to manage oxidative stress. On the other hand, a loss of the cytoskeletal constituents would compromise the structural integrity of RBCs, making them prone to destruction or recognition by cells in the phagocytic system.

Osmotic fragility of the treated RBCs has been widely adopted as a measure or revealing sign of the membrane integrity after undergoing a drug loading process. Numerous reports in the literature indicate changes in osmotic fragility curves after loading RBCs with the hypotonic methods.**Error! Bookmark not defined.**[53] Chiarantini et al. reported that after hypotonic dialysis followed by hypotonic or isotonic washing, RBCs exhibited distinctly different osmotic fragility profiles as well as earlier onset of rupture compared to the normal RBCs.[53] These findings support the assumption that the RBC membranes were considerably weakened during these loading processes. In sharp

contrast, our results demonstrated that RBCs being processed through the LMWP-mediated encapsulation method display a nearly superimposed osmotic fragility profile when compared to that of normal RBCs, with both samples showing an identical onset of at about 150 mOsm/Kg (see Figure 15).

These findings further confirm our hypothesis that the PTD-mediated protein translocation is minimally invasive with no apparent compromise of the RBC membrane.

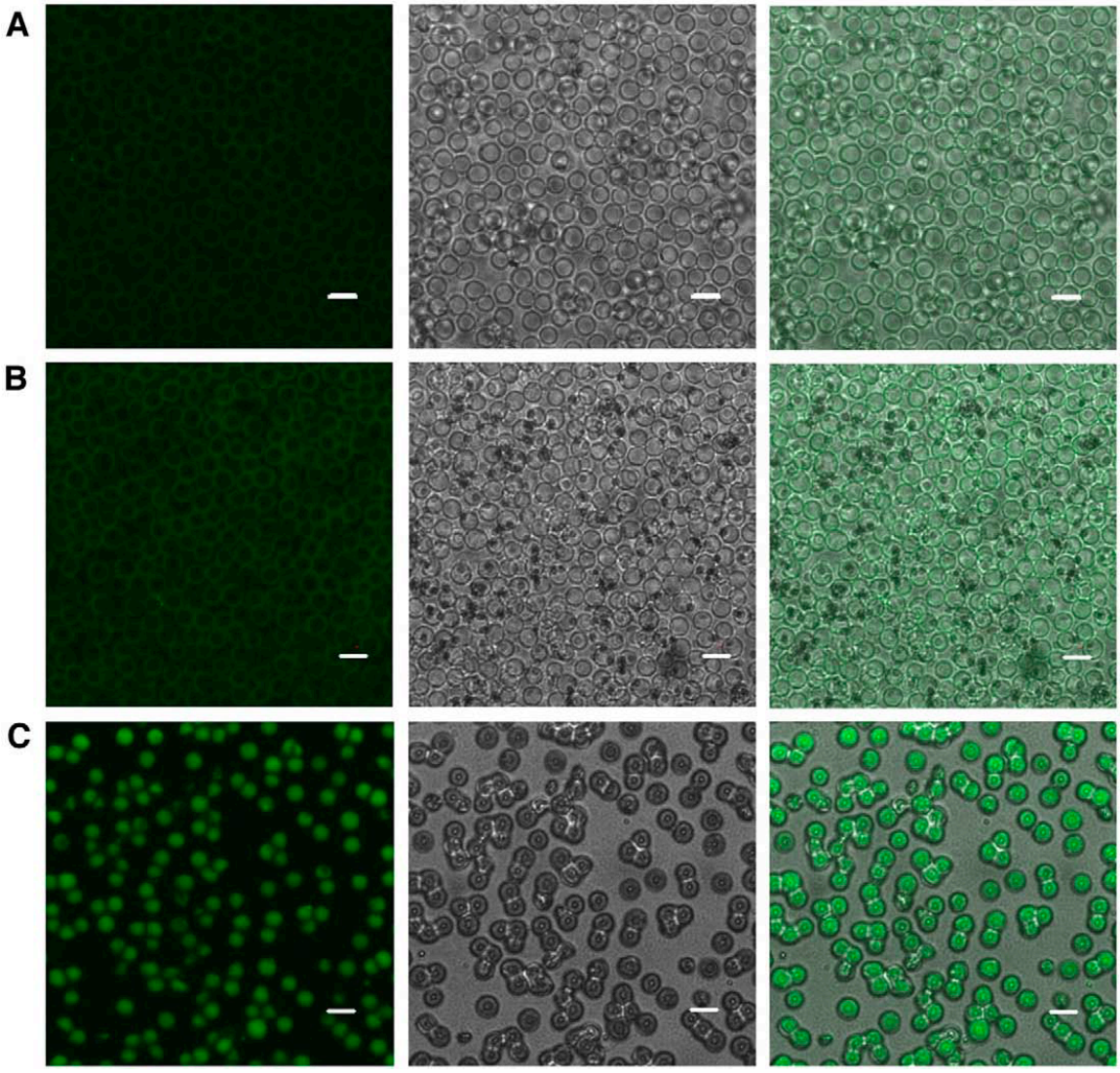


Figure 11. Confocal microscopy images of control RBCs (row A), RBCs incubated with Alexa Fluor 488-labeled ovalbumin (OVA-488; row B), and LMWP-ovalbumin (LMWP-OVA-488; row C). First column: fluorescence mode; second column: DIC mode; third column: superimposition. Scale bar = 5 µm. (Microscope: Carl Zeiss, Jena Germany, LSM 510 META. Objective: 63x, 1.2N. A., Water immersion, Zeiss cat. no. 440668. Temperature: 20 °C. Mounting medium: ProLong Gold, Invitrogen, Carlsbad, CA. Acquisition software: LSM 510 Release Version 4.2 Service Pack 1).

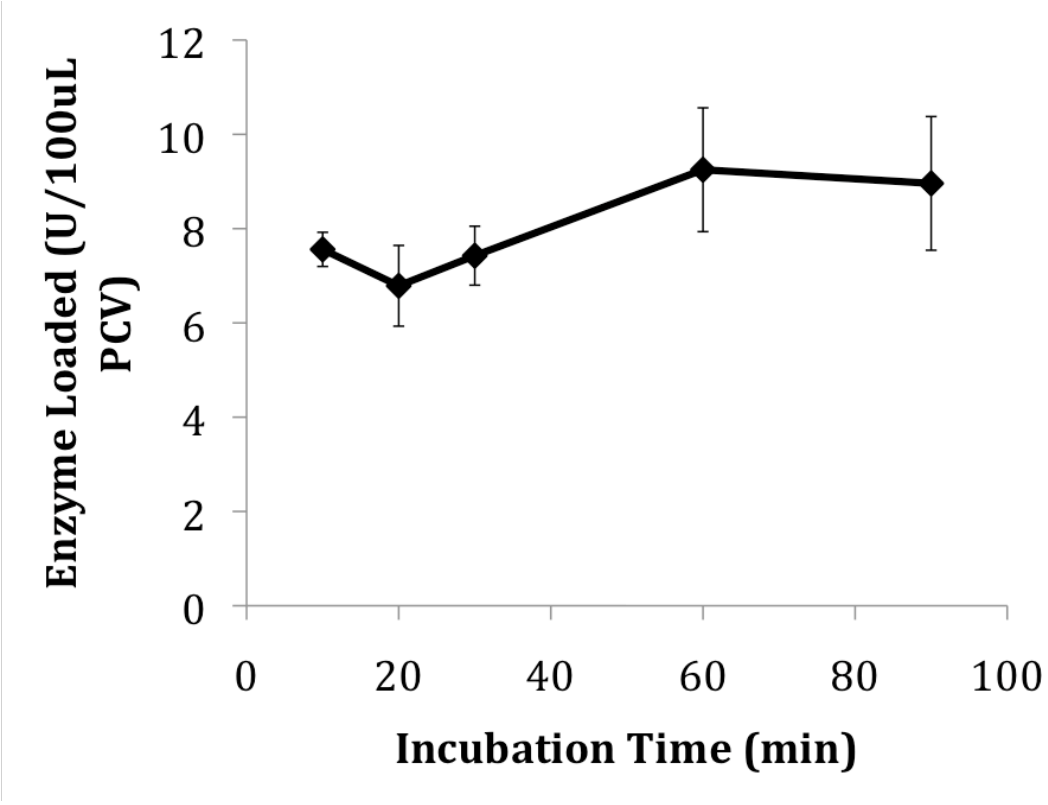


Figure 12. Loading kinetics of LMWP-ASNase into mouse RBCs. LMWP-ASNase solutions at concentration of 100 U/mL was prepared and loading kinetics into mouse RBCs monitored over 90 min period. Statistical analysis indicated no significant difference among values for different time points.

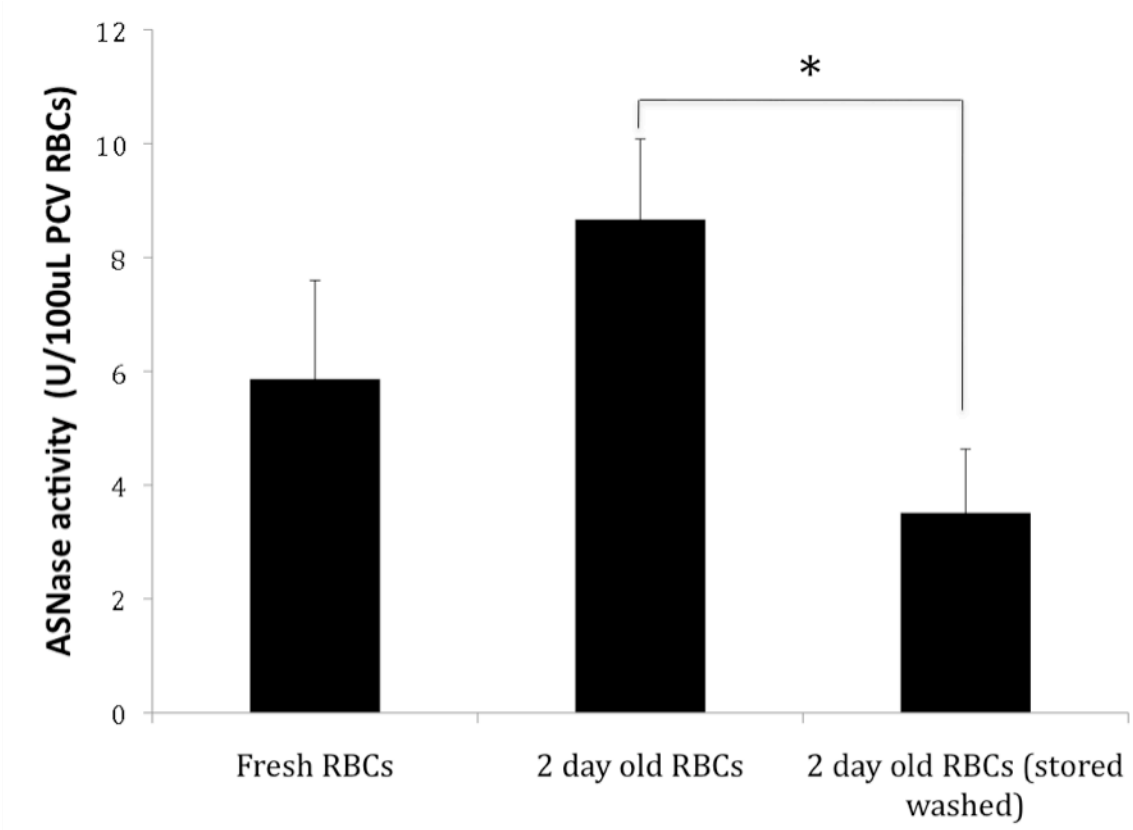


Figure 13. Effect of mouse RBC age on loading. Comparison of ASNase loading into freshly collected RBCs vs. 2-day old RBCs stored as whole blood, or 2-day old but stored washed. (* $p < 0.05$)

Parameter	*Control*	*Loaded*
MCV (fL)	58.3 ± 0.4	57.8 ± 0.4
MCH (pg)	17.3 ± 0.4	16.6 ± 0.3
MCHC (g/dL)	29.6 ± 0.4	28.7 ± 0.6
RDW (%)	17.7 ± 0.2	17.4 ± 0.3

Table 3. Hematological parameters of control (sham loaded) and LMWP-ASNase loaded mice RBCs. MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean cell hemoglobin content, RDW = red cell distribution width.

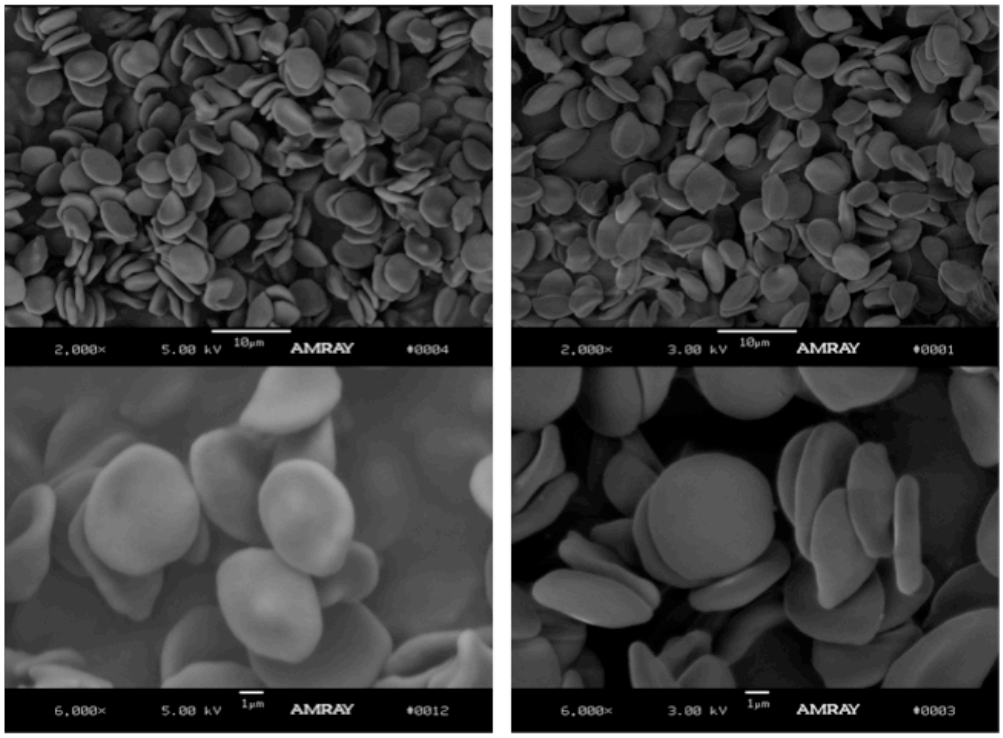


Figure 14. SEM images of control (sham loaded) and LMWP-ASNase loaded mouse RBCs. RBCs were fixed in 2.5% glutaraldehyde in R-HBSS for 1 hour at 4°C. Washed cells underwent dehydration in graded ethanol from 50% to absolute ethanol. Dehydrated RBCs were washed four times with HMDS (hexamethyldisilazane) and air dried overnight. After gold sputtering (Polaron E5100) cells were examined by scanning electron microscope (1910 Field Emission Scanning Electron Microscope, Amray).

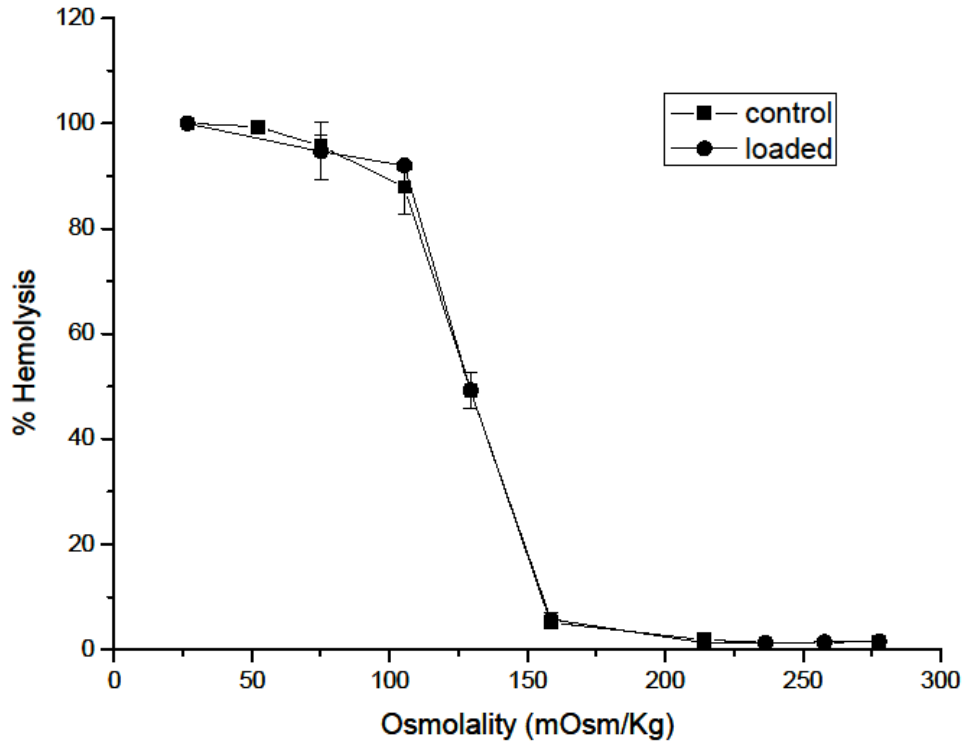


Figure 15. Osmotic fragility curves of control (sham loaded) and LMWP-ASNase loaded sheep RBCs.

	Oxygen Dissociation Parameters		
	Control RBCs	Loaded RBCs	Reference[54]
Hill Coefficient	3.15 ± 0.32	3.07 ± 0.34	3.14 ± 0.12
pO_{50} (mmHg)	37.44 ± 0.03	38.64 ± 0.02	40.0 ± 1.0

Table 4. Oxygen dissociation parameters of control (sham loaded) and LMWP-ASNase loaded sheep RBCs are compared to literature values.

Chapter 4

Pharmacokinetics and Therapeutic Efficacy of RBC-Loaded ASNase

Introduction

In previous chapters we showed successful encapsulation of LMWP-ASNase into RBCs and confirmed that these RBCs are still functionally viable as well as structurally comparable to normal RBCs. The ultimate goal of encapsulating LMWP-ASNase into RBCs is to improve upon pharmacokinetic properties of ASNase, by maintaining original properties of RBCs, and thereby, the therapeutic effect of ASNase. Therefore, additional experiments were undertaken to test therapeutic viability and applicability of encapsulated ASNase.

When considering pharmacokinetic properties of ASNase, it is important to keep in mind that the circulating half-life of native ASNase is 2.4 - 3.3 (± 0.8) hrs in mice[49] which should significantly increase upon encapsulation into RBCs. At the same time when leukemic mice are treated with ASNase-encapsulated RBCs, the life span should significantly increase compared to those treated with free- or hypotonically loaded-RBCs.

In order to compare the efficacy of RBC-loaded and native ASNase against leukemia it was necessary to come up with a mouse strain and corresponding cell line that will respond to ASNase treatment. Horowitz et al mention several murine cell lines that are ASNase-sensitive and express low asparagine synthetase activity along with their origin

of mouse strain.[55] Among those listed the DBA/2 mouse strain with L5178Y cell line was chosen for its wide spread application in examining efficacy of various forms of ASNase.

Materials and Methods

All animal experiments were performed in accordance with the Guide for Laboratory Animal Facilities and Care (NIH publication 85-23, revised 1985) and the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Utah or the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Pharmacokinetics of ASNase Encapsulated in RBCs

RBCs collected from 6 weeks old DBA/2 mice (Harlan, Indianapolis, IN) were treated with LMWP-ASNase conjugates by procedures previously discussed in Chapter 3. The plasma half-life based on enzymatic activity was calculated after intravenous injection of the ASNase-loaded RBCs into the DBA/2 mice. As a control, ASNase was encapsulated into RBCs using the conventional hypotonic rupture / resealing method, according to a previously established procedure[20]. In both groups, each individual mouse was given 8 units of RBC-encapsulated ASNase (n = 4 per group). Total ASNase activity from recipient blood was measured by direct nesslerization.

Determination of Cytotoxicity in Murine L5178Y Lymphoma Cells by MTT Assay

Antiproliferative in vitro screening was performed on the murine L5178Y murine lymphoma cells (American Type Culture Collection, ATCC; Manassas, VA). Cytotoxicity was assessed by MTT assay. The L5178Y cells were suspended into 96-well plates at a density of 1 x 10^5 cells/mL in RPMI 1640 (Gibco, Grand Island, NY)

supplemented with 10 % FBS. Cells were grown for 24 hrs at 37 °C, 5% CO_2, humidified, before ASNase treatments. After 72 hours of incubation with ASNase formulations, cells were incubated with MTT for an additional 4 hrs. Purple formazan product was dissolved in 200 uL DMSO and the corresponding absorbance at 560 nm was measured using a microplate reader. The concentrations required in order to inhibit cell growth by 50% (IC_{50}) were calculated.

Optimal ASNase Treatment Time for Tumor Bearing DBA/2 Mice

L5178Y cells were cultured in RPMI 1640 supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 environment. Each DBA/2 mouse was given 1 x 10^6 L5178Y cells in 0.2 ml RPMI-1640 media by intraperitoneal injection. On day 1, 2, 3, 4, and 5 after tumor implantation a group of mice was selected to receive ASNase (20 U/mouse in 0.1 mL) by intraperitoneal injection and their survival times recorded.

Survival of tumor-bearing mice after administration of LMWP-ASNase-loaded RBCs

L5178Y cells were cultured as described above. DBA/2 mice were then given intraperitoneal injections of 7 x 10^5 cells in 0.1 ml HBSS. Five days after tumor implantation, mice with narrow bodyweight range were selected and divided into two groups: 1) control groups given saline only and 2) RBCs loaded with LMWP-ASNase (n = 5 each). Each mouse in group 2 received intravenous injection (tail vein) of LMWP-ASNase loaded RBCs equivalent to 8 units of free ASNase. The subsequent survival of the tumor-bearing mice was monitored.

Statistical Analysis

All data are expressed as mean ± S.D. The log-rank test was used in the survival study of the tumor-bearing mice. A p-value of less than 0.05 was considered significant.

Results

Prolonged Circulation Half-Life of ASNase Encapsulated in RBCs

Since RBCs loaded with LMWP-ASNase closely resembled normal RBCs in functionality and retained physical properties, we hypothesized that encapsulated ASNase would inherit the same, extended circulation half-life of normal RBCs. To test this hypothesis, we injected DBA/2 mice with LWMP-ASNase loaded RBCs by tail vein, and the circulation half-life of ASNase was determined from the linear portion of the elimination phase. For comparison, we also prepared ASNase-loaded RBCs with equivalent enzyme concentrations using the conventional hypo-osmotic rupture/resealing technique. While both types of RBCs showed a biphasic disappearance of enzymatic activity over time, ASNase loaded into the RBCs by LMWP-mediated procedure displayed a significantly longer circulation half-life (4.5 ± 0.5 days) than that of ASNase loaded into RBCs by hypo-osmotic rupture / resealing method (2.4 + 0.7 days), as depicted in Figure 16, or the free ASNase which had circulating half-life of 3.3 hrs (data not shown).

Verification of Murine L5178Y Leukemia Cell Line Sensitivity to ASNase

Prior to initiating an efficacy study of ASNase in mice model, we wanted to verify that the leukemia cell line to be used, L5178Y, was indeed sensitive to ASNase treatment as this cell line has been known to become insensitive to ASNase treatment.[56] When

these cells were incubated with comparable dose of either native or LMWP-conjugated ASNase for 72 hrs, and cell viability measured by MTT assay, the IC_{50} was determined to be 9.74 IU/mL for native and 1.95 IU/mL for LMWP-conjugated ASNase (see Figure 17) indicating not only that these cells were sensitive to ASNase treatment but also that the conjugation of LMWP increased cytotoxicity of ASNase.

Therapeutic efficacy of RBC-encapsulated ASNase

Upon verification of sensitivity of L5178Y cells to ASNase, the therapeutic efficacy was examined. We injected RBCs loaded with LMWP-ASNase into L5178Y lymphoma-bearing DBA/2 mice to examine the therapeutic efficacy of the encapsulated enzyme. On day five after tumor injection and when symptoms became apparent, 8 IU of RBC-loaded ASNase were intravenously injected. Compared to control animals which received saline injections, the animals treated with LMWP-ASNase loaded RBCs showed a significant increase in mean survival time, almost a 44% increase, from 10.0 ± 1.4 days to 14.4 ± 2.3 days, as shown in Figure 18.

Discussion

One of the proofs of the benefits of the LMWP-mediated cell encapsulation method stemmed from the in vivo pharmacokinetic study of the plasma half-life of the ASNase-loaded RBCs. For comparison, RBCs loaded with ASNase via a hypotonic method were used as a control. Consistent with the findings by other investigators[23, 21, 49] the hypotonic method resulted in changes in morphology and surface structures of many of the treated RBCs and, as a consequence, significantly shortened the circulating half-lives of such cells. An overall half-life of approximately 2.4 days was found for RBCs treated with the

hypotonic method whereas, in contrast, RBCs treated with the LMWP mediated method exhibited significantly prolonged plasma half-life of 4.5 days; almost double the value for the hypotonic treated RBCs (see Figure 16). However, we believe that there is room for improvement because biological carriers like RBCs require more strict measures of handling precautions[57], as evidenced by the fact that the reported half-lives of manipulated RBC in rodents show variable results[19, 20, **Error! Bookmark not defined.**, 58]. Therefore, upon optimization of the RBC encapsulation method via PTD peptide, it is expected that the half-life of enzyme-loaded RBCs will increase compared to the results we have obtained from our preliminary study.

For the ASNase-loaded RBCs to function desirably, another essential requirement is that the entrapped drug must be able to retain its original therapeutic capability. To validate this criterion, we tested the therapeutic functions of the ASNase-loaded RBCs against a L5178Y lymphoma tumor-bearing DBA/2 mouse model. As can be seen in Figure 18, administration of ASNase-loaded RBCs was able to considerably increase the median survival time of the mice (14.4 days), when compared to the median survival time of 10 days observed from the saline-injected control group; an enhancement of the survival time by 44%. It should be quite easy to assess the prowess of the proposed RBC-encapsulation technology in ASNase therapy, after comparing our results with findings by others under similar in vivo conditions. As reported, a nearly 16.7% enhancement in survival time over the control was observed by other investigators following intravenous injection of 8 IU of free ASNase to L5178Y tumor bearing mice.[59] Previously, TAT-ASNase was investigated for potential application for targeted therapy of ALL[60,61]. The same tumor model (L5178Y/ DBA/2) showed that the mean survival time of L5178Y cell

implanted DBA/2 mice treated with free ASNase showed no improvement with respect to an untreated control group[61]. It is also noteworthy that Alpar and Lewis investigated ASNase-loaded in RBCs, which reports impressively longer survival in treated animals.[62] However, in that work, different tumor cell lines and animal models (6C3HED cell lines, C3H mice) were used. The median survival time of the mice in the untreated control group was about 18 days whereas L5178Y implanted DBA/2 mice in our study as well as previous studies[61] showed only 10 days, suggesting the tumor burden in DBA/2 strikes animals more quickly and severely than the C3H mice. The difference is further evidenced by in vitro doubling time of the two cell lines — L5178Y divides more rapidly than 6C3HED cells (data not shown). A more extensive animal investigation designed to further demonstrate the long-term benefits of this new approach in ASNase therapy, such as the alleviation of ASNase-induced toxic and immunologic responses, is currently in progress in our laboratory.

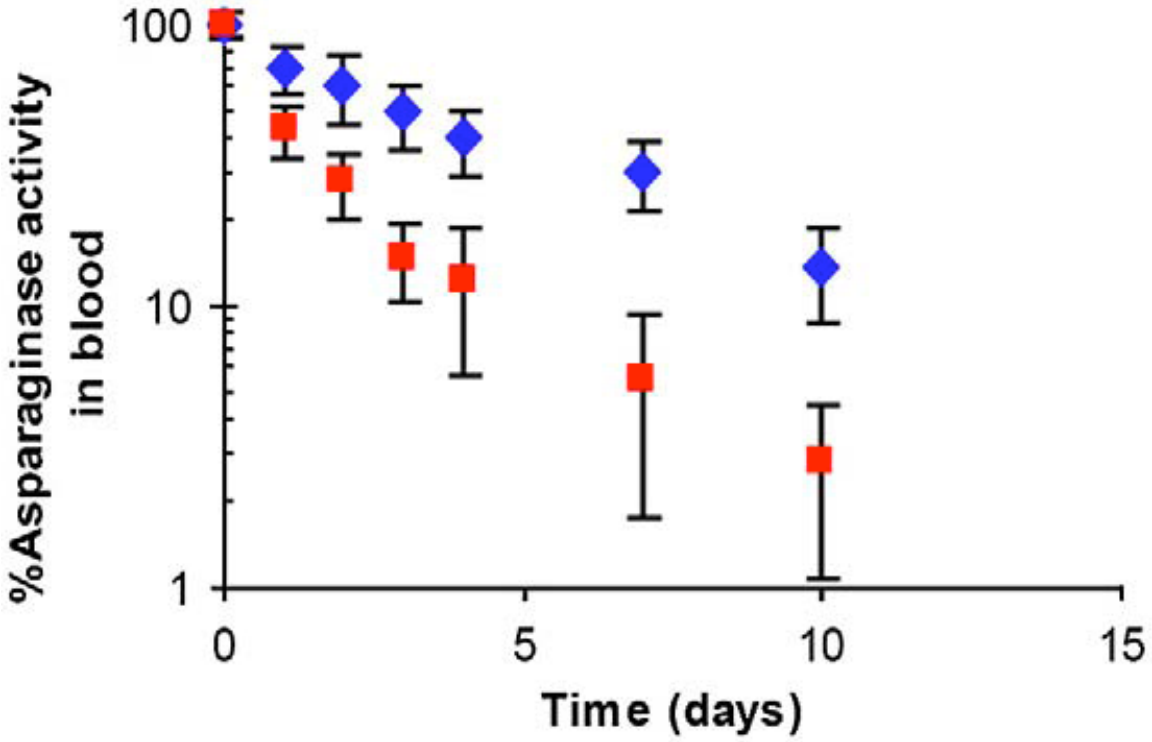
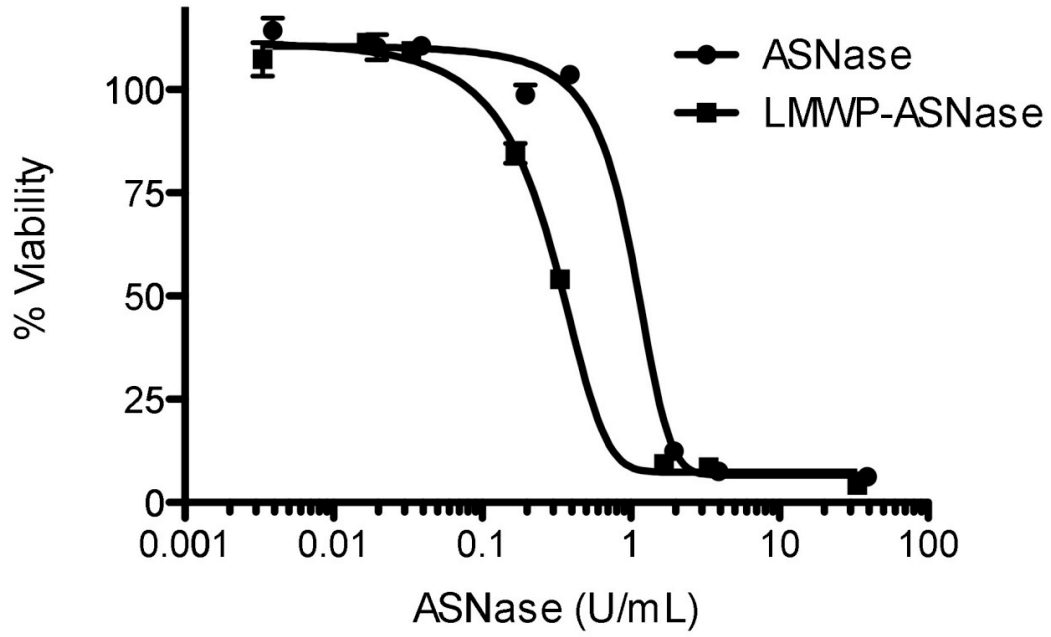


Figure 16. Time profile of ASNase activity in blood in DBA/2 mice (n=4). LMWP-ASNase loaded RBCs (8 IU ASNase per mouse; blue diamond) and ASNase-loaded RBC ghosts (8 IU ASNase per mouse; red square) were given via intravenous injection through tail vein. ASNase activities in whole blood specimens were measured by direct nesslerization.



	ASNase	LMWP-ASNase
IC_{50} (U/mL)	9.74	1.75

Figure 17. Cytotoxicity of native ASNase and LMWP-ASNase. L5178Y cells were incubated with native or LMWP-conjugated ASNase for 72 hrs before MTT assay.

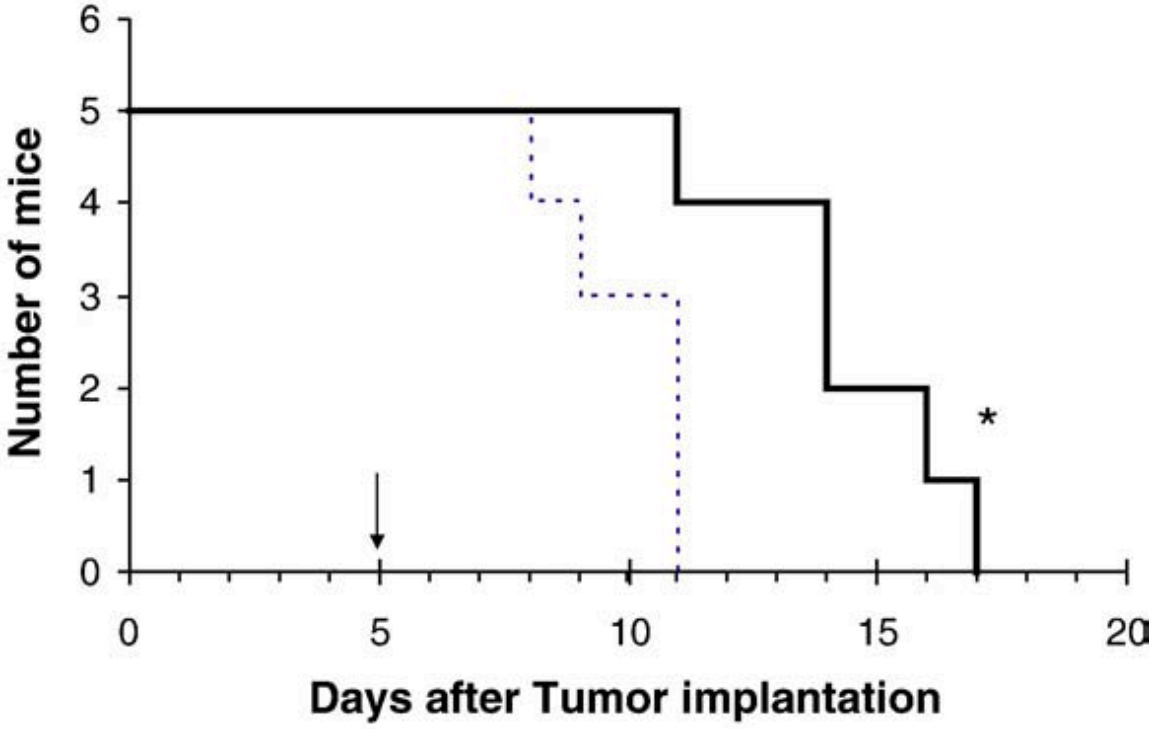


Figure 18. Kaplan-Meier survival curve for DBA/2mice bearing L5178Y lymphoma cells. RBC encapsulated LMWP-ASNase (8 IU ASNase per mouse; solid line) or saline (dotted line) were given on day 5 after tumor implantation when symptom from tumor burden was apparent (n = 5, $*p < 0.05$).

Chapter 5

Conclusions

In conclusion, an innovative method for encapsulation of therapeutically active ASNase into functionally intact RBCs was developed toward enhanced ASNase therapy for ALL. Because of the non-invasive nature of PTD-mediated cell entry, our results showed that the structural and functional integrity of both the loaded ASNase and processed RBCs were completely reserved. RBCs treated by this encapsulation method not only exhibited a long circulating half-life similar to that of untreated RBCs, but also displayed enhanced therapeutic effects of the entrapped protein drug, presumably via protection of the drug by RBCs from possible proteolytic degradation and phagocytic clearance. It should be noted that full preservation of both structure and function of the treated RBCs is of great clinical significance because theoretically this would provide the flexibility of replacing an unrestrictive amount of blood (or RBCs) from the patient with drug-loaded RBCs, should situations warrant such for clinical management.

Furthermore, for second remission treatment of ALL the pegaspargase did not show advantage over native ASNase. However, with RBC encapsulated ASNase, with the RBC membrane acting as a shield to mask the enzyme from the circulation, we can expect a reduced immunogenic effect, which would allow use of ASNase in patients with sensitivity to the enzyme.

Based on this simple encapsulation method, a plasmapheresis-type of blood auto-transfusion system is currently being designed in our lab in which RBCs are separated

from whole blood, processed through the LMWP-ASNase loading process, merged back with other blood components, and finally returned to the patient for in situ drug therapy for ALL.

Overall, this universal method of encapsulation may also be applied to several other protein drugs. Applications of this technology for the treatment of cocaine overdose, oxidative stress, and various types of cancers are currently being pursued in our laboratory.

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