A Study of Zwitterionic Polypeptide-Protein Conjugates

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science in Chemical Engineering

University of Washington

2019

Committee:

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Program Authorized to Offer Degree:

Chemical Engineering

University of Washington

Abstract

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Chemical Engineering

Protein therapeutics has been an attractive approach to serve patients. However, the safety and efficacy of these products are limited by their immunogenicity, low circulation time, and instability. Conjugation is one of the most widely used methods to tackle these problems. PEG has been regarded as a gold standard in pharmaceutical industry for decades. However, problems like organ accumulation, susceptibility to oxidation, low surface packing density, pre-existing and triggered anti-PEG antibodies can cause serious advent effects. Herein, we modify proteins with zwitterionic peptides, aiming to study their *in vitro* binding affinity and *in vivo* pharmacokinetics and immunogenicity. Results show that this is a promising strategy to endow protein drugs with a stealth layer, allowing proteins to escape immune attack, increase half-life with no accelerated blood clearance effect, and exhibit super-low immunogenicity.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Professor Shaoyi Jiang, for his guidance, engagement and inputs throughout this Master Thesis. Furthermore, I would like to thank Professor Qiuming Yu for the support of my final Master defense. Also, I like to thank Dr. Zhefan Yuan and Dr. Priyesh Jain for their help in dimer and polymer synthesis, Bowen Li for his guidance in animal experiments, Patrick James McMullen for his help in HIC and SAS-PAGE characterization, and Yuwei He, and Caroline Tsao for their participation, collaborations, and helpful suggestions.

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Chapter 1. Introduction and Background

1.1 Protein Therapeutics and Current Issues:

Recent years have witnessed significant advances made by protein therapeutics. Since the success of the first recombinant protein therapeutic, human insulin, proteins have emerged as a major new class of therapeutics with nearly 380-marketed pharmaceutical products[1]. With the U.S. Food and Drug Administration Center for Drug Evaluation and Review (CDER) and the Center for biologics Evaluation and Review (CBER) combined together, 62 recombinant therapeutic proteins have been approved since 2011 as shown in Figure 1.1[2]. Therapeutic protein drugs, such as monoclonal antibodies, recombinant human cytokines (e.g. α and β interferon), cellular growth factors (e.g. GM-CSF), hormones (e.g. glucagon), neuromuscular antagonists (e.g. botulinum toxin), and blood products (e.g. clotting factor VIII) are an important class of medicines serving patients who were not treatable by any existing traditional small molecule drugs[2, 3].

Proteins, compared with other forms of therapeutics, have the most dynamic and diverse role of any macromolecule in the body; they can catalyze biochemical reactions, form receptors and channels in membranes, provide intracellular and extracellular scaffolding support, and transport molecules within a cell or from one organ to another[4]. What is more, proteins also elicit high specificity with the molecular targets and present high versatility, providing a wide variety of pharmaceutical targets and showing low toxicity level [5, 6]. Inspired by the many advantages of using protein-vectored treatments, scientists have been exploring and developing different protein therapeutics by either purification from native sources or recombinant DNA technologies to treat

a wide variety of clinical indications, including cancers, autoimmunity, inflammation, exposure to infectious agents, and genetic disorders[2].

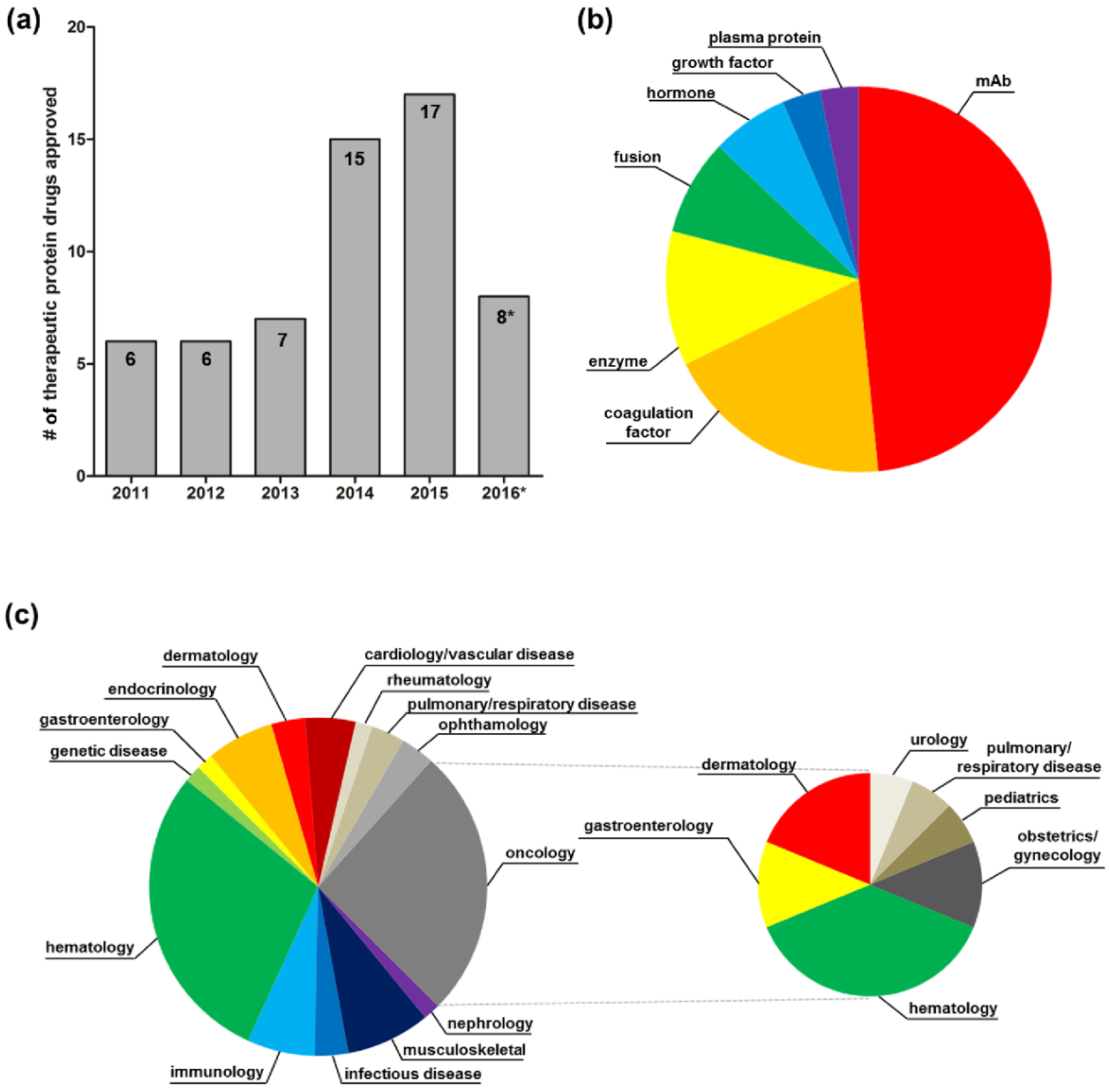

Figure 1.1. U.S. Food and Drug Administration (FDA)-approved therapeutic proteins (2011–2016*)[2].

Although therapeutic proteins are generally considered safe and non-toxic, there are a few drawbacks associated with protein therapeutics, among which high immunogenicity and fast renal clearance are critical concerns in the drug efficacy and toxicity at the user end.

Protein drugs, either purified from native resources or developed via recombinant DNA techniques are often recognized as “non-self” and may elicit unwanted immune responses, generating anti-therapeutic protein antibodies (also known as anti-drug antibodies (ADAs)). This may impact either the pharmacokinetics (PK) of the protein therapeutic (the relationship between the dose and the obtained concentrations in plasma), or the pharmacodynamics (the relationship between systemic concentrations and therapeutic effects), or both. Thus, ADAs may ultimately neutralize the efficacy and/or toxicity profile of therapeutic proteins via accelerated blood clearance (ABC) and cause unwanted toxicity complications[7]. In addition, they are susceptible to proteolytic enzymes in complex environments, such as the human body, causing destruction of drug efficacy[8].

1.2 Current Strategies for Protein Drug Modification

Given the complex issues aforementioned, various technologies have been developed to enhance specific functional attributes of protein drugs without compromising safety and efficacy. For example, the protein conjugation method is currently being used to increase serum half-life, and various protein engineering approaches have been employed to limit drug toxicity and enhance efficacy. Also, current strategies overcoming protein drug immunogenicity and extending blood circulation time have been attempted using different methods, such as humanization process, encapsulation techniques, conjugation, et cetera. Among all the modulation methods, the conjugation of polyethylene glycol (PEG) to these biomacromolecules, a process known as

PEGylation is the most commonly used and well-established one, which alone has brought more than 10 protein drugs into the market[9]. By increasing the overall hydrophilicity and conjugate size, PEGylation prolongs the circulation time through reduction of glomerular filtration, protection from proteolytic enzymes, and coverage of antigenic moieties on the protein surface[10].

Despite this success, there are several shortcomings of PEG as a protein drug carrier that have raised major concerns in the field of nanomedicine. First, although free PEG polymer is believed to be non-immunogenic, it acts as a hapten once bound to the immunogenic protein drugs, generating high immunogenicity accordingly[11]. Emerging pre-clinical and clinical results have also demonstrated the presence of both pre-existing and treatment-induced anti-PEG antibodies (Abs), which has been correlated with the loss of therapeutic efficacy and even has caused fatal side effects to patients[12]. Second, the attachment of PEG to biomacromolecules is also known to reduce protein drug bioactivity due to the disturbance of protein structural integrity[13, 14]. Third, more and more studies have begun to show that the non-degradability nature of PEG causes organ accumulation as shown by *in vivo* animal experiments[15, 16]. Last but not least, PEG is not suitable for long term application as it is susceptible to oxidation[17].

To address these limitations, tremendous efforts have been made by scientists to develop alternatives to PEGylation such as poly(glycerols), poly(oxazolines), poly(amino acids), poly(acrylamides), poly(vinylpyrrolidones), and poly(zwitterions)[18], among which poly(zwitterions) outperform in stabilizing protein activity [19], eliminating immune responses, and extending retention time[20] due to their super hydrophilicity.

1.3 Zwitterionic Stealth Materials for Immune Modulation

Zwitterionic material possesses both cationic and anionic moieties on a single monomer, but is overall charge-neutral. Based on this unique structure, zwitterionic materials can strongly bind water molecules by electrostatically induced hydration and generate a tightly bound water layer, thus achieving outstanding non-fouling properties even in complex biological environment[21]. Over the past several years, various zwitterionic-based materials have been developed and examined both *in vitro* and *in vivo* such as phosphobetaine [22] and poly(sulfobetaine) [23].

Other protein-mimicking polymers like synthetic polypeptides are degradable. Also, they have versatile side chain structures, tunable properties, and numerous functions[24]. Owing to recent advances in synthetic technologies, automated solid phase peptide synthesis (SPPS) has become a facile and powerful tool to generate libraries of polypeptides for numerous applications [25]. The alternating-charged EK polypeptide was shown to stabilize protein *in vitro*[26]. We hence incorporate zwitterionic characteristics with high shielding density polypeptides to cover the immunogenic episodes on a protein surface. What is more, usually functional amine groups are randomly sited on the protein drug surface, causing uneven reaction sites and resulting in partially covered products. The abundance of lysine moieties on zwitterionic EK polypeptide is capable of providing extra amine functional groups, which can be further utilized to increase the surface packing density when used in conjugation via a layer-by-layer graft-to method.

1.4 Solid Phase Peptide Synthesis (SPPS)

The first dipeptide, glycylglycine, was synthesized by Emil Fischer et al. at the beginning of 20th century[27]. Almost half a century later, du Vigneaud et al. in 1953 made the synthesis of

polypeptides possible in homogeneous solution[28]. At that time, the synthesis process was rather time-consuming and technically demanding, requiring separation and purification of all peptide intermediates to ensure good quality control. Hence at that time, it was hard to produce long-chain peptides. In an effort to address these difficulties, a revolutionary breakthrough was made by Bruce Merrifield et al. They pioneered a new heterogeneous synthesis method named Solid Phase Peptide Synthesis (SPPS)[29], which accounts for long chain peptides construction between two phases, an insoluble solid resin support and liquid agents. By this method, long chain peptides can be easily fabricated in a similar way natural ribosome does. To start, the first amino acid of the chain is attached to solid polymer by a covalent bond, and the addition of each succeeding amino acid is completed in a stepwise manner until the desired sequence is assembled. Finally, the peptide is removed from the solid resin and purified via various columns. This greatly simplifies the manipulations and saves time. As a result, SPPS is widely used today. The scheme of a tetrapeptide synthesis process following this synthetic approach is shown in Figure1.2.

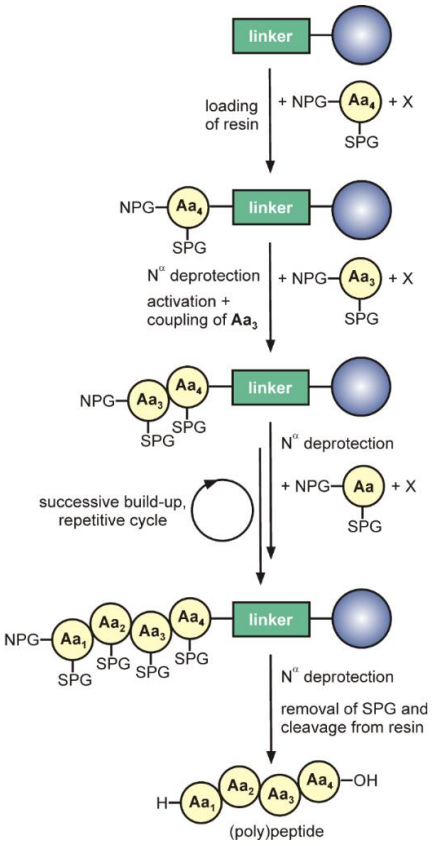

Figure1.2 synthesis scheme of a tetrapeptide assembly by SPPS [25]

In this thesis, we report an *in vitro* synthesis method of a zwitterionic $(EK)_{10}$-C-NH_2 material consisting of alternating anionic Glutamic Acid (E), cationic Lysine (K), and the Cysteine tail providing thiol functional group. Thereafter, zwitterionic PolyEK-CCH and hyperbranched PolyEK-Asparaginase (ASP-EK-T) conjugates were developed by using thiol-ene click chemistry. The *in vitro* synthesis and "graft to" conjugation methods are believed to outperform genetic infusion method which has relatively short sequence, low expression level, and low surface sieving density. Also, the *in vitro* shielding density was characterized by indirect Enzyme-Linked ImmunoSorbent Assay (ELISA) and Hydrophobic Interaction Column (HIC). The *in vivo* pharmacokinetics and immune isolation performance were studied by using a mice animal model.

Chapter 2. Experiments:

2.1 Materials:

All reagents were purchased from commercial sources and used directly unless otherwise specified.

Chemicals

The Fmoc-Glu(t-butyl)-Lys(Boc)-OH (EK Dimer) was synthesized by Dr. Zhefan Yuan and Dr. Priyesh Jain.

Ultrapure Milli-Q water (Millipore, 18.2 MΩ cm) was used for all the experiments. Methanol (anhydrous, ⩾ 99.8%), Ethanol (ACS reagent, ⩾ 99.5%), Sulfuric Acid (>99.999%), N,N′-Dicyclohexylcarbodiimide (DCC), N,N-Dimethylformamide (>99.8%, DMF). Diisopropylcarbodiimide (DIC, >98.0%), Piperidine (>99%), Trifluoroacetic acid (TFA, >99%), 3,6-dioxa-1,8octanedithiol (DODT,>95%), Triisopropylsilane (TIS, >98%), Magnesium sulfate (>98.0%), Phenol (>99.0%), Thioanisole (>99.0%), Dimethyl sulfoxide (DMSO, ⩾ 99.9%), MOPS (>99.5%), Amicon® Ultra-15 Centrifugal Filter Units, Tween 20 (100%), Bovine Serum Albumin (BSA, analytical standard), Phosphate Buffered Saline (PBS, pH 7.4 at 25 °C) were purchased from Sigma Aldrich.

N-α-maleimidoacet-oxysuccinimide ester (AMAS), N-hydroxysuccinimide (NHS), Seeblue Plus 2 Protein Standard, Imject™ Blue Carrier™ Protein (CCH), Pierce™ Bovine Serum Albumin Standard (2mg/mL), HEPES (>99.5%), Carbonate, Bicarbonate, Tris Base (>99.8%), HRP substrate 3,3’,5,5’ tetramethylbenzidine (TMB), Hydrochloric acid (1N) were obtained from ThermoFisher Scientific.

(Nε-tert-butyloxycarbonyl)-l-Lys, Nα-Fmoc-Nε-(tert-butyl)-l-Glu, and Fmoc-Cys(trt)-OH were purchased from aapptec. L-Asparaginase (ASP, purified from E.coli ASI.357, ProSpec), HiTrap™ HIC Selection Kit (GE Healthcare Life Sciences), Asparaginase Activity Assay Kit (BioVision), Anti-Asparaginase antibody (Biotin) (ab34616) (100µg, Abcam), Sodium Chloride (EMD Millipore), HRP-conjugated Mouse IgG-Fc Fragment Antibody (Bethyl), HPR-conjugated Goat anti-Mouse IgM Secondary Antibody (Novus Biologicals USA). Dichloromethane (DCM), Diethyl Ether (anhydrous, Et2O), and acetonitrile were obtained from VWR (West Chester, PA).

Materials:

illustra™ Sephacryl S-1000 Superfine (GE Healthcare Life Sciences), Amicon Ultra centrifugal filter (30K and 100K, EMD Millipore), Corning High-binding 96-Well plates with clear bottom (Sigma Millipore), Corning Non-binding 96-Well Plates (half area, Sigma Aldrich), 96-Well Plates with UV Transparent Flat Bottom (Thomas Scientific), Pierce™ Streptavidin Coated 96-Well Plates (Sigma Aldrich),

Equipment

Liberty Blue Automated Microwave Peptide Synthesizer (CEM Corporation), Gel Permeation Chromatography (GPC, WYATT Orbit Recycle System), CYTATION Imaging Reader (BioTek), Econo-Pac 10DG Desalting Columns (BIO-RAD), Protein Purification Workflow (NGC Chromatography System, BIO-RAD).

2.2 Methods:

2.2.1 Synthesis and Characterization of $(EK)_{10}$-C-NH_2 Polypeptide

$(EK)_{10}$-C-NH_2 was synthesized by Fmoc Solid Phase Peptide Synthesis (SPPS) on Liberty Blue Automated Microwave Assisted Peptide Synthesizer (CEM). Sequence synthesis scale was set at 2.5 mmol on Rink amide MBHA resin (0.6 meq/g substitution). Deprotection was performed in 20% piperidine/DMF solution with machine default microwave conditions. Coupling reactions were performed in the presence of a 5-fold molar excess of reagents [0.2 M amino acid solution (in DMF) with 0.5M DIC (in DMF) and 1.0 M Oxyma (in DMF)] by using 2.5mmol coupling cycle method provided from CEM. Cleavage was performed using 20 ml of cocktail (TFA/phenol/water/thioanisole/EDT;82.5/5/5/5/2.5) for 180 min at room temperature. Following cleavage, $(EK)_{10}$-C-NH_2 was precipitated out and washed with ice-cold anhydrous ethyl ether. Econo-Pac 10DG Desalting Columns were used to furtherly enhance the purity of the products.

The principle of the synthesis process is shown in Figure 2.1.

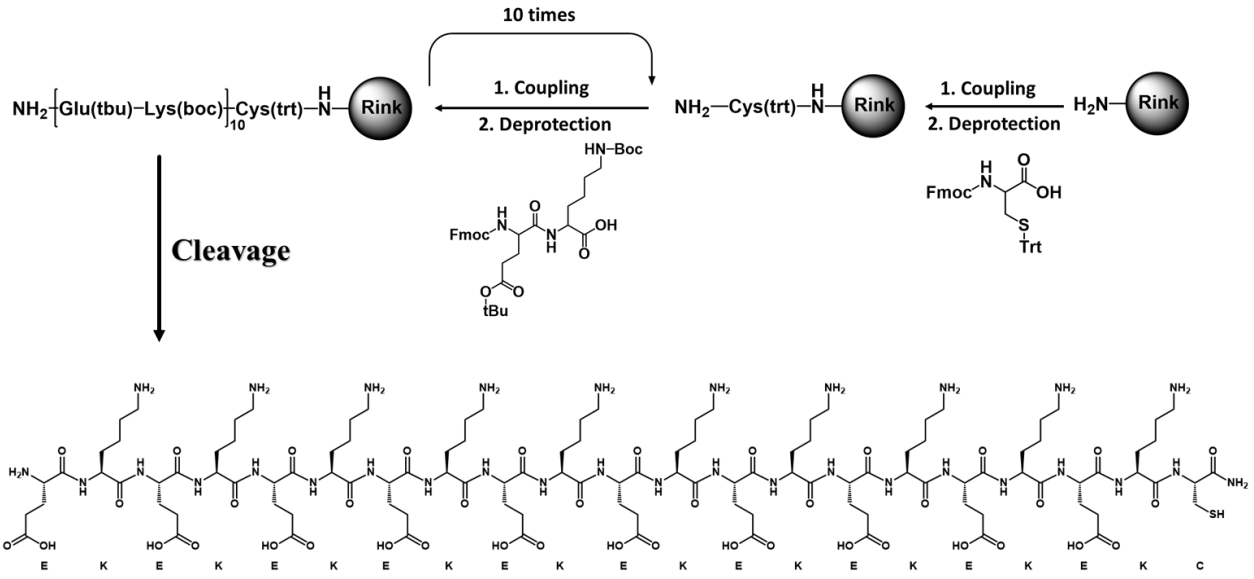

Figure 2.1 Synthesis process of $(EK)_{10}$-C-NH_2 via SPPS method. The cysteine tail was first attached to the Rink Amide Resins (RAM), followed by the step wise alternating attachment of lysine and glutamic acid.

2.2.2 Synthesis and Optimization of Hyperbranched PolyEK-Asparaginase (ASP-EK-T) Conjugate

The hyperbranched PolyEK-Asparaginase conjugate was prepared using a layer by layer method. Amines on native ASP was first activated by a maleimide-NHS bifunctional crosslinker. Two equivalents of AMAS crosslinker to amine groups were dissolved in DMSO (20mg/ml) and added dropwise into ASP solution (2mg/ml in PBS buffer, pH 7.4). Following half hour of stirring, the reaction mixture was ultra-centrifuged for 5 times against fresh MOPS buffer (0.1M, pH7.0) in a protein concentrator tube (MW cutoff: 30k Da) to remove unreacted AMAS and any possible small molecule impurities. Residual solution containing AMAS activated ASP was then combined with $(EK)_{10}$-C-NH_2 stock solution (10 mass-fold excess to ASP, in MOPS buffer) to initiate the first EK layer modification. The conjugation reaction was kept overnight at 4^{o}C and same ultra-centrifuge step was performed to remove excess EK peptide. Purified ASP-EK-Single Layer (ASP-EK-s) conjugate was stored at 2mg/ml in 4^{o}C fridge for further characterization and next conjugation steps. ASP-EK-Double Layer (ASP-EK-d) formulation was prepared by introducing EK peptide to the amine groups originated from 1st EK layer. A similar AMAS activation followed by EK modification procedure was performed and the resulting ASP-EK-d conjugate was purified by ultra-centrifuge (MW cutoff: 100k). By the same method, ASP-EK-Triple Layer (ASP-EK-t) formulation was prepared and purified.

The synthesis processes and final products were illustrated in Figure 2.2.

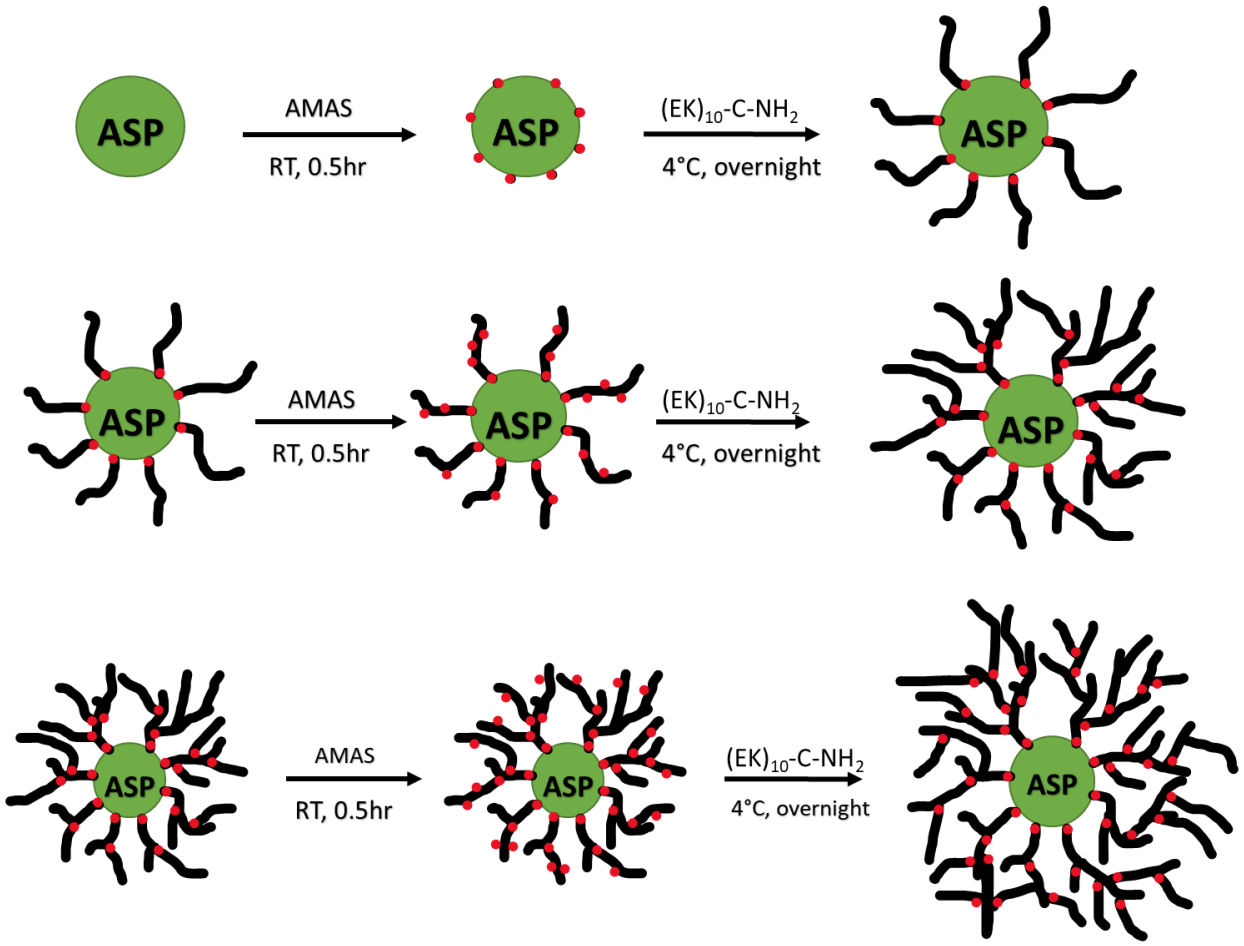

Figure 2.2. Synthesis process of hyperbranched ASP-EK-t conjugate using thiol-ene click chemistry.

Gel Permeation Chromatography (GPC) was performed to characterize the size differences of ASP-EK-S, ASP-EK-D, and ASP-EK-T products. Also, to obtain the final fully covered ASP-EK-T conjugate products, Hydrophobic Interaction Column (HIC) were used to eliminate those not fully encapsulated.

2.2.3 Synthesis and Characterization of CCH-EK Conjugate:

As shown in Figure 2.3, 1:2.5 (amino group and NHS ester ratio) AMAS crosslinker and Imject Blue Carrier Protein (CCH) were stir-reacted for 0.5hr under room temperature. The products were then washed and purified in a protein concentrator tube (MW cutoff: 30k Da) for 5 times. After this, 1:10 (mass ratio) CCH-AMAS and $(EK)_{10}$-C-NH_2 were mixed together in MOPS buffer (0.1M, pH7.0) for overnight reaction under 4°C. Finally, the CCH-EK conjugate were washed for 6 times to eliminate the unreacted $(EK)_{10}$-C-NH_2 and then concentrated to 1mg/ml stock solution for further animal study. The synthesis of CCH-PEG (10K) samples was followed by the same protocol as CCH-EK. 280nm UV absorbance was read to confirm the size increase (CCH is too big to detect the size increase using GPC).

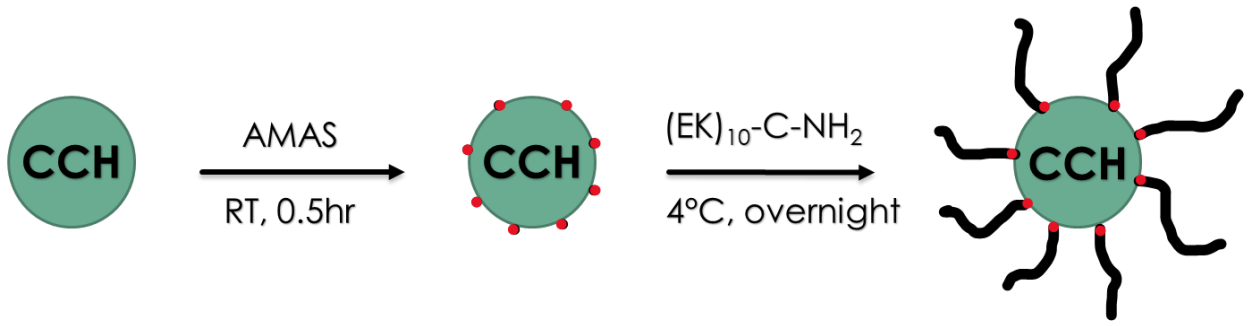

Figure 2.3. The simplified scheme of the synthesis process of CCH-EK conjugate.

2.2.4 *In Vitro* Activity Test of ASP-PolyEK Conjugate

After each synthesis step, conjugate activity was tested to confirm that the protein was still biologically effective. The activity of the conjugates was characterized by using Asparaginase Activity Assay Kit (BioVision) and following the manufacturer's recommended protocol. To be specific, the colorimetric mode was applied, and the sample dilution were confirmed by pilot

experiments to ensure readings are within the standard curve range. 10X dilution was suitable for blood samples collected at 5min, and 5X dilution was applied to blood collected at 1hr, 4hr, 8hr time points. Samples collected at 24hr were diluted 2 times. Other samples were used as collected.

2.2.5 Specific Binding Affinity Characterization

To characterize the molecular sieving ability of the hyperbranched polypeptide, native ASP, ASP-EK-S, ASP-EK-D, and ASP-EK-T conjugate samples were prepared to test their anti-ASP antibody binding affinity. The method used was following the sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) model as reported by previous paper[30]. The detailed process is illustrated in Figure 2.4. Streptavidin-coated 96-well microplates (Pierce) were firstly washed three times with 200 µl wash buffer (25mM Tris, 150mM NaCl, 0.25% bovine serum albumin, 0.05% Tween-20, pH 7.2), after which 100µl of biotinylated anti-asparaginase antibody (10µg/ml) wash buffer was added in each well and incubated for 2hr at room temperature. Then, all wells were washed three times with wash buffer. Subsequently, 100 µl of serial dilutions of native ASP, ASP-AMAS, and ASP-EK-t samples (1mg/ml to 1pg/ml) were incubated for 30min at room temperature. After three times of wash, 100 µl horseradish peroxidase-labelled anti-asparaginase antibody (4mg/ml in wash buffer) was added and incubated for 30 min. After a final 6 wash with, 100µl TMB Substrate Solution was added and incubated for 10min. The absorbance of each well was measured at 450nm right after 50µl 2N HCL stop solution was added.

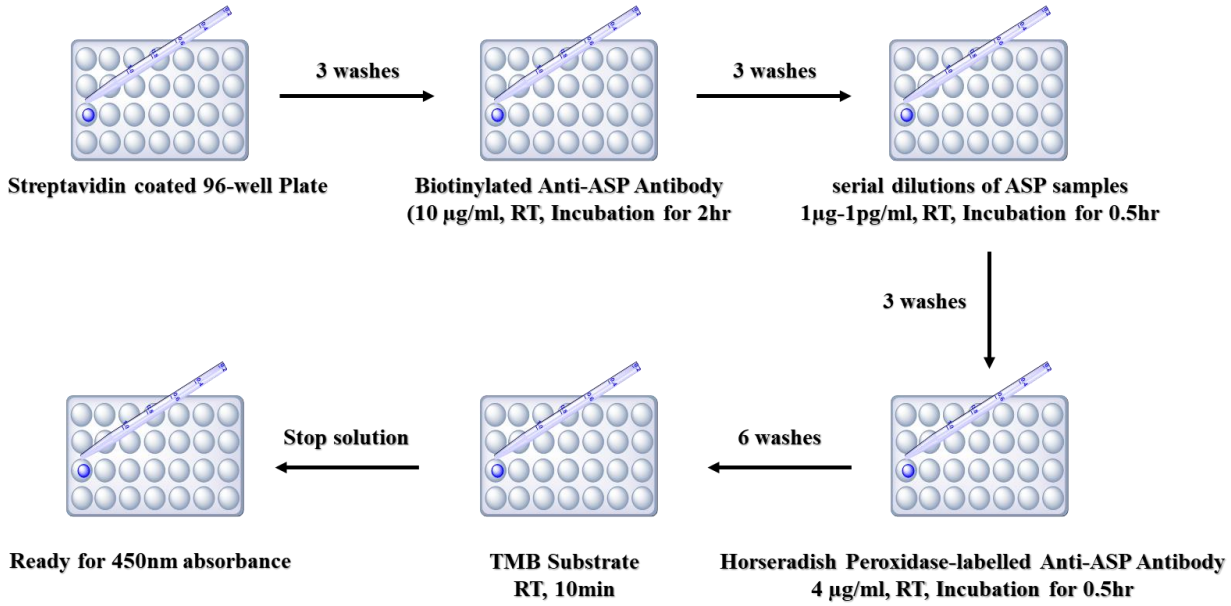

Figure 2.4 Illustration of the *in vitro* sandwich ELISA.

2.2.6 Non-Specific Binding Affinity Characterization

Chromatography was performed in a Pharmacia Fast Protein Liquid Chromatography (FPLC) system (NGC Chromatography System, Bio-Rad) equipped with Butyl HIC column (HiTrap Capto butyl, 5mL, GE). The column was equilibrated with 2M ammonium sulfate in 20mM Tris-HCl (pH 7.4) at a flow rate of 0.5mL/min. Injection of samples was performed using a 1mL loop. Native Asparaginase and EK-Asp conjugates were loaded onto the column and isocratic elution was carried out with 2 M ammonium sulfate in 20 mM Tris-HCl (pH 7.4) at a flow rate of 0.5mL/min. One-milliliter fractions were collected in 1.5mL tubes. The absorbance of the eluate was measured continuously at 280 nm. After elution of unbound or weakly retained species at 2M salt, the ionic strength of the buffer was decreased (Tris-HCl 20mM, pH 7.4) to elute bound species. The fraction pools were concentrated and desalted using ultracentrifuge filter (Ultra-4, Amicon).

2.2.7 Purity and Molecular Weight Characterization via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze purity and molecular weight of ASP-EK conjugate formulations after HIC separation. In general, NuPage Bolt 4-12% Polyacrylamide Gel was used during electrophoresis process. MOPS was used as the loading buffer. Seeblue Plus 2 Protein Standards were used as the ladder. Electrophoresis was performed under 200 voltage for 30min. The gel was rinsed in colloidal blue overnight to visualize protein samples.

2.2.8 Pharmacokinetic and Immunogenicity Study of Hyperbranched ASP-EK-T Conjugate

All animal experiments adhered to federal guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). Animals were randomized to treatment groups at the beginning of each study and a sample size of five animals per group was used. C57LB/6 mice (male, body weight 20–25 g) were purchased from Jackson Laboratories (Seattle, WA).

The pharmacokinetics of native Asparaginase and hyperbranched ASP-EK-t conjugate were studied using two groups of mice. I.V. administrations were performed at the first day of each week for consecutive three weeks. 50μL 12.5mU/ml ASP samples were administered via a tail vein injection method. Blood samples were then collected at 5min, 1, 4, 8, and 24hr time points respectively relative to the injection time. Blood was collected at day 21 and were used for immunogenicity study (IgM and IgG antibody detection) by indirect ELISA. Also, the enzyme

contents in blood serum were estimated by using an Asparaginase Activity Assay Kit. The cartoon of *in vivo* animal experiments is shown in Figure 2.5.

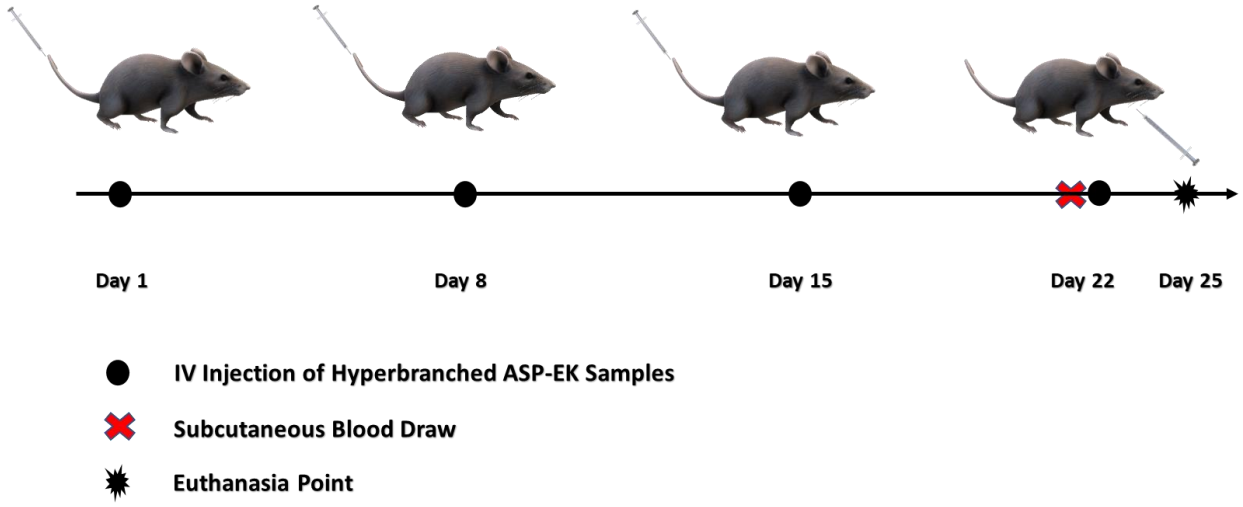

Figure 2.5. Schematic illustration of pharmacokinetics and immunogenicity study with mice model.

In this experiment, anti-ASP, anti-EK and anti-conjugate antibodies were detected. The first step of indirect ELISA is antigen coating. For anti-ASP antibody detection, 100µL native ASP antigen (10µg/mL) solution (prepared in 0.05M carbonate-bicarbonate, pH 9.6 buffer) was added to each well of the 96-well plates. After overnight coating at 4°C, the plates were washed with wash buffer (50mM Tris, 0.14M NaCl, 0.05% Tween 20, 6ml 6M HCl, pH 8.0) three times to remove the uncoated antigens. All wells were then filled with 250µL blocking buffer (50mM Tris, 0.14M NaCl, 1% BSA, pH8.0) for 2hrs incubation at room temperature, after which the plates were washed four times. Then, 100 µL series of diluted mice serum samples (the first antibody, prepared in blocking solution) were added to each well for another 1hr incubation for antibody-antigen binding, after which the wells were washed for five times. Next, 100 µL of secondary antibody, HPR-conjugated Goat anti-Mouse IgM/IgG Secondary Antibody (50000X dilution) was added to each well incubated for 1hr, followed by washing for six times to eliminate the unbound conjugate.

Finally, 100 µL TMB substrate solution was added for a 10min reaction (avoid light, room temperature), and 100 µL stop solution (0.18M H_2SO_4) was added subsequently to stop the enzymatic reaction. Absorbance at 450nm (signal) and 570nm (background) was recorded by the microplate reader. The detections of anti-EK and anti-conjugate antibodies were performed using the same procedure, except the coating antigens were BSA-EK and ASP-EK-T respectively. The antibody measurements were performed in duplicate.

2.2.9 Immunogenicity Study of CCH-EK Conjugate

200 µL 1mg/mL CCH-PEG (10K), and CCH-EK conjugate were administered into two groups of mice respectively by subcutaneous injection method. The administrations of CCH samples were repeated four times with one week as the time interval between each immunization. Blood samples were collected right before injection by subcutaneous blood draw method at day 29 and then centrifuged to get the blood serum for indirect ELISA test. All mouse was euthanized after the fourth week (day 32). The animation of the immunogenicity study is shown in Figure 2.6.

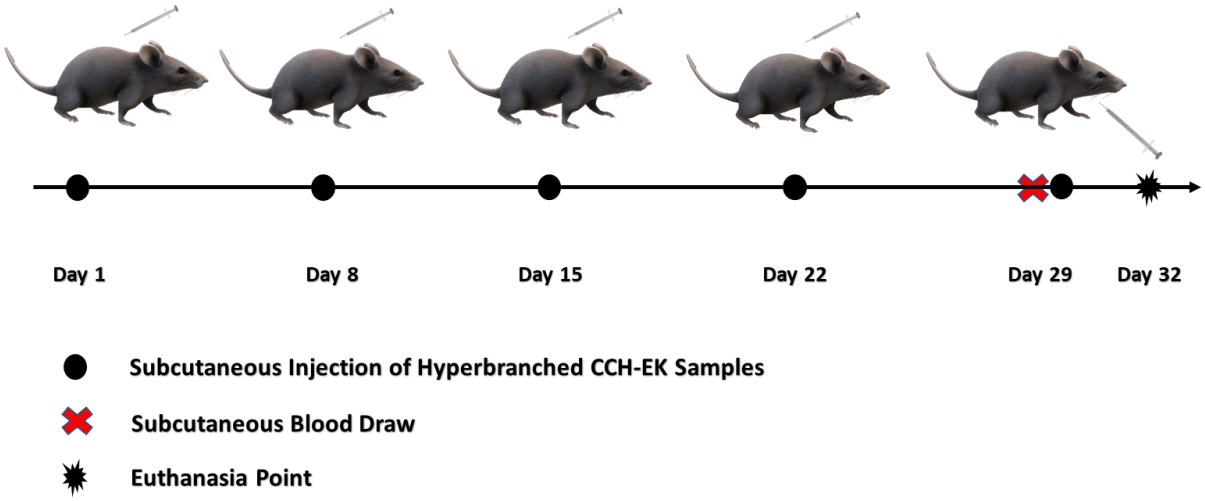

Figure 2.6. Schematic illustration of the immunogenicity study with mice model

For detection of anti-EK antibodies, BSA-EK conjugate was used as the antigen during coating process. BSA-EK conjugate was prepared according to the same procedure as CCH-EK conjugate discussed above, and the indirect ELISA was also performed using the same protocol as described above.

Chapter 3. Results and Discussion

3.1 Synthesis and Characterization of $(EK)_{10}$-C-NH_2 Polypeptide

The automated Solid Phase Peptide Synthesis (SPPS) method is currently the most established method for peptide synthesis in lab settings, which allows the rapid and successive assembly of amino acids into peptide chains on a solid resin support. The porous solid support is composed of resin beads functionalized with amine reactive groups which are capable of chemically coupling the amino acids based on stepwise elongation. Hence, the peptide chain remains covalently attached to the support throughout the synthesis. Also, the application of microwave energy in peptide synthesis not only provide efficient coupling but also yields high purity products in a short time. The excess raw materials, solvent, impurities, and side products can also be easily washed out without the compensation of the desired products. In this thesis, the alternative EK structure renders the zwitterionic property, the cysteine tail provides the thiol functional group for further conjugate synthesis. Also, microwave was incorporated to produce the peptide chain with high degree of yield and low degree of racemization. At the end of the synthesis, the desired polypeptide was cleaved from the support while simultaneously removing all protecting groups by using a prepared solution (TFA/phenol/water/thioanisole/EDT;82.5/5/5/5/2.5).

It is important to note that the cleaved products were of relatively high Polydispersity Index (PDI). Therefore, to obtain the final products with equal molecular weight, the cleaved polypeptide was precipitated using ice-cold anhydrous ethyl ether solvent and purified via Econo-Pac 10DG Desalting Columns. The Gel Permeation Chromatography (GPC) results are shown in Figure 3.1, indicating the desired products.

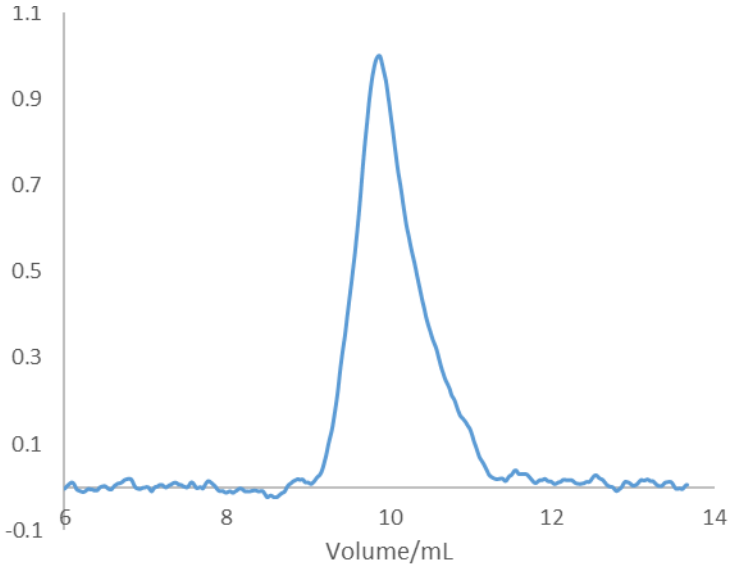

Figure 3.1. The Gel Permeation Chromatography of $(EK)_{10}$-C-NH_2

3.2 Synthesis and Optimization of Hyperbranched PolyEK-Asparaginase (ASP-EK-T) Conjugate

L-Asparaginase has been a key component for the treatment of acute lymphoblastic leukemia (ALL) for nearly 50 years[31]. Due to its non-human nature which elicits severe immune responses and hypersensitivity reactions in patients, the enzyme efficacy is severely impaired and the administered ASP was cleared rapidly through accelerated blood clearance (ABC). Since ASP has enough lysine monomer on the surface to serve as reactive sites, it's easy to do surface modification by using the "graft to" method. Up to now, PEGylation is the most commonly used method to increase serum half-life and shield the immunogenic episodes of ASP. Although this method showed increased serum half-life and lower anti-ASP antibody level in some patients, there are increasing clinical statistics showing that PEGylated ASP also exhibits low efficacy to patients with either pre-existing anti-PEG antibodies or PEG triggered antibodies[32], rendering

their treatment ineffective. The anti-PEG antibodies may even cause lethal side effects to patients. Thus, a new biomaterial with super low immunogenicity and adequate functional groups (enabling high surface packing density) is highly demanded. In comparison to traditional conjugation methods in which neutral linear polymers were covalently attached to protein surfaces, functional and architecturally complex nonlinear polymers, especially polypeptides, offer numerous functional groups for enhancing the potential of therapeutic proteins.

Hence in this thesis, we designed a hyperbranched formulation in which three layers of the polypeptide were conjugated to the surface of ASP via thiol-ene click chemistry. After purification, this triple layer construction ensures full coverage of surface immunogenic epitope and simultaneously increases largely the conjugate size, which results in a longer *in vivo* circulation time. The simplified scheme of the molecular structure is shown in Figure 3.2.

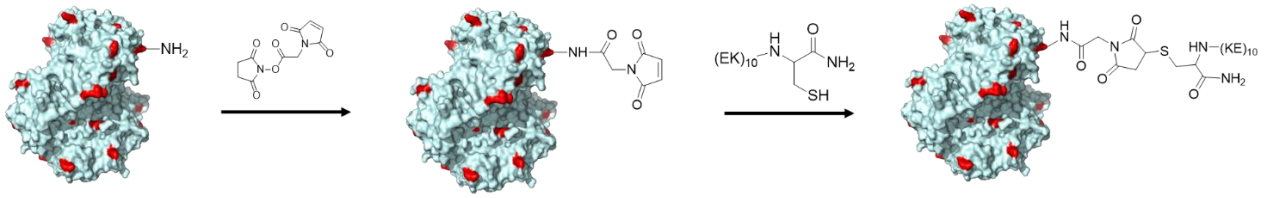

Figure 3.2. The simplified conjugate synthesis principle and process. The red color indicates the lysine group residing on the ASP surface.

The degree of modification (hydrodynamic volume change of the conjugate) after each layer immobilization is shown in Figure 3.3. As it's presented to us, the retention time decreases as the number of conjugated layers increases, indicating the obvious size expansion after each polypeptide conjugation.

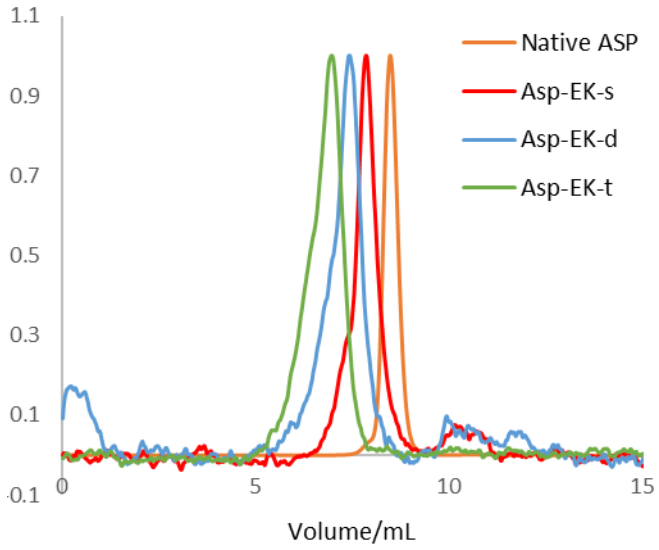

Figure 3.3. Gel Permeation Chromatography of native ASP, ASP-EK-S, ASP-EK-D, and ASP-EK-T conjugates.

3.3 *In Vitro* Activity Test of ASP-EK Conjugates

Although the conjugation method shows great promise in pharmaceutical industries, for protein drugs, particularly enzymes, it is essential to demonstrate that the biological activity of the native protein is not destroyed. Hence, *in vitro* activity testing was performed with native ASP, ASP-AMAS, ASP-EK-t, and PBS buffer (as background). The kinetic results are shown in Figure 3.4. As the reaction progresses, the converted products accumulate, resulting in the linear increase of 570nm absorbance.

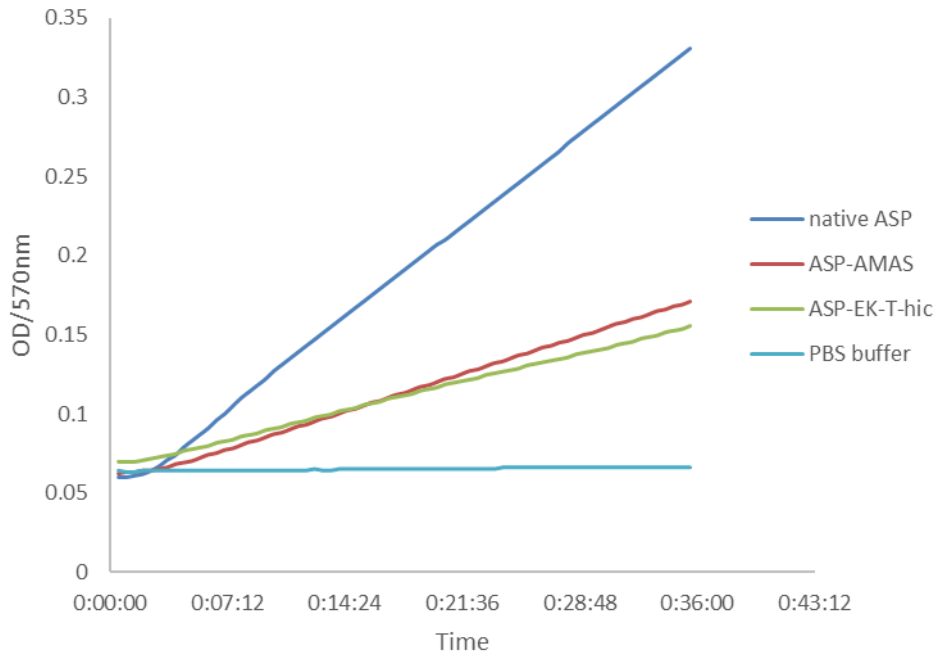

Figure 3.4. The kinetics of ASP enzymatic activity with native ASP, ASP-AMAS, ASP-EK-T, and PBS buffer samples.

As illustrated, the enzymatic activity is demonstrated by the time needed to react the exact number of substrates, and higher slope means higher activity. Within the linear range between 10min to 25min, the ASP-AMAS retains 52.5% activity compared to native ASP after covalent attachment. Even more, it is noteworthy that even after three layers of conjugation, the ASP-EK-t conjugate still had 45.6% of the control specimen's activity, but its recognization by anti-ASP antibodies was more than 1000-times less. Hence, it is reasonable to conclude that the AMAS crosslinker is the primary culprit in decreasing the ASP bioactivity. Compared to the relatively small decrease in activity, the gain in epitope shielding *in vitro* is enormous.

3.4 Specific Binding Affinity Characterization

In order to demonstrate that the three-layer zwitterionic polypeptide-protein conjugates offer significant shielding effects, *in vitro* ELISA testing was carried out and the results are shown in Figure 3.5.

In this experiment, streptavidin-coated 96-wells were used as the template, anti-ASP antibody was employed as the first antibody, and the HRP-conjugated anti-ASP antibody was used as the second antibody. Thus, a higher OD number means more bound ASP samples, which indicates a lower surface shielding capability. Overall, it was concluded that as the number of conjugate layers increases, the ASP binding affinity to anti-ASP antibodies decreases. To be specific, compared to the native ASP which has a detection limit of 10^3pmol/ml, the single layer conjugate significantly reduces the binding affinity with a detection limit of 10^5pmol/ml, but still has a tinny of binding ones. However, the second and third layer almost fully cover the binding episodes of ASP, indicating a satisfactory sheltering effect under mild conditions.

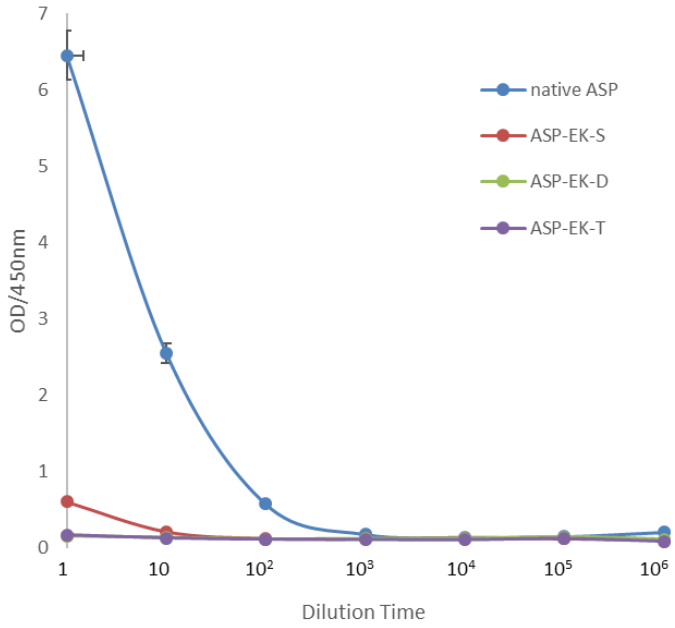

Figure 3.5. *In vitro* anti-asparaginase antibody binding affinity of native ASP, ASP-EK-S, ASP-EK-D, and ASP-EK-T. The original concentration is 1μmol/ml.

3.5 Non-Specific Binding Affinity Characterization

Despite the size increase, there remains a few conjugates which were not fully packed, as shown in Figure 3.6. Therefore, Hydrophobic Interaction Chromatography (HIC) was employed to separate and purify the fully packed conjugates while maintaining biological activity. Once dissolved in the high-salt buffer, the solvation of sample solutes was reduced, and the hydrophobic regions that became exposed were attached to the medium. A series of decreasing salt gradient Tris-HCl buffers were then applied to elute samples from the column, and the fully covered samples were washed out and collected. The HIC results are shown in Figure 3.7.

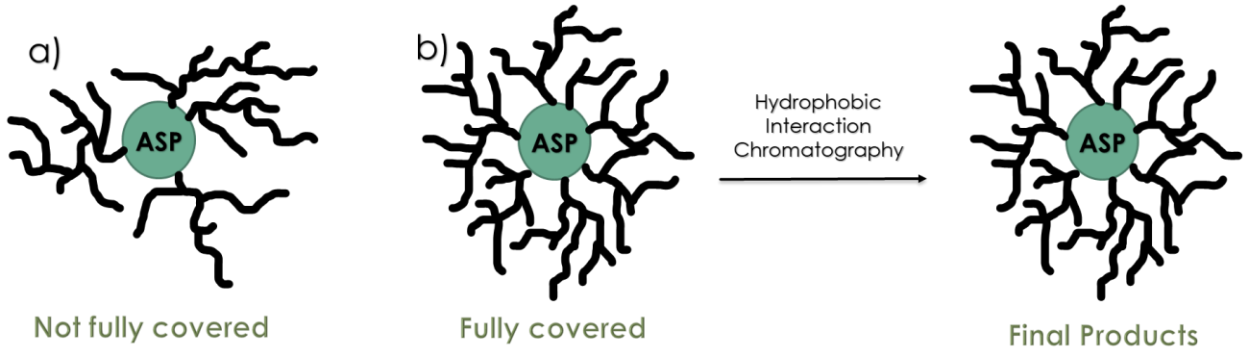

Figure 3.6. a) Asparaginase not fully covered. The conjugate will present immunogenicity when dissolved in high salt buffer; b) the fully covered Asparaginase conjugate.

Compared to the native ASP, which was eluted at 1.13M concentration, the hyperbranched conjugate shows two peaks. The left one represents the fully covered conjugates, it just goes out with the loading buffer, with no non-specific binding affinity. The other one is eluted at 1.76M concentration, which shows a litter non-specific binding. Table 3.1 shows the sample elution gradients, and the three-layer conjugate has the best results.

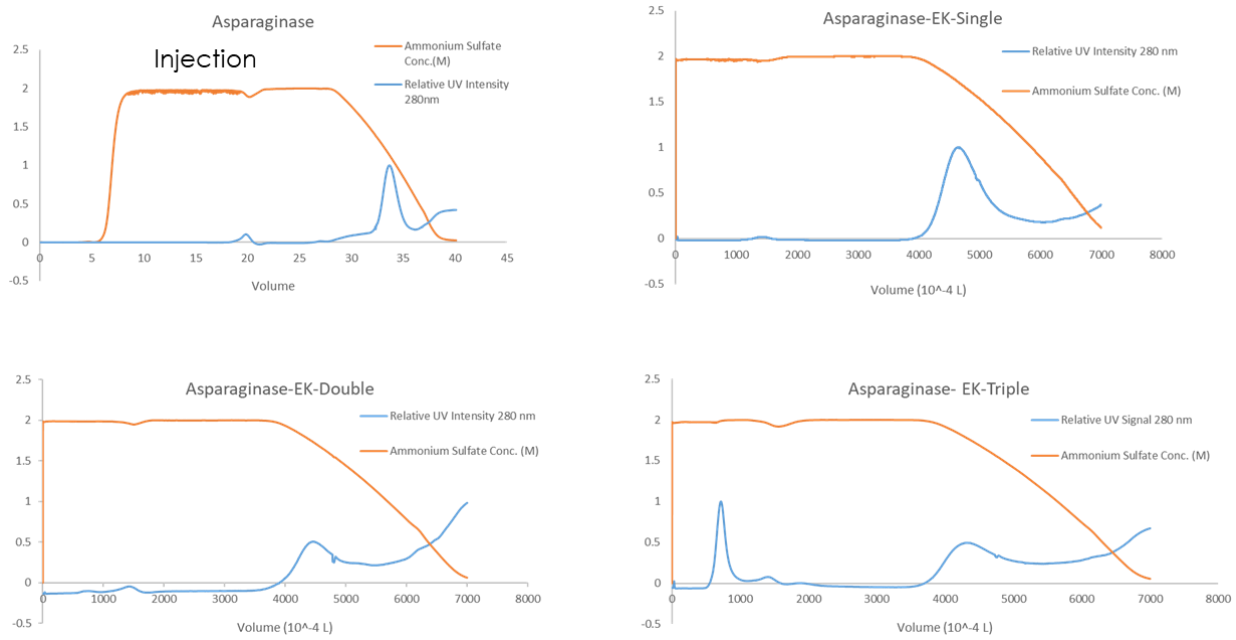

Figure 3.7. HIC results of native ASP, ASP-EK-S, ASP-EK-D, and ASP-EK-T.

Table 3.1 Sample elution gradients

Asparaginase Formulation	**Ammonium Sulfate Concentration at Elution**
Native Asparaginase	1.13 M
Asparaginase-EK-single	1.71 M
Asparaginase-EK-double	1.73 M
Asparaginase-EK-triple	2 M (unbound), 1.76 M

3.6 Purity and Molecular Weight Characterization via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to characterize the increases of molecular weight after each layer conjugation. The mass distribution of the samples is shown in Figure 3.8. Compared to the native ASP (MW 35K), the MW of ASP-EK-triple drastically increases to 198K. Also, it is evident to see the MW increase after each layer

conjugation. Through this method, conjugates with the highest molecular weight were collected for animal experiments.

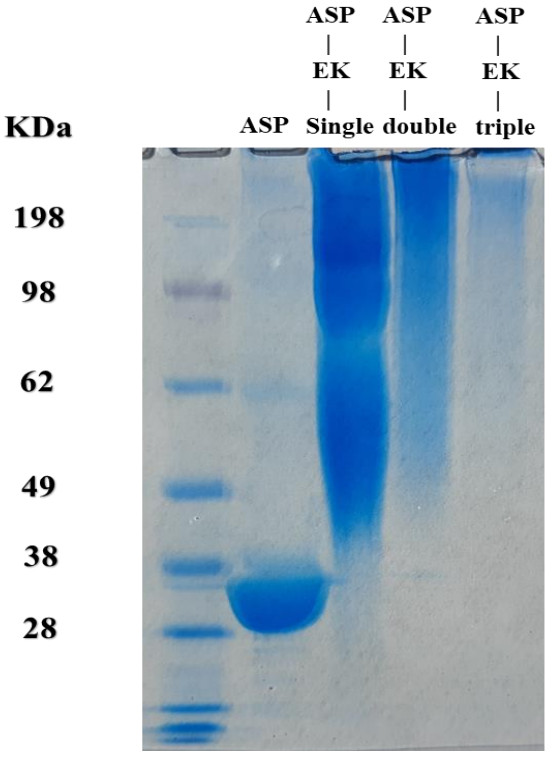

Figure 3.8. SDS-PAGE results of native ASP, ASP-EK-t, ASP-EK-d, and ASP-EK-t samples.

3.7 *In Vivo* Pharmacokinetics and Immunogenicity Study of Hyperbranched ASP-EK-T Conjugate

The serum half-life of therapeutic protein drugs is mainly influenced by two factors: drug size and immunogenicity. Drugs that are too small exhibit fast renal clearance while immunogenicity leads to low drug efficacy and accelerated blood clearance. Hence, it is essential to increase the overall hydrodynamic size and shield the immunogenic episodes on the surfaces of protein drugs. Having demonstrated that the grafting of hyperbranched $(EK)_{10}$-C-NH_2 did not eliminate catalytic activity,

subsequent *in vivo* experimentation was carried out to examine the circulation time of and immune responses towards ASP and the EK polypeptide.

For PK characterization, the Asparaginase Assay Kit and kinetic colorimetry detection were employed to obtain the serum circulation data. The results of both the native ASP group and the ASP-EK-T group are shown in Figure 3.9. Overall, the ASP-EK-t conjugate significantly outperformed the native ASP and sustained bioactivity for longer periods post-injection. To be specific, the ASP-EK-T conjugate maintained superior circulation time even after the third injection, while the native ASP experienced an obvious accelerated blood clearance. Meanwhile, there is no ABC effect observed in the ASP-EK-t group. The extended and unchanged circulation time of ASP-EK-t conjugate after triple administration reveals the non-fouling property and the size increase, which together evade the fast clearance by immune and renal system.

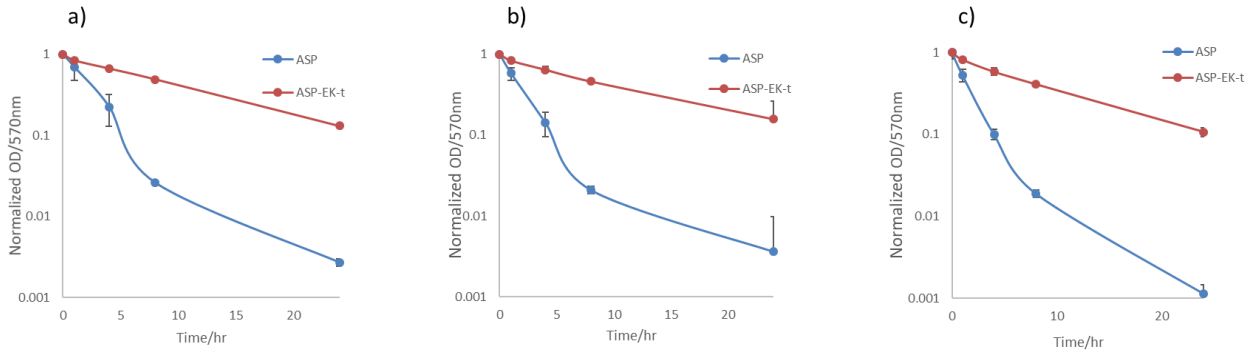

Figure 3.9. a) week 1, b) week 2, c) and week 3 pharmacokinetics of native ASP and ASP-EK-t samples via IV. Injection in mice.

Immunoglobin G (IgG), the most abundant antibody in the body, was detected in mice serum at day 21. The indirect ELISA results are shown in Figure 3.11. The anti-ASP titer in the ASP-EK-t mice group is much lower than the native ASP group, which demonstrated that the immunogenic episodes were fully shielded by the polypeptide. Also, the super low anti-EK titer in the ASP-EK-

t mice group indicates that the synthesized polypeptide is of super low immunogenicity even under complex environments.

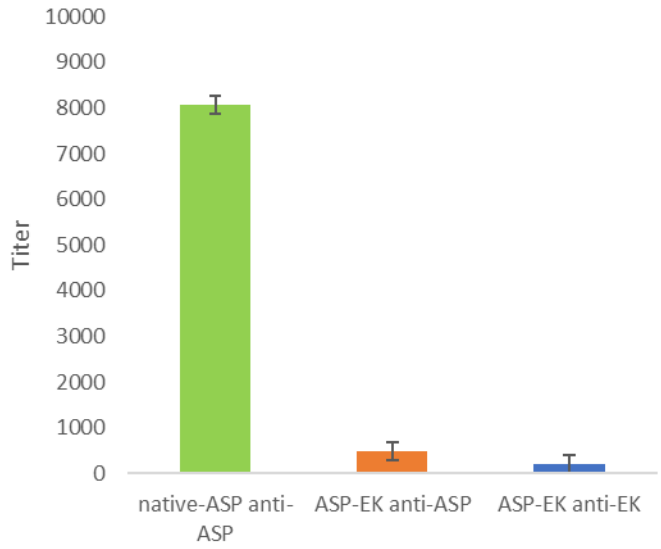

Figure 3.10 Detection of week 3 anti-ASP and anti-EK IgG antibodies in mice serum.

3.8 Synthesis and Characterization of CCH-EK Conjugate:

The synthesized $(EK)_{10}$-C-NH_2 zwitterionic biomaterial with molecular weight (MW) 2.8kDa belongs to the class of small molecule drugs (hapten), whose MW resides in the range of 2-5 kDa and are not usually immunogenic even when administered in the presence of adjuvants, such as organic compounds, (poly)peptides, hormones, lipids, and oligosaccharides. One method to examine the potential immunogenicity of small molecule drugs is to attach them to a highly immunogenic protein carrier and stimulate immune responses. If results only show the antibodies towards immunogenic protein without that towards polymer, then it can be concluded that the compound possesses an immune isolation property.

Imject Blue Carrier Protein, a highly soluble, mollusk-derived concholepas concholepas hemocyanin (CCH, extremely stable heterodidecameric structure composed of two large

polypeptide subunits with molecular weight 404/351KDa respectively), is one of the most commonly used hapten carriers due to its large size, numerous immunogenic epitopes, superior solubility, and abundance of lysine residues for conjugation. Together, these features can ultimately increase the likelihood of stimulating hapten-specific antibodies.

After demonstrating that the ASP-EK-T conjugate does not show immunogenicity towards either proteins or polypeptides, it is desirable to further confirm that when attached to a highly immunogenic protein carrier, the $(EK)_{10}$-C-NH_2 still shows no hapten effects. Hence in this thesis, CCH was employed as the carrier protein, and the $(EK)_{10}$-C-NH_2 was covalently conjugated to the CCH by using AMAS crosslinker chemistry. CCH-PEG conjugate was used as the control group. The results were confirmed by 280nm absorption.

3.9 Immunogenicity Study of CCH-EK Conjugate

The IgM and IgG isotype antibodies were detected at week 4, and the results are shown in Figure 3.11. As expected, strong immune responses were induced by PEGylation (IgM titers 1:1600; IgG titers 1:1600). By contrast, the anti-polymer response observed for CCH-EK conjugate was negligible (IgM titers<200; IgG titers <200), thus demonstrating its effectiveness in ameliorating humoral immune responses. This greatly increases the possibility in long-term therapies with low administration frequency.

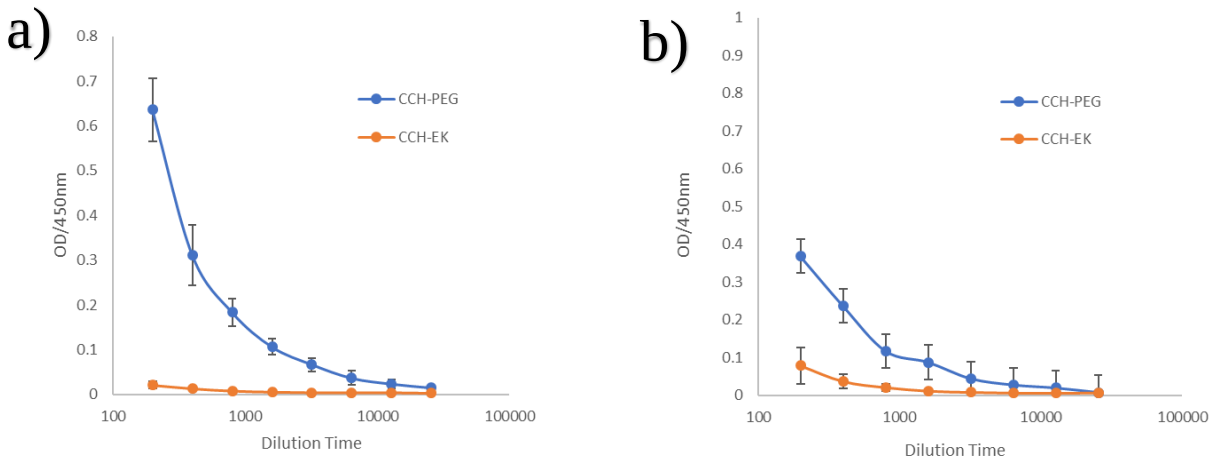

Figure 3.11. Detection of anti-EK IgM and IgG in mice serum. a) Week 4 anti-IgM antibody; b) Week 4 anti-IgG antibody.

Chapter 4. Conclusions

In summary, we successfully synthesized a well-defined functional zwitterionic $(EK)_{10}$-C-NH_2 polypeptide and developed a series of methods to prepare ASP-EK-t hyperbranched conjugates and CCH-EK conjugates for *in vivo* studies. The conjugate activity was measured by Asparaginase Activity Kit (colorimetric mode) while the surface shielding capacity was characterized by HIC and *in vitro* Binding Affinity Test. The *in vivo* performance was then studied by using a mice model, focusing on pharmacokinetics and immunogenicity. Our data showed that ASP-EK-t hyperbranched conjugates were fully covered with no immunogenic episodes observed. Furthermore, the synthesized conjugate retained 45% *in vitro* enzymatic activity compared to the native ASP, while possessing a sustained circulation time which surpasses the performance of the native ASP. In addition, our immunogenicity study shows no antibody detection towards both ASP and polypeptide at week 3, indicating the stealth property of the synthesized $(EK)_{10}$-C-NH_2 and its high surface packing density. Finally, CCH protein carrier was employed as a model protein to explore the non-fouling limit of the polypeptide, and the results show that $(EK)_{10}$-C-NH_2 possesses no hapten effect even when attached to a highly immunogenic protein. Overall, this work provides a novel zwitterionic polypeptide with super-low immunogenicity and multi-functionality. Future research is to explore the application of $(EK)_{10}$-C-NH_2 for other protein conjugates.

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