A Therapeutic Uricase with Reduced Immunogenicity and Improved Pharmacokinetics

Chenjue Tang

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Abstract

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Chenjue Tang

Chair of the Supervisory Committee:
Boeing-Roundhill Professor, Dr. Shaoyi Jiang
Chemical Engineering

Due to the lack of uricase, humans are not capable of oxidizing uric acid in their bodies. High level of uric acid in serum may cause crystallization in joints and acute inflammation with severe pain, known as gout. Uricase is a protein of the promising therapeutic value for patients with gout, but it still meets several challenges, such as the short blood-circulation life, instability and inherent immunogenicity of an exogenous enzyme. Chemical conjugation of poly (ethylene glycol) (PEG) is one of the methods to address this issue, but pre-existing and induced PEG-specific antibodies can cause adverse side effects. Here, we reported a novel hydrophilic polypeptide for uricase modification. We studied the thermal stability and in vivo pharmacokinetics of this polypeptide-protein conjugates. Results show improved thermal stability and prolonged in vivo half-life circulation and a feasible method for highly immunogenic therapeutic proteins.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1. INTRODUCTION

1.1 PROTEIN THERAPEUTICS AND CURRENT ISSUES

Treatment with therapeutic proteins has emerged as a promising approach since early 1980s when the first recombinant human therapeutic protein, Humulin, was created by Genentech in the treatment of diabetes mellitus [1]. Recently developed protein therapeutics target patients with cancers, genetic disorders, autoimmunity, or exposure to infectious agents. Protein therapeutics can be classified based on molecule categories, including enzymes, antibody-based drugs, growth factors, interleukins, interferons and Fc fusion proteins [2, 3]. A small part of therapeutic proteins is directly obtained from native source while most are produced from organisms such as bacteria, insect cells, mammalian cells and plants and the choice of a production system depends on production cost or protein properties. [4-7] Recombinant proteins have several advantages over native proteins. First, the production processes of recombinant proteins are usually more efficient and cheaper. Second, recombinant proteins containing human gene sequences may have higher specificity and lower immunogenicity. Third, recombinant proteins can help patients to avoid exposure to potential human diseases. Last but not the least, recombinant proteins can possess improved functions via a recombinant DNA technology over non-recombinant proteins[8].

Comparing to small molecule drugs, therapeutic proteins also have many advantages, such as therapeutic proteins have more specific and complex functions, less potential to interact with normal physiobiological process, higher tolerance and less chance to induce immune response if therapeutic proteins can also be derived from the human body[9]. In addition, the U.S. Food and Drug Administration (FDA) approved duration of therapeutic proteins may also be shorter than that of small molecule drugs. According to the FDA database, more than 240 therapeutic proteins

and peptides have been approved for clinical trials[10, 11] and 62 recombinant therapeutic proteins have been approved since 2011 (Figure 1.1) [12].

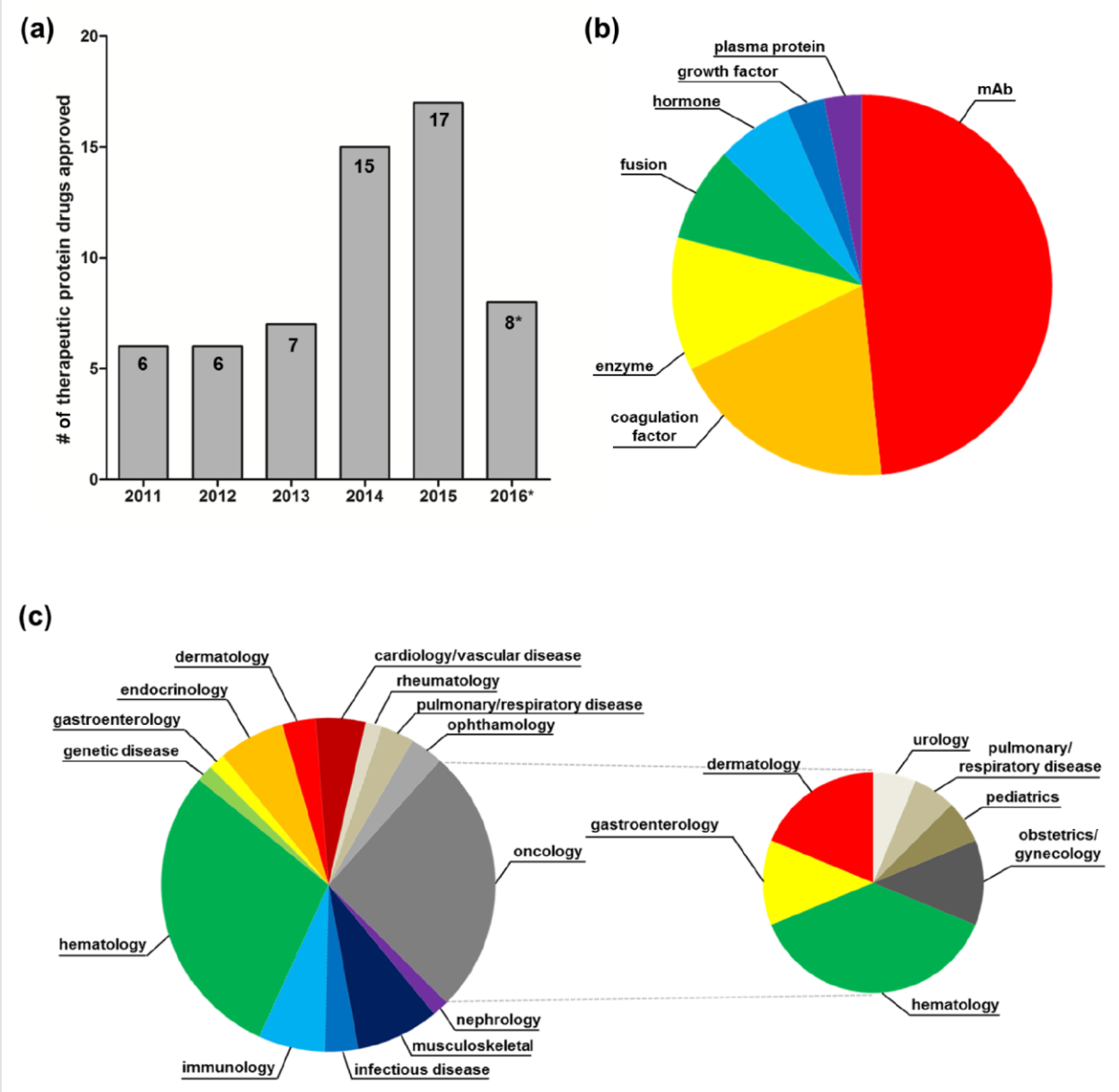

Figure 1.1 FDA-approved therapeutic proteins by year 2011-2016 (a) Number of therapeutic protein FDA approved. (b) Distribution of therapeutic proteins FDA approved. (c) Distribution of FDA-approved therapeutic proteins by therapeutic area (Left) and in area for oncology drugs (Right).[12]

Despite the advantages of protein therapeutics mentioned above, both recombinant and non-recombinant proteins are still facing some pressing issues. First, the complex structure of proteins makes them unstable and vulnerable under physiological conditions. Additionally, any proteins from non-self sources could be recognized by the immune system, elicit the immune responses and be cleared by anti-drug antibodies (ADA), which could result in either poor pharmacokinetics (PK) or pharmacodynamics (PD) or both, ultimately causing efficacy issues[13]. To sustain the concentration of protein drugs in serum to achieve expected effects, increasing dosages are required for certain patients while this could lead to severe adverse effect and even threaten patients' lives[14].

1.2 PROTEIN CONJUGATION

To address the pressing issues aforementioned, many promising strategies, including Fc fusion proteins and antibody therapeutics, have been applied to protein therapeutics to endow them with better efficacy, improved safety and delivery efficiency and reduced toxicity [15]. Among all the methods, protein conjugation to chemicals is the most commonly used and well-established technology as a post-production modification methodology in protein therapeutic field. The most commercially successful chemical involved with this technology is poly (ethylene glycol) (PEG), and the conjugation process is known as PEGylation. By increasing the molecular weight and hydrodynamic volume of protein by a factor of five to ten via PEGylation, protein conjugates can avoid rapid renal clearance, and thereby increase their circulation time in serum. Extended half-life of protein conjugates enables less frequent drug administration[16]. Additionally, serum circulation half-life of protein conjugates can be finely tuned by varying the amounts, molecular weight and branching degree of conjugated PEG[17]. Another benefit of PEGylation is the ability of reducing the immunogenicity of protein and preventing the aggregation. The attached PEG

molecules can attract surrounding water molecules and form a hydration layer to increase overall hydrophilicity, shield the antigenic moieties on the surface from immune system recognition and prevent protein conjugates from aggregation, opsonization, enzymatic degradation, and phagocytosis by mononuclear phagocyte system (MPS) [18].

Although more than ten PEGylated protein therapeutics have been approved by FDA since the first PEGylated drug, Adagen, was commercially available on the market in 1990[19]. Some drawbacks raised people's concern in the field of nanomedicine. First, free PEG polymers are regarded as non-immunogenic material and induce no antibodies in serum, but once they are attached to immunogenic protein, they behave as haptens and trigger production of anti-PEG antibodies, which could induce rapid clearance in subsequent administration[20]. Besides that, some researches also showed that PEGylated liposomes may cause hypersensitivity reactions by activating complement system[21]. For some therapeutic proteins such as asparaginase and uricase, PEGylation can even result in loss of bioactivity due to distortion effects on protein structure[22]. Last but not the least, the nature of non-degradability and susceptibility to oxidation of PEG polymer make this material not suitable for long term application *in vivo*[23].

1.3 ZWITTERIONIC POLYMER IN IMMUNOGENICITY REDUCTION

Zwitterionic materials are a category of materials containing both cationic and anionic parts with high dipole moments but can still stay in neutral charge states[24]. Unlike the hydration layer of PEG formed via hydrogen bonding, zwitterionic polymers can attract water molecules by electrostatic effect and develop a strong hydration layer around the polymers due to the unique structure, which also makes zwitterionic materials possess better capability of resisting nonspecific protein adsorption, preventing the formation of capsules after implantation *in vivo*, avoiding the recognition and clearance of immune system and increasing the stability of enzyme in urea-rich

conditions without sacrificing its bioactivity[25-29]. These materials are widely spread in nature including cell membranes and proteins, and were successfully synthesized in 1950s for the first time. Carboxybetaine (CB) and sulfobetaine (SB) are two representatives of synthesized zwitterionic materials developed in recent years, and polyCB (pCB) and polySB (pSB) have been applied as alternatives to PEG through the conjugation to amine groups on protein surface (Figure 1.2). Comparing to PEG, zwitterionic materials can provide better stabilizing characteristics, prevent the production of anti-PEG antibodies and will not lead to activity loss after conjugation[14, 25].Although zwitterionic materials demonstrate superhydrophilicity, better non-fouling and protein-stabilizing property, they still suffer from the same problem, non-degradability, as PEG [30, 31].

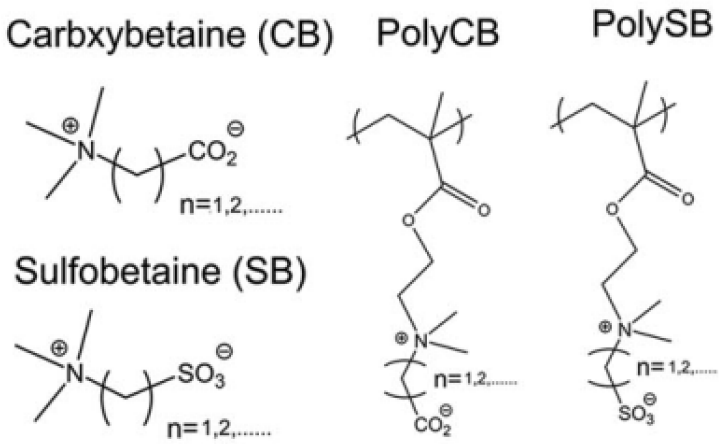

Figure 1.2. The molecular structures of CB and SB moieties and polyCB and polySB[31].

Polypeptides consisting of positively charged lysine (K) and negatively charged glutamic acid (E) could be another solution to PEG. Poly(EK) possesses similar zwitterionic structure as pCB or pSB because of the repeated EK sequences (Figure 1.3), thus poly(EK) can also possess superhydrophilicity and biocompatibility as well as biodegradability[32]. Compared with

conjugation to pCB or PEG, conjugation to poly(EK) can provide extra amino groups, which is another advantage, especially for those proteins containing insufficient amino groups on the protein surfaces for conjugation. Abundant amine groups on poly(EK) can also be utilized as the substrate for next-layer conjugation. Therefore, the conjugation density can be dramatically elevated through this layer-by-layer graft to methodology to cover the episodes on protein surfaces and lower the immunogenicity.

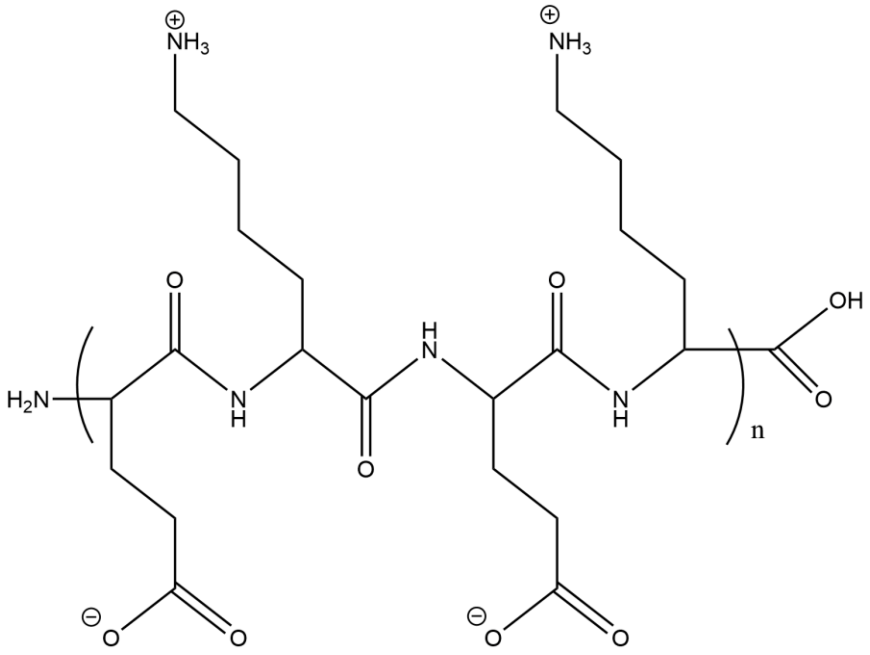

Figure 1.3 Structure of the EK peptide, showing alternating charges on each residue.

Chapter 2. EXPERIMENT

2.1 MATERIALS

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

Chemicals:

Uricase from Candida sp. (>4.2U/mg), N,N′-Dicyclohexylcarbodiimide (DCC), N,N-Dimethylformamide (DMF, >99.8%), piperidine (>99%), Diisopropylcarbodiimide (DIC, >98.0%), sodium sulfate, trifluoroacetic acid (TFA, >99%), dimethyl sulfoxide (DMSO, ⩾ 99.9%), Amicon® Ultra-15 centrifugal filter units, phosphate buffered saline (PBS, pH 7.4 at 25 °C) were purchased from Sigma Aldrich.

N-hydroxysuccinimide (NHS), N-α-maleimidoacet-oxysuccinimide ester (AMAS), Amplex™ Red Uric Acid/Uricase Assay Kit, carbonate bicarbonate, hydrochloric acid (1N) and hydrochloric acid (12M) were purchased from ThermoFisher Scientific.

Rink amide MBHA resin, Fmoc-Glu(tBu)-OH (>99%) and H-Lys(Boc)-OH (>99%) were obtained from Aapptec.

Dichloromethane (DCM), hexane, diethyl ether (anhydrous, Et2O), ethyl acetate and acetonitrile were obtained from VWR (West Chester, PA)

Materials:

Amicon Ultra centrifugal filter (cutoff: 30K and 100K, EMD Millipore), 96-Well Plates with UV Transparent Flat Bottom (Thomas Scientific), Corning Non-binding 96-Well Plates (Sigma Aldrich)

Equipment:

Gel Permeation Chromatography (GPC, WYATT technology), multimode plate reader (Cyt3M, BioTek), benchtop centrifuge (5804R, Eppendorf), Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM corporation).

2.2 METHODS

2.2.1 *Synthesis and characterization of EK dimer*

Fmoc-Glu(tBu)-OH (42.5g, 0.1 mol) and NHS (13.8g, 0.12 mol) were dissolved in anhydrous acetonitrile (400 mL) under nitrogen. Then DCC (22.7 g, 0.11 mol) was added and the mixture was stirred at RT overnight. In a separate round-bottom flask, H-Lys(Boc)-OH (25.0 g, 0.102 mol) was stirred in a mixture of $NaHCO_3$ 6.8%/acetonitrile 1:2 (900 mL) and then the mixture was poured dropwise into the former solution. The reaction was kept stirring for 24h at RT before adjusting the pH to 6 (1N HCl). All precipitants were filtered, and acetonitrile was evaporated. Aqueous layer was acidified to pH=1 by 12M HCl and extracted with DCM (400mL*3). All organic layers were combined, washed with water (1 × 300 mL) and dried with anhydrous Na_2SO_4. The crude was crashed out in excess hexane and recrystallized twice in an ethyl acetate/hexane solution. The EK dimer with protection group (52.6 g, 73.9 mmol, 73.9%) was obtained as a white powder. 1H NMR characterization of EK dimer was performed in $CDCl_3$ solvent.

2.2.2 *Synthesis of $(EK)_{10}$-C-NH_2 polypeptide (pEK)*

$(EK)_{10}$-C-NH_2 was synthesized by solid phase peptide synthesis (SPPS) with the automated microwave peptide synthesizer. Peptide synthesis started on the amino group of Rink amide MBHA resin (at 2.5 mmol) with the cysteine, and then EK dimers. Coupling reactions were facilitated at 40 W for 5 mins with 5-molar excess of amino acid activated by reagents dissolved

in DMF (Amino acid: DIC: Oxyma=2:5:10). Deprotection of Fmoc group on amino acid was performed by washing the resin with piperidine/DMF solution (volume ratio=1: 4) twice per cycle. Washing steps were performed with DMF after each coupling reaction and deprotection operation. When cysteine was coupled on the resin and deprotected, it was substituted with EK dimer in coupling steps until the completion of polypeptide synthesis. Cleavage was performed by immersing polypeptide-loaded resin in 10 ml of cocktail reagent (TFA: water: phenol: thioanisole: EDT=82.5: 5: 5: 5: 2.5) for 3 h at RT. Eventually, polypeptide was crashed out from cleavage reagent and washed with ice-cold anhydrous ethyl ether for at least 5 times.

2.2.3 *Synthesis and purification of Uricase-pEK conjugates*

The synthesis of Uricase-pEK conjugate was achieved by using a layer-by-layer method. Amino groups exposed on the surfaces of native uricase were activated firstly by N-α-maleimidoacet-oxysuccinimide ester (AMAS), a maleimide-NHS bifunctional crosslinker. Two molar equivalents of AMAS to exposed amino groups on native uricase were dissolved in DMSO (40mg/ml) and added into the protein solution (2mg/ml, PBS, pH=7.4) dropwise, followed by mixing for 0.5 h at RT. The reaction solution was ultrafiltrated using Amicon ultra centrifugal filter with 30K molecular weight (MW) cutoff and refreshed with PBS buffer for at least 5 times to remove unreacted AMAS and other small molecule impurities produced with native protein. The absorption of filtrate in ultrafiltration tube was characterized at 280 nm with the multimode plate reader and compared with absorption of fresh PBS buffer to avoid existence of unreacted AMAS in solution before next step. Concentrated uricase solution then was added into the $(EK)_{10}$-C-NH_2 solution (2 molar-fold excess to amino groups exposed on uricase, in PBS buffer, pH=7.4, 80mg/ml) for the first layer conjugation. The reaction solution kept mixing to react at 4 ℃ overnight and ultrafiltrated to remove free pEK in the solution with 100K MW cutoff and refreshed

with PBS buffer for at least 5 times. The purified URI-pEK-single layer conjugates (URI-pEK-s) were stored at 4 ℃ for further conjugation. The second and third layer pEK were conjugated to the amino groups originated from the previous pEK layer, and similar procedures of activation, purification and conjugation were performed to prepare the URI-pEK-double layer conjugates (URI-pEK-d) and URI-pEK-triple layer conjugates (URI-pEK-t). The conjugation process was illustrated in Figure 2.1.

Hydrophobic interaction column (HIC) were used to separate the fully covered URI-pEK-t conjugates from the mixture due to the different hydrophilicity of native uricase and pEK peptide. Gel permeation chromatography (GPC) was performed to characterize the size differences between the native uricase and URI-pEK-t products.

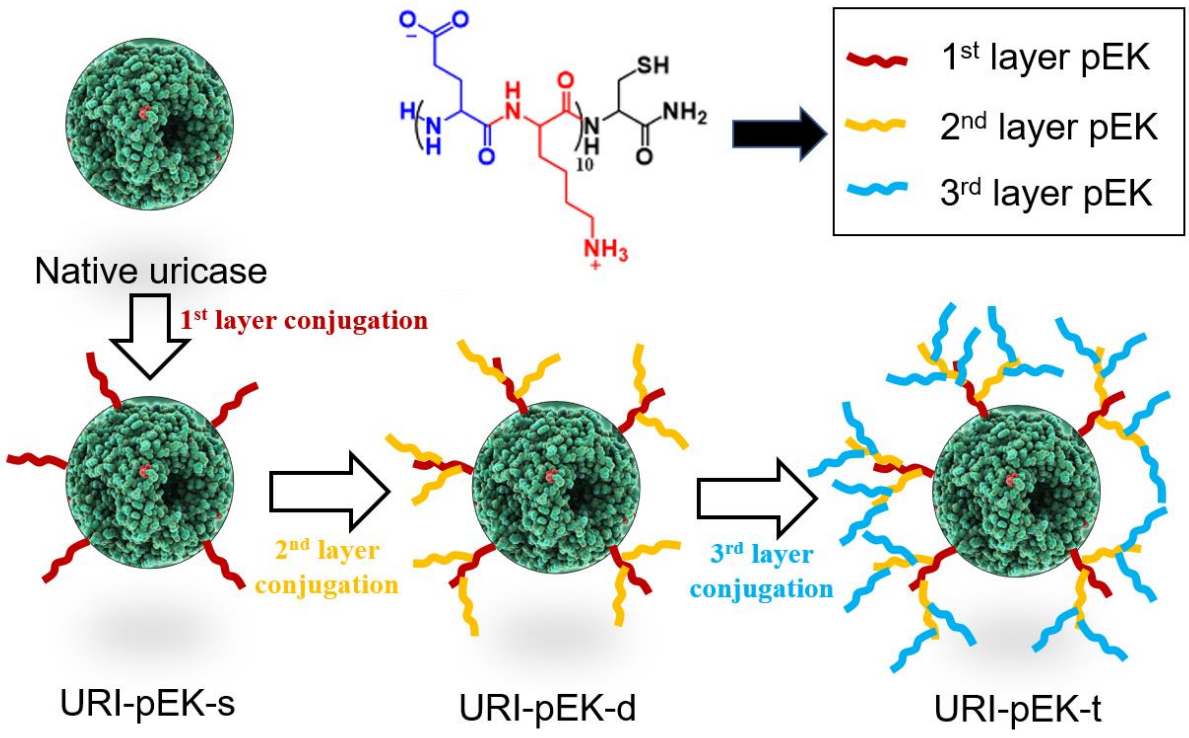

Figure 2.1. Illustration of the pEK peptide layer-by-layer conjugation method. The second and third layer pEK were conjugated to the amino groups originated from previous pEK layer.

2.2.4 *In vitro activity test of URI-pEK conjugates*

In vitro activity test was performed after each layer conjugation step to confirm the enzyme was still bioactive by using Amplex™ Red Uric Acid/Uricase Assay Kit to characterize the activity of conjugates. To be specific, uricase can convert uric acid to allantoin, hydrogen peroxide (H_2O_2) and CO_2. The H_2O_2, together with the pre-existing horseradish peroxidase (HRP), reacts with the Amplex® Red reagent to produce resorufin, which can absorb and emit at around 571 nm and 585nm, respectively. All samples were set up according to the protocol from manufacturer and incubated at 37 ℃ for 30 mins, and then fluorescence was measured by using the multimode plate reader to excite at 540 nm and detect emission at 590 nm.

2.2.5 *Thermal stability of URI-pEK conjugates*

Native uricase and URI-pEK conjugates were incubated in PBS buffer solution, pH 7.4 at 55℃ in water bath and a concentration of 2mg/ml. Both protein solutions were sampled at 0, 10, 20, 30, 60, 90 mins and refrigerated at 4 ℃ for further activity characterization. Activities were measured with the same protocol from the manufacturer mentioned in section 2.2.4. Values were recorded and normalized for the data comparison.

2.2.6 *Pharmacokinetic study of URI-pEK conjugates*

All animal experiments obeyed federal guidelines and were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC) under protocol #4203-01. Animals were randomly divided into two groups at the beginning of each study and a sample size of five animals per group was used, and one group for native uricase and the other group for URI-pEK conjugates. C57LB/6 mice (male, body weight 20–25 g) were purchased from Jackson Laboratories (Seattle, WA).

At day 0, all weights of mice were recorded to ascertain the amount of administration of 10 U/ml and the dosage was set at 5 U/kg mice. The baseline sera were sampled from each animal before injection. The native uricase and conjugates were administrated into the mice via intravenous (I.V.) injection. The blood was sampled at 5 min, 3h, 6h, 24h, 48h and 72h post injection and were treated by sitting at RT for 0.5 h to clot and centrifuged to obtain the clear sera for further characterization. Activities were measured with the same protocol from the manufacturer mentioned in section 2.2.4 and all values were calculated and normalized for the data comparison. The pharmacokinetics study process was illustrated in Figure 2.2.

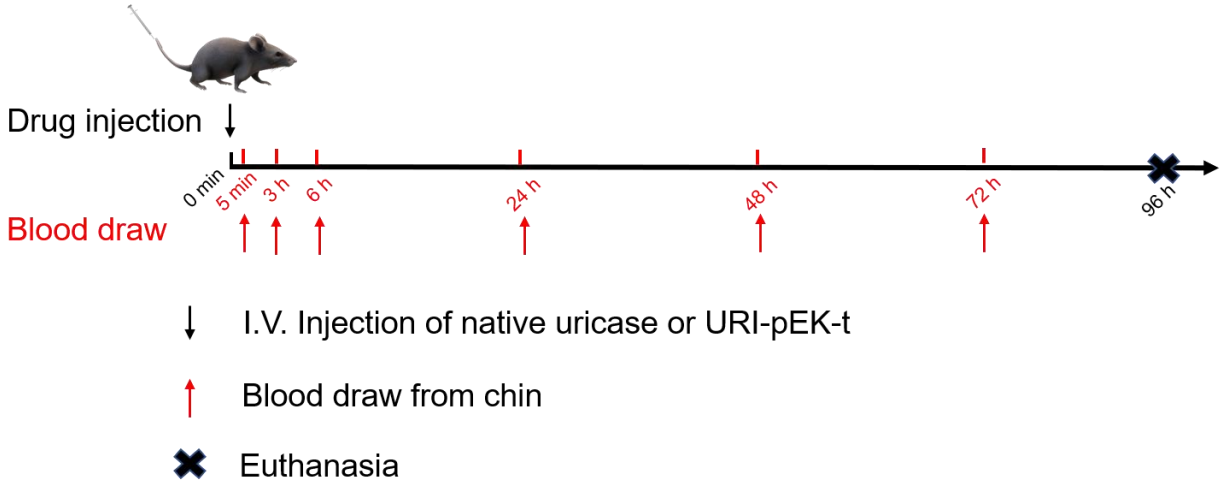

Figure 2.2. Schematic illustration of pharmacokinetics study of native uricase and URI-pEK-t in a mouse model.

Chapter 3. RESULTS AND DISCUSSION

3.1 SYNTHESIS AND CHARACTERIZATION OF EK DIMER

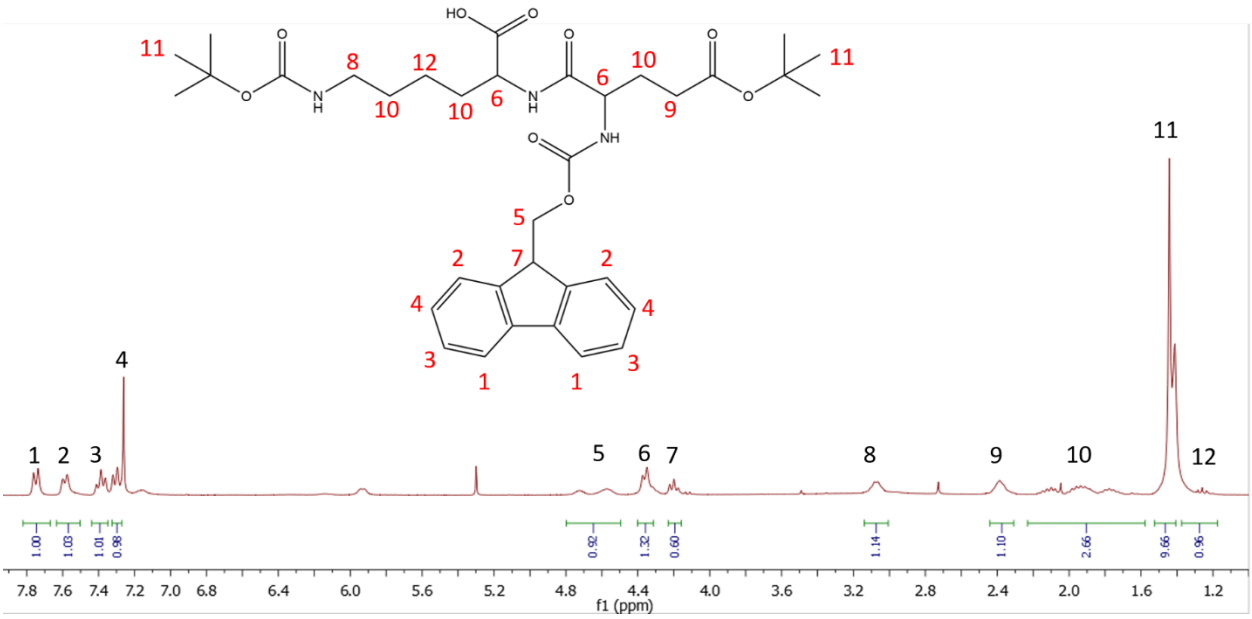

Figure 3.1. ^{1}H NMR spectra of EK dimer.

(CDCl3, 300 MHz) δ (ppm): 7.76 (2H, d), 7.60 (2H, d), 7.38 (2H, t), 7.29 (2H, t), 4.8-4.5 (2H, m), 4.45-4.25 (2H, m), 4.19 (1H, m), 3.07 (2H, m), 2.77 (2H, m), 2.2-1.7 (6H, 3m), 1.44–1.41 (18H, 2s), 1.25 (2H, m). According to the NMR spectra above, all peaks could be assigned to related hydrogen atoms based on the chemical shifts and splitting, and the ratio of areas under the peaks almost equaled to the ratio of amounts of hydrogen atoms in different chemical environments, which suggested we successfully synthesized EK dimer we designed.

3.2 GEL PERMEATION CHROMATOGRAPHY (GPC) CHARACTERIZATION OF URI-PEK CONJUGATES

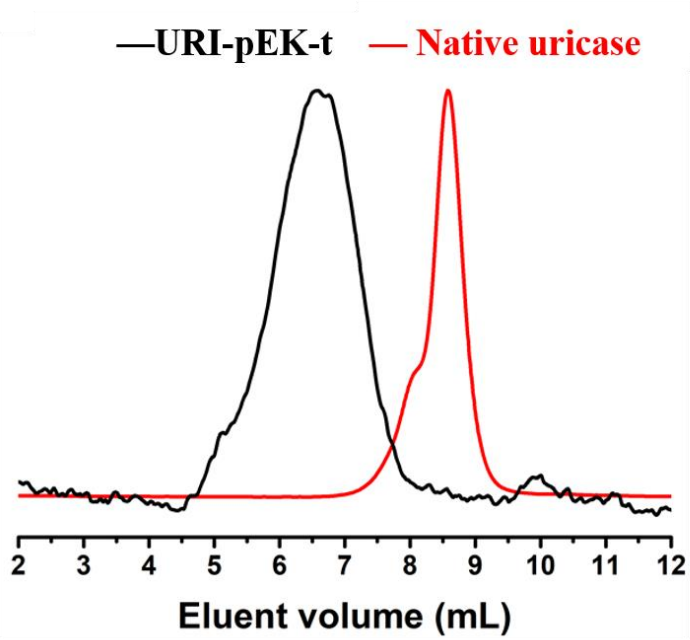

Figure 3.2. GPC curves of native uricase and URI-pEK-t.

GPC analysis (Figure 3.2) showed that the curve of URI-pEK-t obtained significant left shift compared to native uricase, indicating an apparent increase in size of uricase. Triple layer of pEK can stabilize the protein structure and meanwhile can ensure full coverage of immunogenic surfaces and increase the hydrodynamic size of uricase to avoid rapid renal clearance (molecular cutoff: 30-50kDa), and thereby increase their circulation time in serum.

3.3 IN VITRO ACTIVITY TEST OF URI-PEK CONJUGATES

Table 3.1 In vitro activity test results

	Native uricase	URI-pEK-s	URI-pEK-d	URI-pEK-t
Activity/U	33.70	16.14	6.62	2.77
mass/mg	7.37	3.7	2.71	1.73
Unit activity/(U/mg)	4.57	4.36	2.44	1.60

From the table 3.1 above, we can know that the activities of conjugated uricase could decrease as the layers of conjugated pEK increase and the URI-pEK-t conjugate still reserve 35% of initial activity. The activity loss could easily happen during the process of protein conjugation because uricase is quite a sensitive and fragile protein. Slight changes in pH, temp, ion strength in the buffer and even overlong conjugation time could cause disrupt the protein structures and result in the loss in bioactivity. But this activity loss can be avoided by optimizing the conjugation conditions to achieve high retained bioactivity after the chemical modification on uricase since the URI-pEK-s possessed almost the same bioactivity as the native uricase.

3.4 THERMAL STABILITY OF URI-PEK CONJUGATES

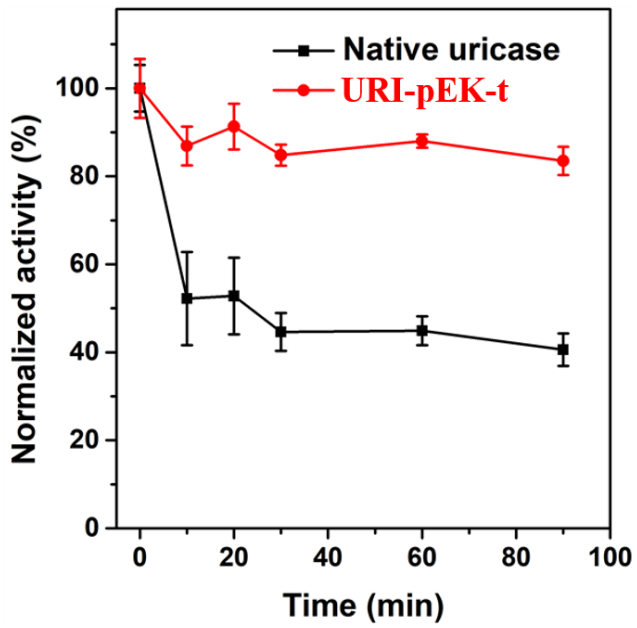

Figure 3.3. Thermal stability study of modified uricase vs. native uricase, as retained activity after incubation at 55 °C for 90 mins.

As shown in the Figure 3.3, URI-pEK-t exhibited exceptional thermal stability and still had 86.9% of original activity over 90-min incubation, but native uricase showed time-dependent activity loss and the activity dropped to 40% of its initial activity after the incubation. It was observed that pEK can stabilize uricase again thermal denaturation and resist the activity loss at increased temperature by enhancing the hydrophobic interactions within the protein and reducing the interaction between the protein surface and water. High stability is vital for enzyme storage or transportation and can lower the production cost.

3.5 PHARMACOKINETIC STUDY OF URI-PEK CONJUGATES

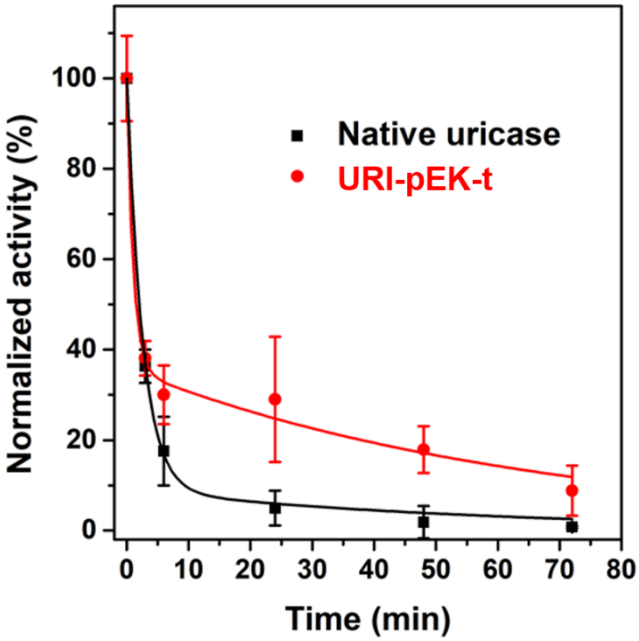

Figure 3.4. PK curves of native uricase and URI-pEK-t.

The PK profile of 1st dose of URI-pEK-t as well as native uricase is shown in Figure 3.4 and all concentration-time curves match the two-compartment model. The URI-pEK-t formulation exhibited an extended circulation time and outperformed the native uricase post-injection. The

half-life ($t_{1/2}\beta$) of URI-pEK-t is 45.2h, 2.8-fold higher than the 16.2 h $t_{1/2}\beta$ of native uricase and the area under curve (AUC∞) of URI-pEK-t is 4.5-fold that of the native enzyme, indicating its significantly higher systemic availability. These data reveal that increased size and non-fouling property both assist the protein evade the clearance by renal system and immune system to achieve the prolonged circulation.

Chapter 4. CONCLUSIONS

In summary, we successfully synthesized a neutrally charged EK dimer, prepared a novel functional polypeptide, $(EK)_{10}$-C-NH_2, and achieved hyperbranched EK polypeptides conjugated to uricase using a layer-by-layer method. The protein conjugates were purified by HIC and activity tests were performed by employing the Amplex™ Red Uric Acid/Uricase Assay Kit. Our data showed that URI-pEK-t was fully encapsulated by 3 layers of hydrophilic polypeptide with no immunogenic surfaces exposed. The thermal stability of uricase was obviously improved via its chemical conjugation to pEK, and the retained bioactivity of URI-pEK-t was twice that of native uricase. There was an activity loss during the conjugation process, but it can be improved by optimizing the conjugating conditions. Furthermore, compared to native uricase, URI-pEK-t possessed prolonged half-time and showed great potential for therapeutic protein in the future. Overall, this work demonstrates a novel zwitterionic polypeptide with ultra-hydrophilicity and multifunctionality.

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