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Nanocapsules of therapeutic proteins with enhanced stability and long blood circulation for hyperuricemia management

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ABSTRACT

Among a broad spectrum of medical treatments, protein therapeutics holds tremendous opportunities for the treatment of metabolic disorders, cancer, autoimmune diseases and etc. Broad adaption of protein therapeutics, however, still remain challenging, not only because of poor protein stability, but they also experience fast clearance after administrated and elicit immune responses, resulting in undesirable biodistribution and short blood residence time. In this study, we demonstrate a novel protein delivery method via encapsulating therapeutic proteins within thin shells of poly(*N*-vinylpyrrolidone) (PVP), which leads to significantly improved protein stability, reduced macrophage uptake, prolonged circulation time and reduced immunogenicity. Exemplified with urate oxidase (UOx), the enzyme used for hyperuricemia treatment, as-formed UOx nanocapsules, n(UOx), exhibits enhanced stability, more significant therapeutic effects, and a more than 10-fold improvement in circulation time when compared with native UOx. This technology not only demonstrates the use of UOx nanocapsules for hyperuricemia management, but also provides a general approach for a broad spectrum of therapeutic proteins for *in vivo* applications.

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1. Introduction

Almost all biological processes are mediated and regulated by proteins or enzymes in living organisms, whereas the loss or dysfunction of certain proteins may result in disorders of metabolic processes and diseases [1,2]. Among a broad spectrum of medical treatments, protein therapeutics holds tremendous opportunities for the treatment of cancer, metabolic disorders, autoimmune diseases and etc. [3,4,5]. However, broad applications of protein therapeutics still remain challenging, not only because these proteins commonly suffer from poor stabilities, but they also experience fast clearance once administrated and elicit immune responses, resulting in undesirable biodistribution and short *in vivo* circulation time [6]. To circumvent these issues, extensive efforts have been made to construct the delivery vehicles [7], such as incorporating therapeutic proteins in liposomes [8], conjugating polymers onto therapeutic proteins [9], and encapsulating therapeutic proteins with polymers [10,11]. Note that one of the most widely employed polymers is poly(ethylene glycol) (PEG) [9,12], which has been successfully adapted to various therapeutic proteins [13]. However, there are increasing evidences of anti-PEG antibodies for patients who have received PEGylated therapeutics, resulting in accelerated clearance and reduced effectiveness [14,15]. Developing novel delivery vehicles for therapeutic proteins, which can achieve prolonged circulation time and low immunogenicity, is of great significance.

Herein, we report a delivery method for therapeutic proteins with significantly prolonged circulation time by encapsulating the proteins within thin layers of poly(vinylpyrrolidone) (PVP). PVP is a biocompatible, non-cytotoxic and FDA approved polymer that has been broadly used as drug carriers and plasma substitutes [16,17]. To demonstrate the design, UOx, commonly used for the management of hyperuricemia [18,19], is used as the model protein. As depicted in Scheme 1, UOx is firstly conjugated with polymerizable acrylate moieties, followed by a free-radical polymerization with *N*-vinylpyrrolidone (NVP) as the monomer and *N,N′*-methylenebisacrylamide (BIS) as the crosslinker. Through noncovalent interactions, the monomers and crosslinkers are enriched around the protein molecules, and a thin shell of network polymer is formed around individual proteins, leading to the formation of UOx nanocapsules denoted as n(UOx). Each nanocapsules contains a UOx core within the PVP shell, which stabilizes the protein without

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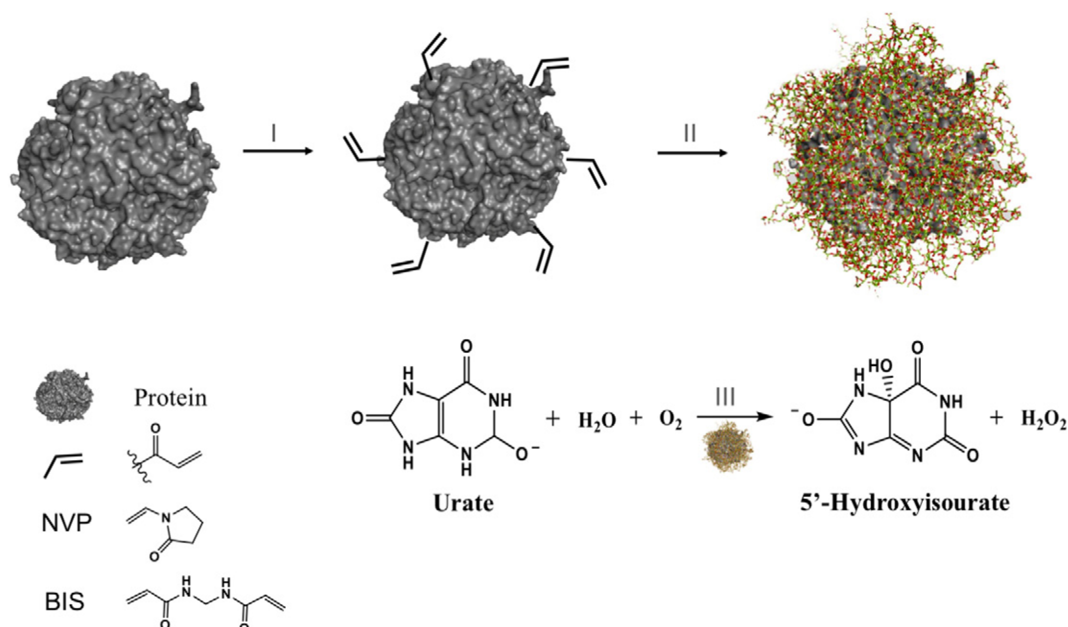

Scheme 1. Design and synthesis of UOx nanocapsules. **I**, modification of UOx by conjugating the UOx with polymerizable acryl groups; **II**, polymerization of a thin network poly(*N*-vinylpyrrolidone) around the UOx with *N*-vinylpyrrolidone (NVP) as the monomer and *N*,*N*′-methylenebisacrylamide (BIS) as the crosslinker; **III**, reaction of urate to 5-hydroxyisourate catalyzed by n(UOx).

limiting the transport of its substrate, urate. The PVP shells not only allow the effective transport of urate molecules to the UOx cores ensuring effective enzymatic reactions, but also significantly prolong the circulation time of the nanocapsules *in vivo*, providing an effective therapeutic treatment for hyperuricemia and chronic gout.

2. Materials and methods

2.1. Materials

HeLa and THP-1 cells were purchased from American Type Culture Collection (ATCC). The Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640 growth medium and Penicillin/Streptomycin (P/S) were obtained from Invitrogen (Shanghai, China). Fetal Bovine Serum (FBS) was obtained from Thermofisher Inc. (Shanghai, China). Fluorescence dyes including Rhodamine B isothiocyanate was obtained from Sigma-Aldrich (Shanghai, China). FITC-NHS was purchased form thermos fisher scientific Inc. Urate oxidase (UOx) extracted from *E. coli* was obtained from Beijing Institute of Biotechnology (Beijing, China). Uric acid assay kit was purchased from Shanghai Rongsheng Biotech Co., Ltd. Sodium phosphate dibasic, sodium bicarbonate, and dimethyl sulfoxide (DMSO), phorbol myristate acetate (PMA), uric acid, hypoxanthine (HX), lithium carbonate, borax anhydrous, boric acid, *N*-hydroxylsuccinimide ester, *N*,*N*′-methylenebisacrylamide, ammonium persulfate (APS), *N*-vinyl pyrrolidone (NVP), PVP (Mw 10000), bovine serum albumin (BSA) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. Bicinchoninic acid disodium salt hydrate (BCA) and copper sulfate were purchased from Sigma-Aldrich and used as received. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), and 3,3′-Dioctadecyloxacarbocyanine perchlorate (DIO) were purchased from Sigma and used as received. Cell Counting Kit-8 was obtained from Dojindo Laboratories. Clear polystyrene 12-well and 96-well tissue culture plates were obtained from Corning Costar. Male Balb/c and Kun Ming (KM) mice were purchased from Chinese Academy of Sciences (SLAC Laboratory Animal Co. Ltd., Shanghai, China). Goat Anti-Mouse IgM, IgG, and IgE monoclonal antibody conjugated with horse radish peroxidase (HRP) were purchased from Southern Biotech (USA). All other reagents and solvents were purchased from Sigma-Aldrich (Shanghai, China) and used as received.

2.2. Synthesis and characterization

2.2.1. Synthesis of UOx nanocapsules

Acryloylation of UOx: all buffers were degassed before use. 5 mg of UOx at 2.5 mg/mL in 100 mM borate buffer (pH 8.5) were reacted with 21.5 μL of 20% (w/v) of *N*-acryloxysuccinimide (NAS) in DMSO (molar ratio of NAS to UOx, 17:1) for 4 h at 4 °C and the potassium oxalate monohydrate (100 mM) was added into the mixture to preserve the active site of the native UOx. The solution was stirred gently and the vial was covered with parafilms during the reaction. Subsequently, the solution was thoroughly dialyzed against 50 mM phosphate buffer (pH 7.4) at 4 °C using a 12–16 kDa dialysis tubing. Polymerization of *N*-vinyl pyrrolidone shell: 5 mg UOx with polymerizable acryl groups was diluted with 50 mM phosphate buffer, and the final concentration of UOx in the reaction mixture was 1 mg/mL. 115 mg NVP as the monomer and 15 mg BIS as the crosslinker were added to the protein solution. Before the initiation of polymerization, the potassium oxalate monohydrate (100 mM) was added into the mixture to preserve the active site of the native UOx. After the solution was sparged with N_2 for 20 min, the free radical polymerization was initiated by APS (10%, w/v) and TEMED. The vial sparged with N_2 was covered and the mixture was stirred gently for 4 h in ice bath. After polymerization, the reaction mixture was dialyzed in 50 mM phosphate buffer (pH 7.4) at 4 °C to remove unreacted small molecules, with a 12–16 kDa dialysis tubing.

The other proteins such as glucose oxidase (GOx), enhanced green fluorescent protein (EGFP), L-asparaginase (L-Asp) and BSA (bovine serum albumin) are encapsulated using the above synthesis route.

2.2.2. Synthesis the polymer of BIS-PVP

BIS-PVP shell polymer was synthesized according to the method of nanocapsules without adding the protein. Briefly, 115 mg NVP and 15 mg BIS were initiated by TEMED and APS in 5 mL of 50 mM phosphate buffer. It is worth noting that *N*-(3-Aminopropyl) methacrylamide (APM) was also polymerized in this polymer (mole ration of NVP: APM = 50: 1) and used for conjugating FITC. The polymer

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