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# Study of Uricase-Polynorbornene Conjugates Derived from Grafting-from Ring Opening Metathesis Polymerization

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## ABSTRACT

PEGylation has been the 'gold standard' in bioconjugation due to its ability to improve the pharmacokinetics and pharmacodynamics of native proteins. However, growing clinical evidence of hypersensitivity reactions to PEG due to pre-existing anti-PEG antibodies in healthy humans have raised concerns. Advancements in controlled polymerization techniques and conjugation chemistries have paved the way for the development of protein-polymer conjugates that can circumvent these adverse reactions while retaining the benefits of such modifications. Herein, we show the development of polynorbornene based bioconjugates of therapeutically relevant urate oxidase (**UO**) enzymes used in the treatment of gout synthesized by grafting-from ring-opening metathesis polymerization

(ROMP). Notably, these conjugates exhibit comparable levels of bioactivity to PEGylated UO and demonstrate increased stability across varying temperatures and pH conditions. Immune recognition of conjugates by anti-UO antibodies reveal low protein immunogenicity following the conjugation process. Additionally, **UO** conjugates employing zwitterionic polynorbornene successfully avoid recognition by anti-PEG antibodies, further highlighting a potential replacement for PEG.

# INTRODUCTION

Protein therapeutics dominate the pharmaceutical market and are forecasted to surpass a market size of 490 billion USD by 2029.[1] Over the last decade we have seen a notable increase in FDA-approved biologics.[2] This progress can be attributed to high specificity of proteins that reduce off-target effects or interference with normal biological processes unlike small molecule drugs.[3] However, challenges of proteins as therapeutic agents include immunogenicity, low solubility, and low stability caused due to protease degradation and immune surveillance. 'PEGylation' or covalently modifying proteins with hydrophilic polyethylene glycol (PEG), a technique pioneered by Abuchowski et. al. in 1977 has been the golden standard in addressing these challenges.[4–6] PEGylation enhances protein solubility and stability while mitigating immunogenicity.[7–9] As of October 2023, there are 28 FDA approved PEGylated protein therapeutics used for the treatment of diseases such as cancer, diabetes and hepatitis and many more in various stages of development.[10] However, growing instances show clinical evidence of adverse effects attributed mainly to pre-existing anti-PEG antibodies recognizing either the polymer backbone or the methoxy end group of the polymer.[11,12] Studies indicate that up to 72% of humans who have not been treated with PEGylated therapies still have pre-existing anti-PEG antibodies.[13–24] These elevated occurrences are believed to result from extensive exposure to PEG and PEG derived substances through common everyday products. This may become a more pressing concern in the future since SARS-CoV-2 mRNA vaccines used PEGylated lipids and were shown to induce anti-PEG antibodies.[21,25]And these anti-PEG antibodies lead to accelerated blood clearance, reduced half-life, loss of protein activity, and hypersensitivity reactions challenging the safety and therapeutic efficacy of pegylated therapeutics.[14,26] Clinical trials of PEGylated

protein therapies such as pegloticase[27], pegaspargase[28], and pegnivacogin[29,30] exhibited clear indications of reduced efficacy and adverse reactions impacting the treatments and sometimes leading to termination of the trials.

Advancements in new chemical and biochemical technologies including controlled polymerizations, conjugation chemistries[6,31–33] and genetic engineering (XTEN[34], PASylation®[35,36], HESylation[37,38]) have facilitated developments of new safer and effective approaches beyond PEGylation.[23,39] Among these, controlled polymerization techniques such as atom transfer radical polymerization (ATRP), reversible-addition fragmentation chain transfer (RAFT), ring-opening metathesis polymerization (ROMP) and nitroxide mediated polymerization (NMPs) have enabled the synthesis of polymers with low dispersity, allowing precise control over chemical functionality, properties, architecture and molecular weights.[40–43] Polymers synthesized using these techniques including polyglycerols[44], poly(oligo(ethylene glycol) methyl ether methacrylate)[45], poly(N-(2-hydroxypropyl)methacrylamide)[46], polyoxazolines[47] and polycarboxybetaines[48] have been extensively studied as potential alternatives to PEGylation in bioconjugation.[49] Furthermore, new bioconjugation chemistries have allowed for more control and precision.

Bioconjugation of proteins is accomplished primarily through two approaches: grafting-to and grafting-from bioconjugation.[50–54] Grafting-to bioconjugation, notably employed in PEGylation of proteins, involves a covalent linkage of preformed polymers equipped with reactive end-group functionalities to complementary amino acid residues within the proteins. While the grafting-to technique allows for the synthesis of well-defined polymers and facilitates thorough characterization of the polymer component, it requires the addition of excess of polymers in the reaction. Hence, given that the grafting-to technique involves reaction between macromolecules, purification can pose challenges due to similar sizes of reacting polymers, unmodified proteins, and pure conjugates. In contrast, the grafting-from technique requires the modification of proteins with an initiator thereby creating protein macroinitiators capable of initiating polymerization of monomers directly from the protein surfaces. This method has been widely explored in recent years due to advantages which include high grafting-density and simplified purification, facilitated by

the substantial size differences between small-molecule reactants like monomers and initiators, and protein-polymer conjugates.[55,56] However, grafting-from bioconjugation requires polymerization conditions that are compatible with biomolecules, usually under aqueous conditions.

ROMP has been of great interest as it is fast and functional group tolerant which allows for the design of diverse functional polymers.[43,57] Development of water-soluble ROMP catalysts have broadened the scope of ROMP in biomacromolecules and living materials.[58–62] Recent studies established the significance of chloride ions in maintaining ROMP catalysts activity in aqueous solution, which enabled controlled polymerizations at near-neutral pH.[63,64] Following this development, we demonstrated the synthesis of polynorbornene bioconjugates by grafting-from ROMP from protein surfaces such as lysozyme and Qβ bacteriophage.[65] These polynorbornene-based protein conjugates not only reduced protein immunogenicity but also evaded detection by anti-PEG antibodies. In this study, we have extended our technique to synthesize protein-polymer conjugates of urate oxidase (**UO**) or uricase, a therapeutically relevant enzyme used for the treatment of gout, which has been increasing in prevalence in the population.[1,66] Notably, the FDA-approved PEGylated **UO** (Pegloticase or Krystexxa®) has shown clinical evidence of hypersensitivity reactions attributed to pre-existing anti-PEG antibodies, necessitating aggressive anti-inflammatory therapy and changes in patient treatments.[67,68] We demonstrate grafting-from ROMP for the synthesis of active **UO** conjugates with higher stability and lower immunogenicity. We also study the immune recognition of the newly synthesized **UO** conjugates.

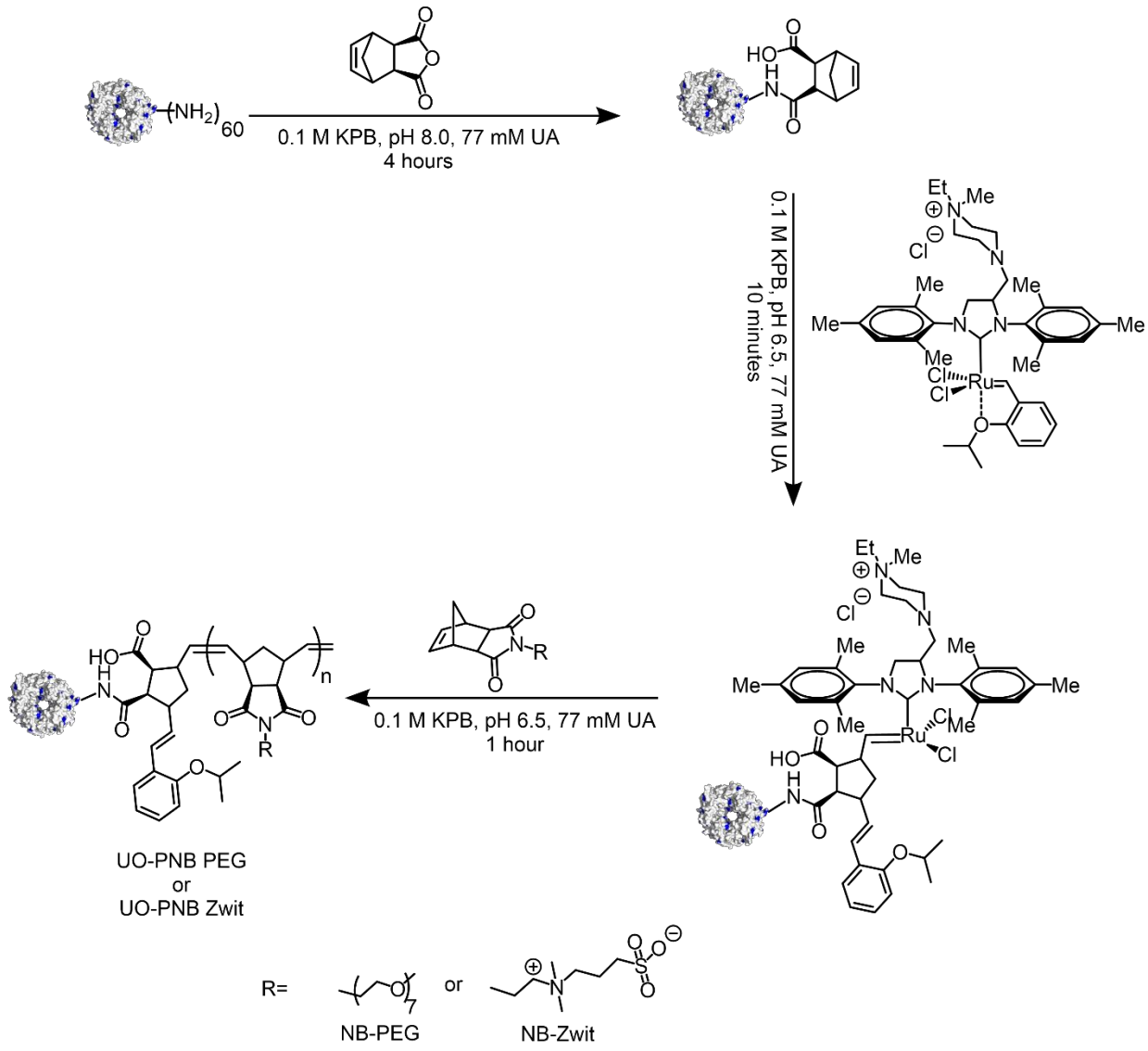
# RESULTS AND DISCUSSION

## Synthesis and characterization of UO conjugates

Uricase or urate oxidase (**UO**) enzyme is a homotetrameric protein (~140 kDa) that catalyzes the conversion of uric acid (UA) to allantoin. The **UO** utilized in the study was obtained from *Bacillus fastidiosus* and each monomer had a molecular weight of ~37 kDa as confirmed by liquid chromatography-electrospray ionization-time-of-flight mass spectrometry (LC-ESI TOF MS) analysis (**Figure S1**). **UO** has 15 lysine residues per

monomer which were chosen as sites to initiate grafting-from polymerization. To enable grafting-from bioconjugation, lysine residues of **UO** were initially modified using cis-norbornene-exo-2,3-dicarboxylic anhydride (NBDA) to incorporate norbornyl groups and yield **UO-NB** (**Reaction Scheme S1**). Optimizing the number of modified lysine residues was crucial to prevent precipitation of **UO-NB** from solution due to increased hydrophobicity. Various NBDA equivalents were tested, as per **Table S1,** for optimization. Precipitation was observed when 7.5 equivalents of NBDA per lysine residues was used, which resulted in an average 10 lysine modifications per protein monomer. For further investigation and future polymerizations, **UO-NB** with 3-5 lysine modifications (26 ± 6.7%) per monomer (**Figure S2**) was used. This optimized **UO-NB** was achieved when using 5 equivalents of NBDA per lysine residue.

**UO-NB** was characterized using size exclusion chromatography and the optimized **UO-NB** formulation exhibited an anomalously higher retention volume compared to the native enzyme, likely attributed to interactions between norbornene and the column material (**Figure S3**). Such anomalous behavior has been previously observed in our studies and could be attributed to interaction between the norbornene modification and the column material.[60,65] Importantly, there was no observed loss of enzymatic activity post-modification, confirming that the higher retention volume was not due to degradation during the chemical modification process (**Figure S4**). It is worth highlighting that the optimized **UO** modification and all the grafting-from polymerizations that followed were carried out in the presence of an excess of UA, serving as a "site-protecting agent."[69–71] The presence of UA molecules temporarily occupying the active sites of **UO** effectively reduces the likelihood of modification of lysine residues within the active sites.




**Synthesis scheme 1.** Synthesis of **UO** conjugates by aqueous grafting-from ROMP technique.

The **UO** macroinitiator (**UO-MI**) was synthesized from **UO-NB** by reacting with water soluble Ru-based ROMP catalyst, AquaMet, for insertion of a ROMP-active Ru-center onto the surface of the protein, which facilitated the polymerizations of norbornene based monomers. Two water-soluble norbornene monomers were synthesized and used to study grafting-from **UO** conjugation: **NB-PEG** and **NB-Zwit. NB-PEG** has an oligoethylene glycol side chain with seven ethylene glycol (EG) repeating units and **NB-Zwit** has a sulfobetaine side chain. Anti-PEG recognition was reported to be proportional

to PEG chain length and studies have indicated that grafting PEG-like brush polymers with up to nine EG repeat units results in minimal antibody recognition.[72] Thus, we hypothesized that **UO** conjugates derived from **NB-PEG** would exhibit significantly reduced recognition compared to long-chain PEG polymers. Furthermore, superhydrophilic zwitterionic polymers have shown great potential in minimizing immune responses in protein conjugates without compromising their therapeutic efficacy.[73]

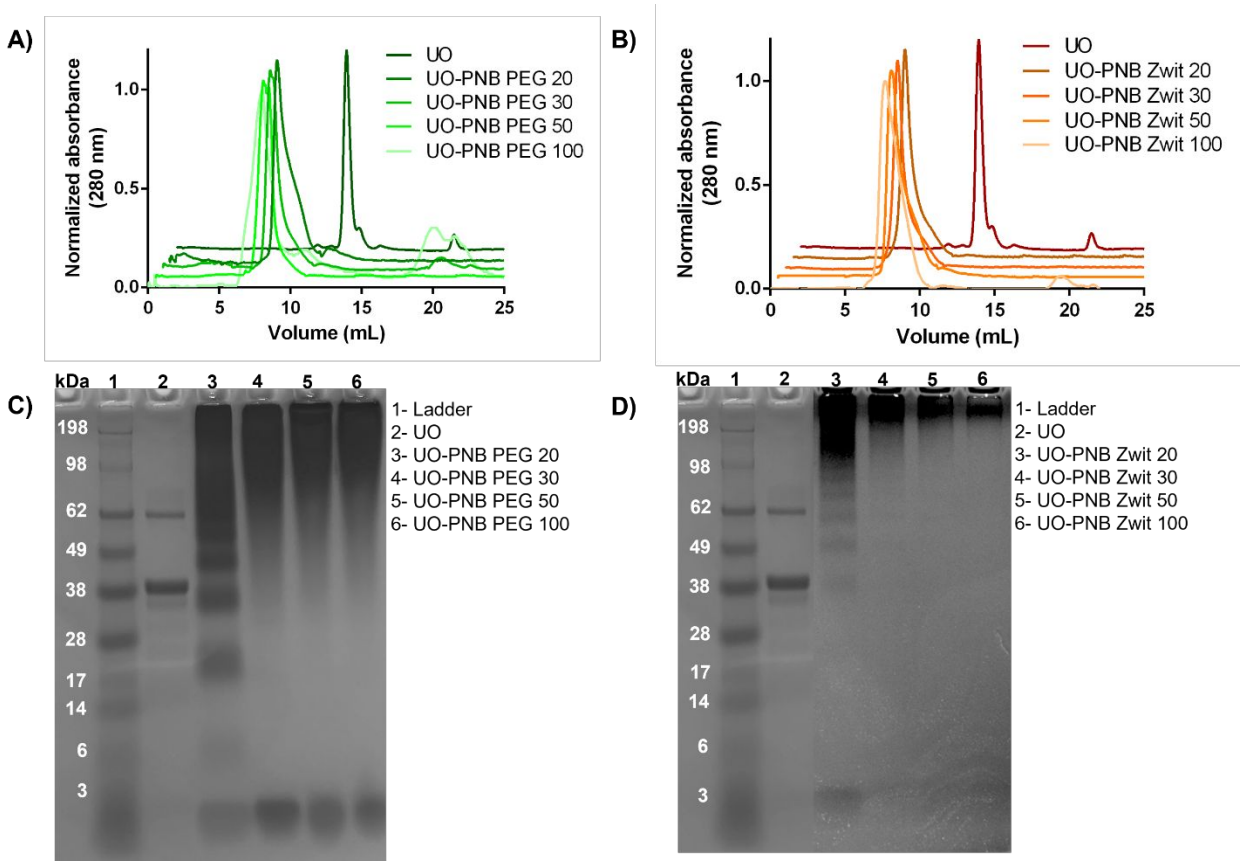



**Figure 1.** Characterization of grafting-from **UO-PNB** conjugates

**A)** FPLC of **UO-PNB PEG** conjugates **B)** FPLC of **UO-PNB Zwit** conjugates **C)** Reducing SDS-PAGE of **UO-PNB PEG** conjugates **D)** Reducing SDS-PAGE of **UO-PNB Zwit**

Varying monomer loadings of 20, 30, 50, and 100 equivalents of **NB-PEG** and **NB-Zwit** per **UO** monomer were used for grafting-from polymerization. Variable stoichiometries were used to study the effect of increasing polymer molecular weight on enzyme activity

and immune responses and to validate controlled polymerization. Conjugates formed after the addition of 100 equivalents of monomers were highly viscous with low solubility in aqueous buffers. All conjugates were purified by fast protein liquid chromatography (FPLC) to remove unreacted **UO** and monomers. Purity of the conjugates was confirmed through characterization using FPLC and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1). **UO** enzyme eluted at retention volume of 12 mL and conjugations resulted in a lower elution volume where the peaks appeared at volumes 7- 10 mL. It is important to note that neither macroinitiator formation nor polymer initiation is quantitative, resulting in a distribution of polymer attachments that, at present, is nearly impossible to resolve quantitatively. This phenomenon in combination with the inherent dispersity of polymerization leads to a distribution of molecular weights throughout the population.

Furthermore, purity was assessed through denaturing SDS-PAGE. The SDS-PAGE analysis of denatured **UO** revealed bands at 38 kDa and 62 kDa representing the monomer and a weaker band indicating dimers, respectively. Purified conjugates displayed broader bands at higher molecular weights, with no bands corresponding to **UO**. **UO-PNB PEG 20** and **UO-PNB Zwit 20** exhibited multiple bands, this is likely due to a grafting-through mechanism at very low monomer concentrations (~6 eq per initiator). In addition to Coomassie blue stain, PAGE gels of **UO-PNB PEG** conjugates were also visualized using iodine (**Figure S5**). The intensity of bands increased with higher **NB-PEG** monomer equivalents confirming an increase in degree of polymerization. However, it's important to note that UO-PNB PEG 100 exhibited reduced band intensity, primarily due to its limited solubility in aqueous buffer. The increasing degree of polymerization with monomer equivalents shows the living nature of the grafting-from polymerization.

To study the activity and immune recognition of grafting-from **UO-PNB** conjugates, we additionally synthesized a control PEGylated UO by grafting-to bioconjugation, **UO-PEG (Reaction Scheme S2, Figure S6-S7)**. **UO-PEG** was synthesized by covalently conjugating a linear 10 kDa N-hydroxysuccinimide-methoxy polyethylene glycol (mPEG-NHS), which targeted lysine residues. For comparison, the synthesis was optimized to obtain a similar number of modified lysine residues. 2,4,6-Trinitrobenzene Sulfonic Acid

(TNBS) assay was used to quantify free amines and indicated approximately three modified lysine residues per UO monomer after PEGylation (**Table S2**).

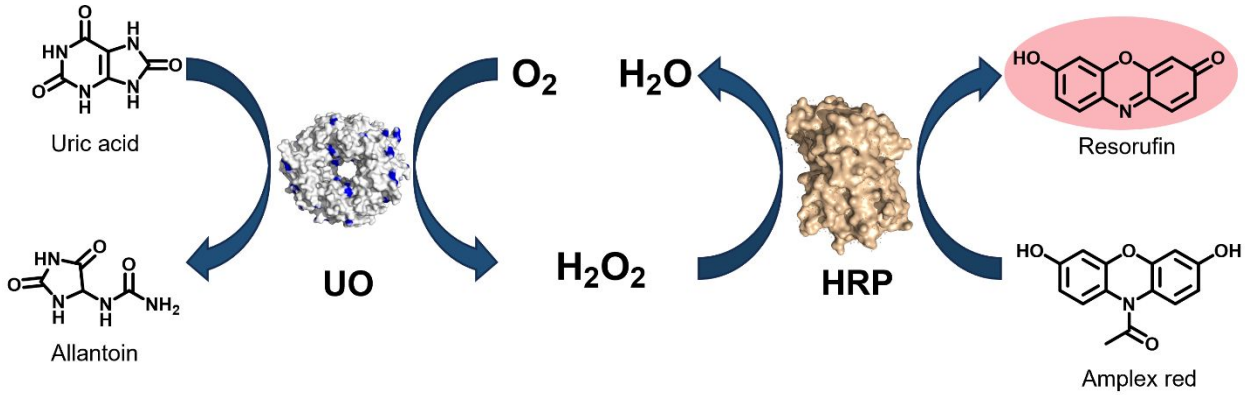



**Figure 2.** Schematic illustration of uric acid/uricase activity assay using amplex red reagent.

## Activity of UO conjugates

The activity of **UO** and **UO** conjugates was determined by measuring the conversion of UA using Amplex red. **UO** enzyme catalyzes the conversion of UA to allantoin (**Figure 2**). Hydrogen peroxide ($H_2O_2$) generated during this conversion further co-catalyzes the oxidation of amplex red reagent to produce fluorescent resorufin, using horseradish peroxidase (HRP) in the assay mixture. The activities of all the **UO** conjugates are shown in **Figure 3.** The analysis of **UO** conjugate activities indicated that significant enzyme activity remained intact following covalent modification with polymers (**Figure 3A**). Specifically, grafting-to **UO-PEG** retained 78% of its activity, and all the **UO-PNB** conjugates generated via grafting-from ROMP exhibited similar levels of enzymatic activity. Increasing the degree of polymerization did not notably affect the retained enzyme activity in **UO-PNB-PEG** conjugates, more pronounced effects were observed in the case of **UO-PNB-Zwit** conjugates. **UO-PNB PEG** conjugates showed up to 73% activity and **UO-PNB Zwit** conjugates had activity ranging from 54%-82% compared to **UO**.

Further examination of **UO** activity under varying temperature and pH conditions was conducted to investigate the impact of polymer conjugation on **UO** stability. All PNB conjugates showed enhanced thermal stability following polymer conjugation (**Figure**

**3B**). Activities at 30°C were considered as 100% for both **UO** and its conjugates. As the temperature increased, there was a clear decline in activities of UO and all UO conjugates. **UO** exhibited a consistent and sharp reduction in activity with increasing temperature. However, this decline in activity was mitigated in all conjugates, indicating that the polymers conjugated to **UO** acted as a protective coating, shielding **UO** from denaturation. While both **UO** and **UO-PEG** lost all activity at 60°C, all **UO-PNB** conjugates still retained 45% activity and eventually lost all activity at 80°C. Additionally, the impact of pH on **UO** activity was explored across a range of pH values from 4 to 10 (**Figure 3C**). All **UO** and UO conjugates lost all activity at pH 4. The highest activity of all **UO** and UO conjugates was observed at pH 10. The improved stability of **UO-PNB** conjugates has the potential to extend the shelf life of protein therapeutics. This advantage becomes particularly valuable in situations where proteins are subjected to various stressors throughout the processes of manufacturing, transportation, and extended storage periods.

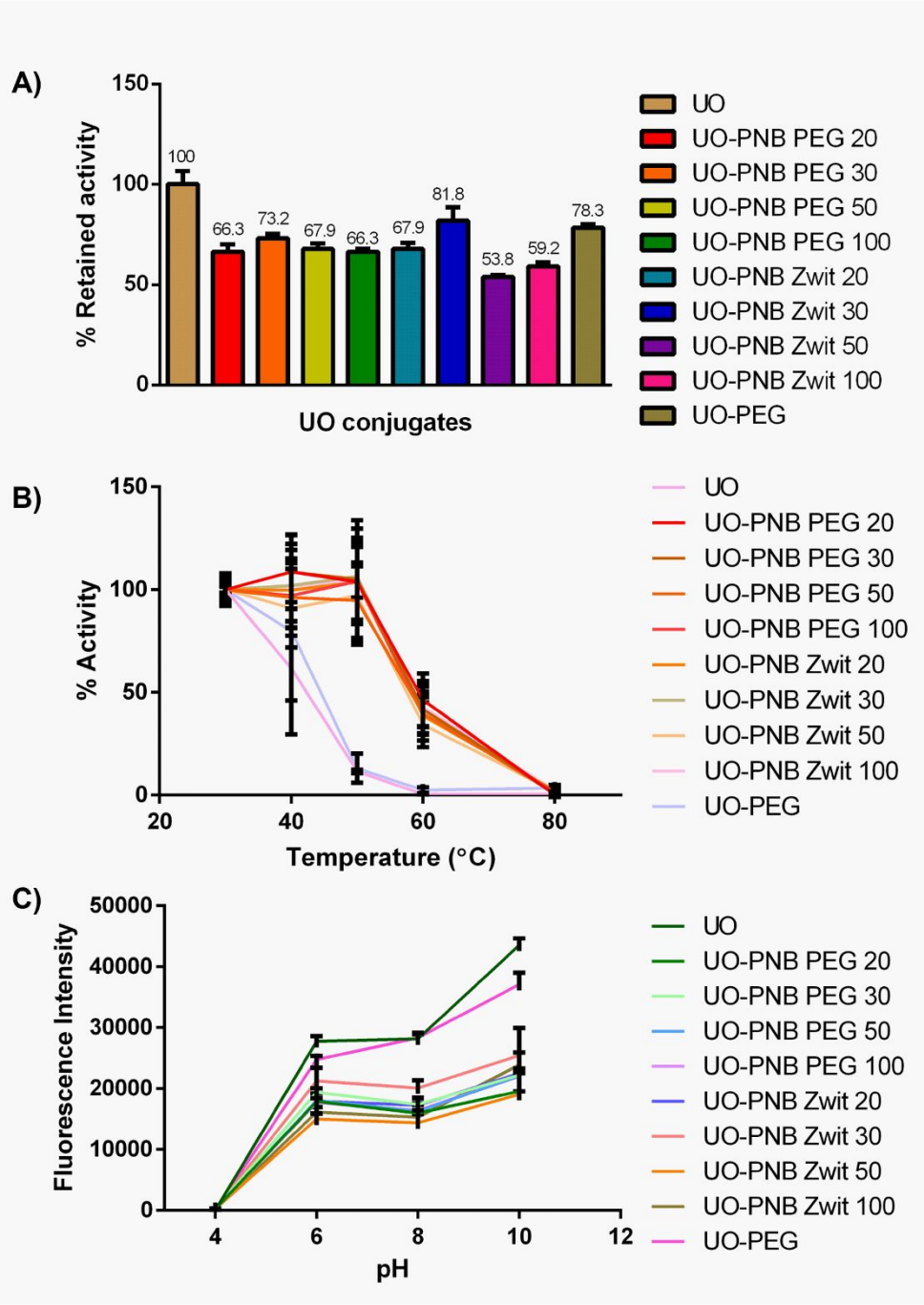



**Figure 3.** Activity of **UO** conjugates

**A)** Retained activity of **UO** conjugates. All activities are relative to the activity of **UO** before conjugation. **B)** Activity of **UO** and **UO** conjugates measured at varying temperature The activities were measured at 30, 40, 50, 60 and 80 °C. Activity of each sample at 30 °C was considered 100%. **C)** Activity of **UO** and **UO** conjugates measured at varying pH The activities were measured at pH 4, 6, 8 and 10.

## Recognition of UO conjugates by antibodies

The immune recognition of both **UO** and **UO** conjugates was assessed using enzyme-linked immunosorbant assay (ELISA), as indicated in **Figure 4**. Immune recognition was tested using two different antibodies: anti-UO antibodies, which specifically target and detect the **UO** enzyme component, and anti-PEG antibodies, employed to investigate the recognition of polymers by pre-existing anti-PEGs. Polymer conjugation decreases the protein immunogenicity by masking the immunogenic sites. Upon covalent polymer conjugation to **UO**, a significant reduction in **UO** immunogenicity was evident, as observed across all **UO** conjugates (**Figure 4A**). Notably, grafting-to conjugation of PEG to **UO** led to a substantial 5-fold decrease in **UO** immunogenicity. **UO-PNB PEG** conjugates showed similar or better effects compared to **UO-PEG** by reducing the immunogenicity of UO by 75-85%. Although **UO-PNB Zwit** conjugates showed higher immune recognition compared to **UO-PEG** and **UO-PNB PEG**, there was a notable 60-70% reduction compared to **UO**. Furthermore, in both **UO-PNB PEG** and **UO-PNB Zwit** conjugates, immune recognition by anti-UO antibodies decreased with an increase in the degree of polymerization.

**Figure 4B** illustrates the immune recognition of **UO** and **UO** conjugates by anti-PEG antibodies. Contrary to our hypothesis, **UO-PNB PEG** conjugates exhibited substantial immune recognition, with approximately 90% recognition when compared to **UO-PEG**. This recognition showed slight increases with higher **NB-PEG** monomer loadings. In the case of **UO-PNB Zwit**, recognition by anti-PEG antibodies was greater than **UO** but remained below 10% when compared to **UO-PEG**. Additionally, there was no change in immune recognition with an increase in **NB-Zwit** monomer loadings.

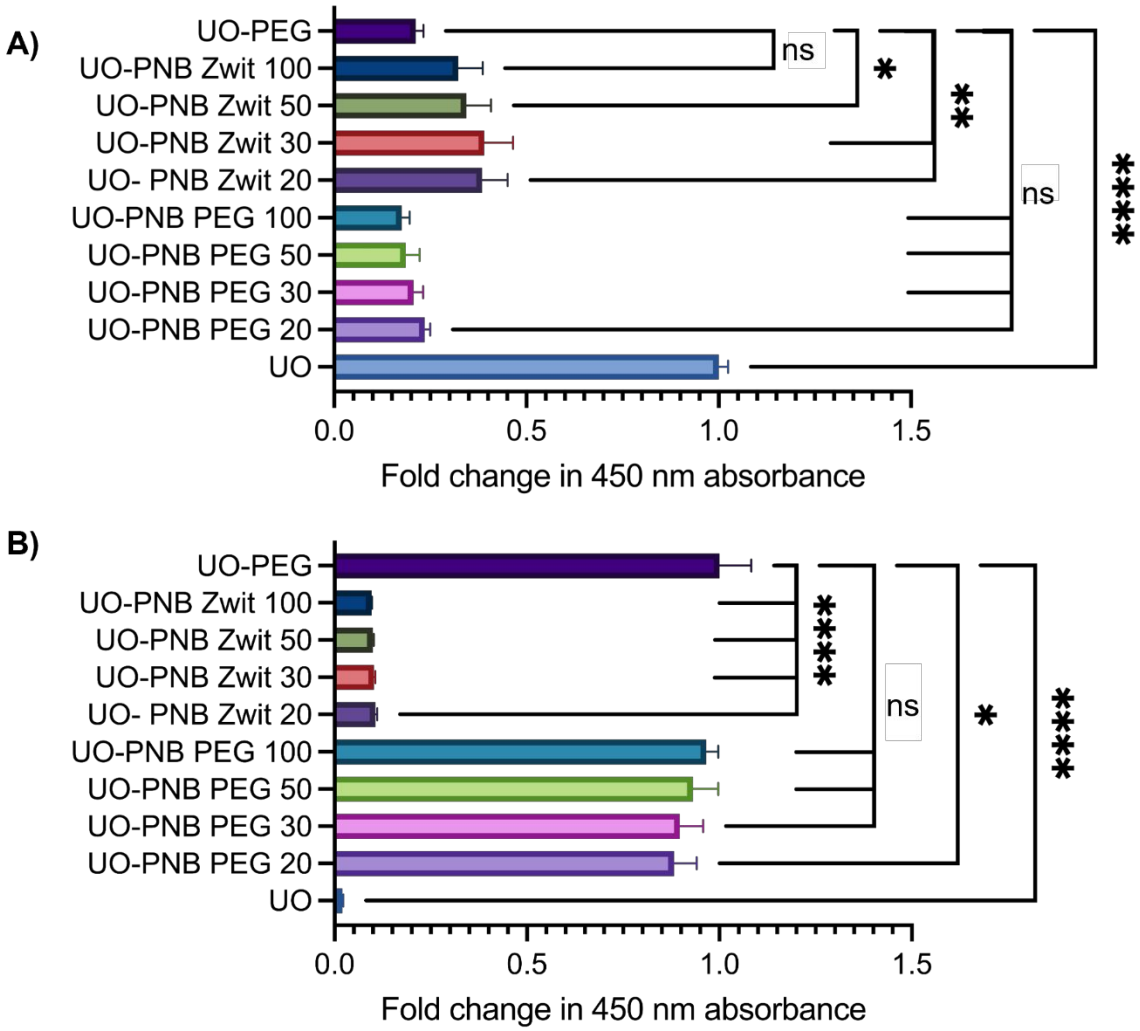



**Figure 4.** ELISA immunoassay to study the immune recognition of **UO** and **UO** conjugates by **A)** anti-UO antibodies **B)** anti-PEG antibodies.

The absorbances at 450 nm were normalized by the signal for unmodified uricase (UO) and the statistical significance values were determined using multiple comparisons in a one-way ANOVA (GraphPad Prism 10.0.2 (232)). **** signifies a statistical threshold of < 0.0001, ** signifies a statistical threshold of $p < 0.01$, * signifies statistical threshold of < 0.1 and n.s. signifies no difference..

# CONCLUSION

In this study, we successfully used a grafting-from ROMP technique to synthesize water-soluble polynorbornene conjugates of **UO, UO-PNB**. The degree of polymerization could be controlled based on stoichiometry showing the living nature of the technique. Grafting-from **UO-PNB** conjugates synthesized in the presence of excess uric acid retained

significant bioactivity. **UO-PNB** conjugates also showed higher thermal and pH stability compared to native UO and PEGylated UO, **UO-PEG.** Immune recognition studies of **UO-PNB** conjugates showed reduction in protein immunogenicity when compared to UO-PEG. In addition, UO conjugates derived from zwitterionic monomer showed no anti-PEG recognition, as would be expected. However, we show that **UO** conjugates of PEG-like brush polymers of PNB with seven repeat units showed significant reactivity with pre-existing anti-PEG antibodies. With rising interests in developing polymer alternatives to PEG in bioconjugation, **UO-PNB Zwit** demonstrates great promise. Zwitterionic polynorbornenes therefore could be a potential PEG alternative and require further investigation.

# METHODS

## Materials

NB-Zwit and NB-PEG monomers were synthesized as previously described.[65] Uricase enzyme from *Bacillus fastidiosus*, mPEG-Succinimidyl Carboxymethyl Ester-10 K, Aquamet were purchased from Strem Chemicals and uric acid was purchased from Sigma Aldrich. cis -Norbornene-exo -2,3-dicarboxylic anhydride was purchased from TCI Chemicals. 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA or TNBS), Invitrogen™ NuPAGE™ MES SDS Running Buffer (20X), Invitrogen™ SeeBlue™ Plus2 Pre-stained Protein Standard, GelCode™ Blue Safe Protein Stain were purchased from Thermo Fisher Scientific. mPAGE™ 4-12% Bis-Tris Precast Gel, 10x8 cm, 12-well was purchased from Millipore Sigma. 4X Bolt LDS sample buffer was purchased from life technologies. β-mercaptoethanol was purchased from Sigma Aldrich. Anti-uricase antibody was purchased by Rockland, anti-PEG antibody was purchased from Sigma, and the secondary antibodies were purchased from Invitrogen.

## Instrumentation

Mass spectroscopy was performed using an Agilent 6230 LC-ESI-TOF MS featuring an Agilent Jet Stream Thermal Focusing technology. The samples were dissolved in 100 mM potassium phosphate buffer (KPB) buffer, pH 6.5 at 1 mg/mL.

Fast Protein Liquid Chromatography (FPLC) was performed using a GE Healthcare AKTA™ purifier equipped with a Superdex 200 Increase column (10/300) GL and UV-Vis detector operating at 280 nm. For all FPLC samples, the mobile phase was PBS 7.4 pH at a flow rate of 0.4 ml/min. UO-PNB PEG and UO-PEG conjugates were filtered using 0.4 μm PVDF filter before injection. UO-PNB Zwit conjugates were spin-filtered before sample injection.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples were performed on mPAGE™ 4-12% Bis-Tris Precast Gel (140 V, 35 min) using an Invitrogen™ NuPAGE™ MES SDS Running Buffer. The samples were prepared with 4X Bolt LDS sample buffer and β-mercaptoethanol followed by denaturation at 100 ˚C. All gels were stained using GelCode™ Blue Safe Protein Stain. UO-PNB PEG/UO-PEG samples were additionally stained by iodine solution. The gels were washed using DI water to destain. The gels stained with Coomassie blue, destained for 30 mins-4 hours and iodine-stained gels destained for 15-20 mins. Concentration of protein solutions were obtained using Bradford assays with BSA as standards. Biotek Synergy HT microplate reader was used to obtain UV-vis absorbances. Particle sizes were obtained by dynamic light scattering (DLS) using a Zetasizer Nano series (Malvern Instruments Ltd.) All samples were at 0.1-0.5 mg/mL in 10 mM KPB buffer, pH 6.5, with 100 mM NaCl and spin-filtered before measurements. ELISA immunoassay was used to study the immune recognition of UO and UO conjugates by anti-UO and anti-PEG antibodies.

## Synthesis of UO-NB.

UO (20 mg, 0.58 μmol, 1 equivalent) was dissolved in 10 mL of 100 mM KPB buffer, pH 8.0 in a 20 mL glass scintillation vial. Cis-5-norbornene-exo-2,3-dicarboxylic anhydride, NBDA (42 mg, 0.26 mmol, 300 equivalents) was dissolved in DMSO at 50 mg/mL and added slowly into the UO solution while maintaining the initial pH by the addition of 1N NaOH solution. The reaction mixture was allowed to react 24 hours. The product was then dialyzed using a 10 kDa MWCO dialysis tube against 10 mM KPB, pH 6.5. Dialysis removes the unreacted NBDA and enables solvent switching. The dialyzed product was concentrated to a final volume of 1 mL using 10 kDa MWCO centrifuge spin-filters.

## Synthesis of grafting-from UO-PNB conjugates

To UO-NB (7.7 mg, 0.22 μmol, 1 equivalent) in 3.85 mL of 10 mM KPB buffer, pH 6.5, 100 mM NaCl in a 2 mL Eppendorf, AquaMet (9.72 mg, 12 μmol, 5 equivalent) dissolved in DI water was added. The mixture was vortexed and allowed to react for 10 minutes obtaining UO-MI (protein macroinitiator). The mixture was centrifuged at 10000 G for 5 minutes to remove unreacted AquaMet and other insoluble components. The supernatant was equally divided into five 10 kDa MWCO centrifuge tubes. The samples were centrifuged three times, each after the addition of 10 mM KPB buffer, pH 6.5, 100 mM NaCl for the removal of excess AquaMet from the supernatant. To the purified UO-MI, monomers NB-PEG (20 equivalents/UO monomer, 6.2 mg, 12 μmol; 30 equivalents/UO monomer, 9.3 mg, 12 μmol; 50 equivalents/UO monomer, 15.5 mg, 30.2 μmol; 100 equivalents/UO monomer, 31.2 mg, 60.5 μmol) or NB-Zwit (20 equivalents/UO monomer, 4.3 mg, 12 μmol; 30 equivalents/UO monomer, 6.5 mg, 12 μmol; 50 equivalents/UO monomer, 10.8 mg, 30.2 μmol; 100 equivalents/UO monomer, 21.6 mg, 60.5 μmol) dissolved in 10 mM KPB buffer, pH 6.5, 100 mM NaCl was added and allowed to react for 40 minutes. The conjugates were centrifuged three times with the addition of 10 mM KPB buffer, pH 6.5, 100 mM NaCl in the 10 kDa MWCO centrifuge tube to remove unreacted monomers. Further purification of conjugates was performed using FPLC.

## Synthesis of grafting-to UO-PEG conjugates

UO (2 mg, 0.057 μmol, 1 equivalent) was first dissolved in 100 mM borate buffer, pH 8.0 in a 20 mL glass scintillation vial. mPEG-NHS 10 kDa (45.7 mg, 4.6 μmol, 5 equivalent/lysine) was dissolved in the same buffer and added into the vial. Conjugation proceeded for 4 hours. The conjugate mixture was then concentrated using a 4 mL 10 kDa MWCO centrifuge filter. The conjugate was purified using FPLC.

## TNBS assay

The assay was used to find the degree of modifications of grafting-to UO-PEG conjugates. 100 uL of 0.2 mg/mL UO and UO-PEG solution in 100 mM borate buffer, pH 8.0 was mixed with 50 uL of 0.1 % (w/v) (diluted fresh from 5 % (w/v) in $H_2O$ solution using 0.5 M borate buffer, pH 8.5) in a 96-well plate. The mixture was incubated for 2

hours at 37 ˚C in dark. The absorbance was detected at 335 nm using a microplate reader and compared to unmodified UO to determine the number of modified lysines.

## Uricase activity of UO conjugates

The uricase activity was measured based on the fluorometric detection of resorufin, a red-fluorescent oxidation product generated when $H_2O_2$ reacts with Amplex Red reagent in the presence of horseradish peroxidase (HRP).

*Prepare the working solution for the assay*: 3.93 mL of 0.5 M Tris HCl buffer, pH 7.5, 20 µL of 100 U/mL HRP in 0.5 M Tris HCl buffer, pH 7.5, 1 mL of 5 mM UA and 50 µL of 10 mM Amplex red reagent dissolved in DMSO was mixed to prepare 5 mL of the working solution. Note: Amplex red reagent is light sensitive.

50 µL of UO, UO conjugates (5 mU/mL) and blank (0.5 M Tris HCl buffer, pH 7.5) were added to a black 96-well plate in triplicate. To these sample solutions, 50 µL of working solution was added. Fluorescence was measured after incubating at 25 ˚C for 30 minutes using excitation in the range of 530-560 nm and emission was detected at 590 nm. The activity of UO was considered 100% and the activities of conjugates were normalized with the activity of UO.

## Stability of UO and UO conjugates at different pH

50 mM sodium citrate was used to prepare solutions of pH 4 and 6. 50 mM Tris HCl was used to prepare pH 8 and 10. 50 µL of UO, UO conjugates (5 mU/mL) and blank (0.5 M Tris HCl buffer, pH 7.5) were added to a black 96-well plate in triplicates. To these sample solutions, 50 µL of working solution was added. Fluorescence was measured after incubating at 25 ˚C for 30 minutes using excitation in the range of 530-560 nm and emission was detected at 590 nm.

## Stability of UO and UO conjugates at different temperatures

UO, UO conjugates (5 mU/mL) and blank (0.5 M Tris HCl buffer, pH 7.5) in a 1.5 mL Eppendorf tubes were incubated at 30, 40, 50, 60, and 80 ˚C for 10 minutes. 50 µL of samples were transferred immediately to a black 96-well plate in triplicates. To these sample solutions, 50 µL of working solution was added. Fluorescence was measured

after incubating at 25 ˚C for 30 minutes using excitation in the range of 530-560 nm and emission was detected at 590 nm.

### ELISA Assay

ELISAs were designed as previously described.[71] The antibody lot information is provided: For uricase detection, goat-derived anti-uricase antibody from Rockland (200-101-0925, Lot 37209) was used as a primary antibody and donkey anti-goat IgG (H + L) horseradish peroxidase (HRP) conjugates from Invitrogen, (A15999, Lot 967125) was used as a secondary antibody. For PEG detection, rat-derived anti-PEG antibody (MABS1962, clone rAGP6, Lot 3724609 from EMD Millipore Sigma) was used as a primary antibody and goat anti-rat IgM HRP from Invitrogen (31476, Lot XF3603641) was used as a secondary antibody. The absorbances at 450 nm were normalized by the signal for unmodified uricase (UO) and the statistical significance values were determined using multiple comparisons in a one-way ANOVA (GraphPad Prism 10.0.2 (232)).

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