Study of a Novel Zwitterionic Material and Its Biological Applications

Jinrong Ma

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Shaoyi Jiang,

Qiuming Yu

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Jinrong Ma

University of Washington

Abstract

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Jinrong Ma

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

Zwitterionic materials have been demonstrated to have excellent non-fouling properties for a wide range of biomedical and engineering applications. Inspired by naturally occurring osmolytes, our group have developed new zwitterionic molecules. In this work, one of these new zwitterionic materials has been studied for its non-fouling properties when it is coated on a surface via atom transfer radical polymerization (ATRP) and prepared as a hydrogel and for its ability to protect proteins when it is conjugated to a highly immunogenic protein such as uricase. Results are compared with those from poly(ethylene glycol) (PEG) and other zwitterionic materials whenever possible. Results show that this new material based on a naturally occurring osmolyte is able to effectively prevent non-specific protein adsorption and to maintain protein stability and stability.

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Content

1. Introduction 1
    1.1 Zwitterionic Materials and Their Biological Applications 1
    1.2 Bioinspired Zwitterionic Materials and Zwitterionic Trimethylamine *N*-oxide Derivatives 1
    1.3 Living Radical Polymerization of Zwitterionic Monomers 3
    1.4 Development of Zwitterionic Polymer Conjugates 6
2. Experiment Methods 10
    2.1 Materials 10
    2.2 Synthesis of TMAO Monomer 11
    2.3 Living Radical Polymerization of TMAO Monomer 12
    2.4 TMAO Polymer-Protein Conjugate 14
    2.5 Surface Coating and Method of SPR 15
    2.6 Preparation of TMAO hydrogel and Method of ELISA 16
3. Results and Discussion 18
    3.1 Synthesis of TMAO Monomer 18
    3.2 Living Radical Polymerization of TMAO 19
    3.3 TMAO Conjugation of Different Proteins 23
    3.4 Characterization of TMAO Surface Coating 27
    3.5 Protein Adsorption on TMAO Hydrogels 30
4. Conclusions 33
5. Reference 34

1. Introduction

1.1 Zwitterionic Materials and Their Biological Applications

Zwitterionic materials refer to materials which possess moieties with both cationic and anionic groups. These kinds of materials are distinguished by high dipole moments and highly charged groups, but are still charge neutral [1]. This unique molecular structure endows zwitterionic materials the ability to strongly bind water molecules via electrostatically induced hydration. The repulsive hydration force from surface water layer enables zwitterionic materials to repel nonspecific protein adsorption [2]. Recent studies showed that besides non-fouling properties, zwitterionic materials are able to increase protein stability under harsh conditions without scarifying protein bioactivity [3] and induce no immunological responses *in vivo* [4].

1.2 Bioinspired Zwitterionic Materials and Zwitterionic Trimethylamine *N*-oxide Derivatives

Zwitterionic materials are often derived from naturally occurring zwitterions, especially from osmolytes, in **Fig. 1**. For example, the zwitterionic structure of carboxybetaine methacrylate (CBMA) is similar to that of glycine betaine, which is one of nature osmolytes in living organism. Similarly, sulfobetaine methacrylate (SBMA) is mimic from taurine and methacryloyloxyethyl phosphorylcholine (MPC) is from lipid head groups outside cell membranes.

Among various kinds of osmolytes, trimethylamine *N*-oxide (TMAO) ranks the

first, followed by glycine betaine, trehalose and ectoine to protect protein stability and bioactivity [5]. Thus, it is expected that zwitterionic TMAO has strong hydration similar to other zwitterionic materials and is among the most hydrophilic materials known. Compared with other nature-mimicking zwitterionic molecules, TMAO monomer has many unique properties and advantages. Structurally, TMAO is a small zwitterionic molecule in which the oxygen and nitrogen atoms are negatively and positively charged. The $N^+ - O^-$ group makes TMAO "zwitterionic" in nature. For TMAO, the $N^+ - O^-$ distance of 1.34 Å is 4.05 Debye [6], meaning that 90% of the dipole moment of TMAO is generated by the $N^+ - O^-$ part. This large dipole moment of the $N^+ - O^-$ part characterizes the super-hydrophilicity of TMAO. Zwitterionic polymers developed until now have repeating units where the two chargers are separated by alkyl spacer. Qing *et al.* [7] have shown that the spacer affects non-fouling property. While TMAO has a unique structure containing a very compact zwitterionic $N^+ - O^-$ unit with no spacer between chargers. Thus, zwitterionic TMAO derivative-based N-oxide monomer (or TMAO monomer) is expected to possess excellent hydrophilicity among various nature mimicking zwitterionic monomers. Surface Plasma Resonance (SPR) and Enzyme-Linked ImmunoSorbent Assay (ELISA) were used to show the non-fouling property of this promising novel material.

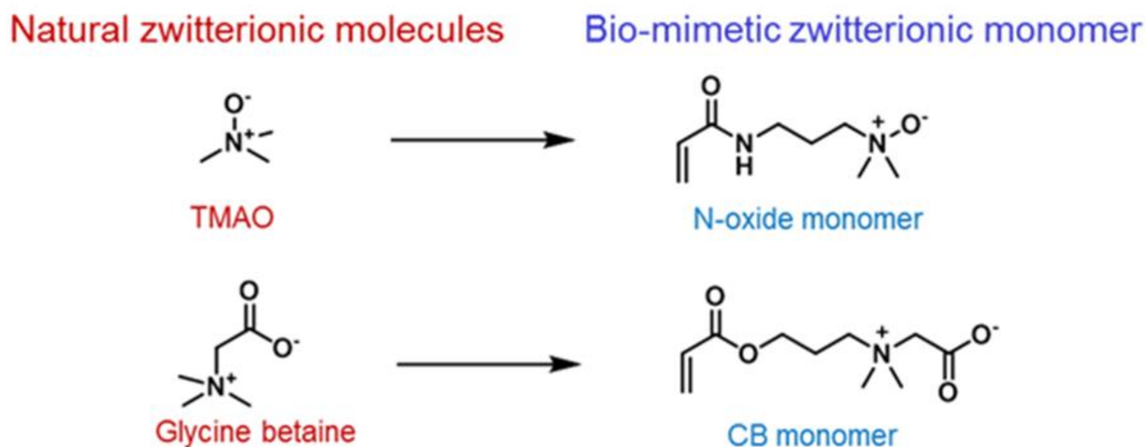


Figure 1. Naturally occurring zwitterionic small molecules and polymerizable monomers.

1.3 Living Radical Polymerization of Zwitterionic Monomers

For any potential applications of zwitterionic polymers, it is highly desirable to precisely control their molecular weights with narrow polydispersity index (PDI). Nowadays, controlled living radical polymerization techniques have been widely used to obtain well-defined polymers with pre-designed compositions, topologies and functionalities. Among all living polymerization techniques, atom transfer radical polymerization (ATRP) has been widely employed for polymerization of numerous functional monomers [8, 9, 10, 11]. ATRP works the best for hydrophobic monomers, wherein the monomers are usually inert towards its catalyst system and transition metal forms a complex with certain ligands, which controls the polymerization behavior by controlling the amount of free radical [12]. However, it becomes more complicated in the ATRP of polar monomers, especially zwitterionic monomers with nitrogen. ATRP of zwitterionic TMAO monomer poses challenges since it contains active groups and is much more reactive. For example, the complexation between the

monomer and the transition metal may lead to the loss of catalyst activity. The existence of high protonic solvent will lead to Cu(I) complexes disproportionation [13]. Here we optimized the bulk ATRP conditions of this challenging monomer, and the well-controlled polymer obtained was then used for TMAO polymer-protein conjugates.

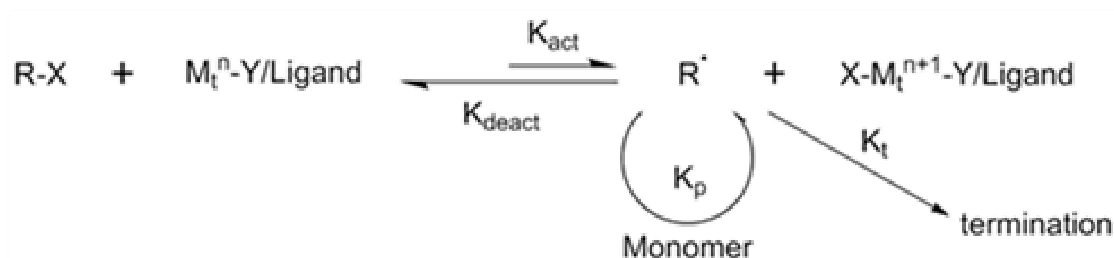


Figure 2. Transition-Metal-Catalyzed ATRP mechanism

Controlled radical polymerization can also be applied to fabricate functionalized surfaces covered with polymer brushes of controlled thickness [14,15,16,17]. Compared with free radical polymerization, controlled radical polymerization provides the narrow PDI of polymer chains and therefore promotes the uniformity of surface polymer brushes. To prepare stable coating, chemical bonding between polymer brushes and surfaces are preferred. Typically, surface coating can be categorized into “graft from” and “graft to” methods [2, 18]. “Graft-to” method involves the preparation of a polymer with a surface-adhesive moiety, and the direct attachment of the polymer onto the surface. While “graft from” method involves the immobilization of initiators on the surface and polymerization of a monomer from the immobilized initiators such

as via ATRP. This surface initiated control radical polymerization is also called surface-initiated ATRP (SI-ATRP) [19]. In SI-ATRP, chain growth begins with an ATRP initiator, which is immobilized on a substrate. The surface initiator will allow monomer polymerization to start from the surface and then form polymer brushes. Compared with “graft-to” method, (graft-from) SI-ATRP will promote the surface packing density, uniformity and stability of the coating layer. SI-ATRP can be applied to various surfaces, such as gold, silicon and clay by immobilizing initiators onto these surfaces first. In this work, we implemented this technique to coat a gold surface and explored the surface behavior of coated TMAO polymer brushes.

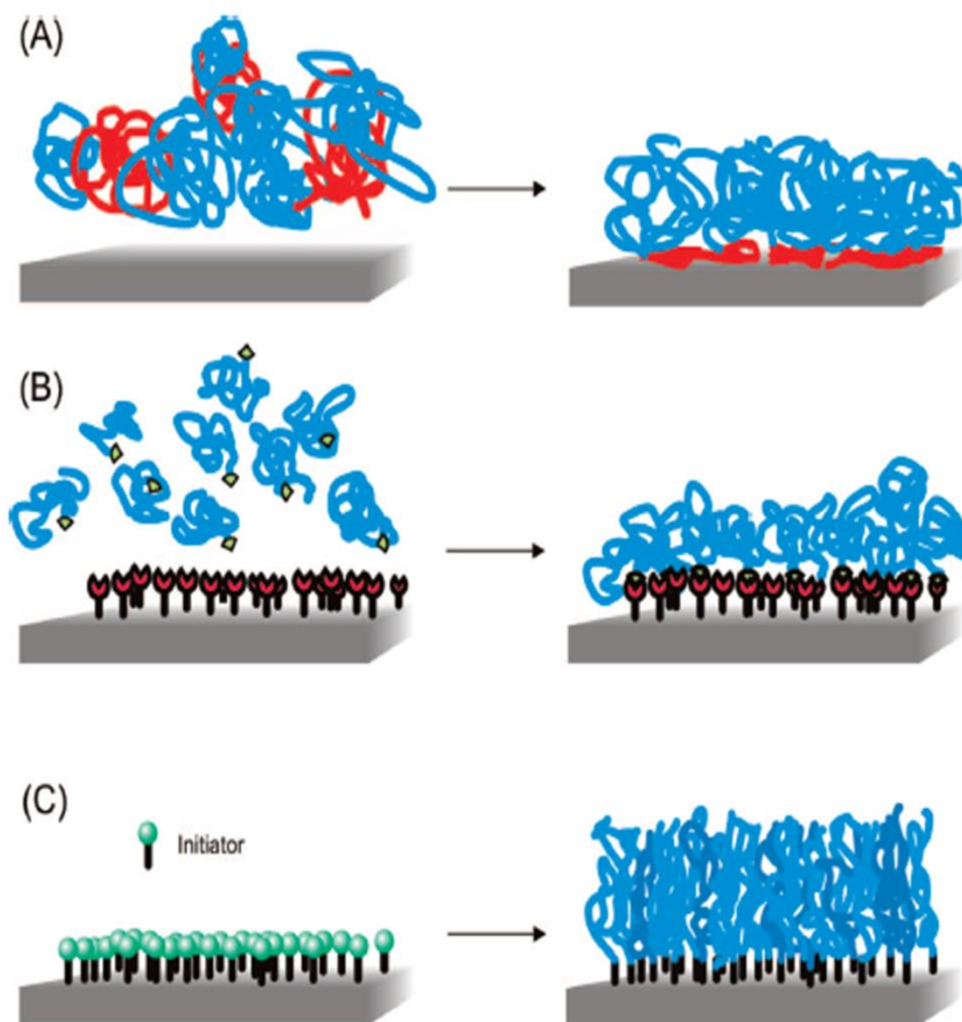


Figure 3. Synthetic strategies for the preparation of polymer brushes: (a) physisorption of diblock copolymers via preferential adsorption; (b) chemisorption via reaction of appropriately end-functionalized polymers with complementary functional groups at the substrate surface (grafting to approach); (c) polymer brushes grown via surface-initiated polymerization techniques (grafting from approach). [19]

1.4 Development of Zwitterionic Polymer Conjugates

Protein is the most important molecule to living species and human beings. It can do almost everything inside human body, such as controlling metabolism, defending bacteria and viruses and building our tissues and organs. Protein is widely accepted as an excellent therapeutics candidate to alleviate diseases not easily treated with

traditional chemical drugs [20,21,22,23]. More than 60 protein drugs are already in the market and hundreds of proteins drug are being evaluated in different phases of clinical trials.

However, the immunogenicity of many protein drugs not only limits their therapeutic efficacy, but also threatens patients' lives with adverse effects including anaphylaxis and infusion reactions. Therefore, only minimally immunogenic proteins can be applied into human therapy. Numerous efforts have been made to humanize non-human sourced proteins, but limited success have been achieved compared with the bright initial prospects [4]. Chemical conjugation of polyethylene glycol (PEG) has been the most commonly used method to solve the immunogenic issue of protein. Although beneficial as a successful method, PEG is also known to adversely affect protein binding affinity, therefore reducing overall bioactivity [2]. For example, the bioactivity of PEGylated interferon-α2a was reported to drop to 7% as compared with its native form [4]. Apart from decrease in activity, the PEG triggered immune response is another issue for PEGylation. It is reported that patients developed anti-PEG antibodies after repeated administration of PEGylated therapeutics [3]. A report showed the Pegloticase (krystexxa), a PEGylated uricase product caused more than 40% of refractory chronic gout patients receive this drug-triggered high-level anti-polymer antibody and consequently became non-responders to its treatment [24]. Furthermore, another issue is due to pre-existing anti-PEG antibodies. These findings raise severe concern upon the further application of PEGylation and an alternative approach is urgently needed to cope with such an alarming issue.

Based on previous studies of PEGylation, it is believed that PEGylation issues are tightly correlated with the amphiphilic nature of PEG [25,26,27], as **Fig. 4b**. Thus, we hypothesize that a highly hydrophilic material should solve this problem. Recent studies witnessed zwitterionic material emerging as a significant class of extremely hydrophilic biomaterial [28,29,30,31]. The high surface hydration renders zwitterionic material as an excellent candidate for protein modification. The interaction between proteins and zwitterionic materials is shown as **Fig. 4c**. Andrew *et al*. showed that poly(carboxybetaine) (pCB) protein conjugates were able to maintain the stability of enzyme α-chymotrypsin without scarifying its binding affinity [32]. S.Liu *et al*. found that the stealth layer of polyzwitterions can protect protein therapeutics from host immune recognition without reducing enzymatic activity or inducing polymer-specific antibody production [3]. P. Zhang *et al*. demonstrated that pCB polymer network mesh coating on a protein can effectively improve its stability, extend its pharmacokinetic (PK), and mitigate its immune response with superior [4]. As TMAO is regarded as a highly hydrophilic zwitterionic material, it is believed TMAO polymer conjugate will do an excellent job in enabling a conjugated protein with great stability, extended PK and lower immune responses.

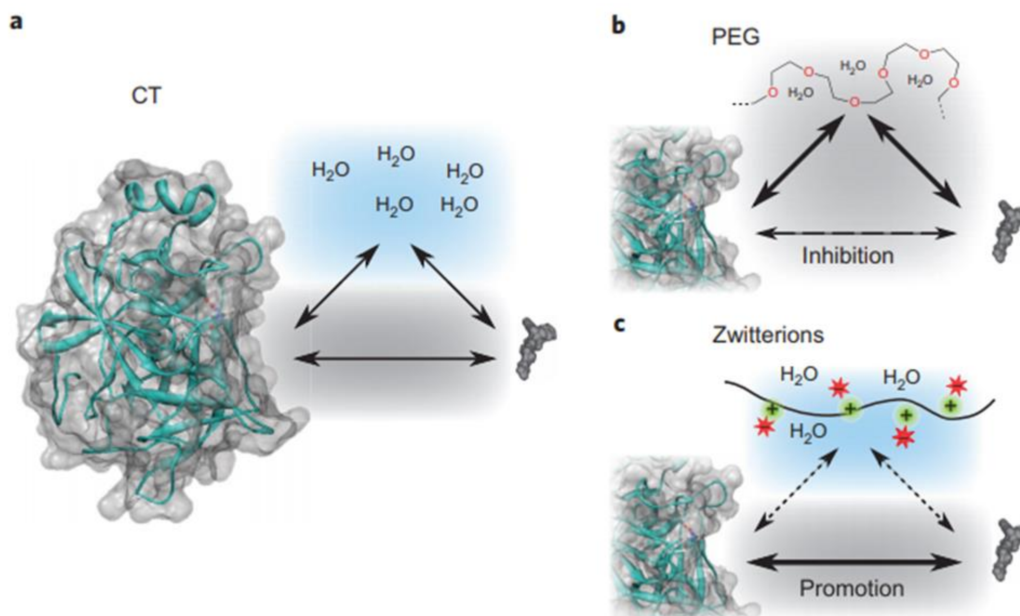


Figure 4. Mechanism of how PEG and pCB polymers influence binding affinity. a). Relationship between enzyme and substrate without polymer. b). PEG impedes affinity by reducing enzyme–substrate hydrophobic–hydrophobic interactions as a result of its amphiphilic characteristics. c). Super-hydrophilic pCB has a strong effect on the structure of water, creating a local environment that increases enzyme–substrate hydrophobic–hydrophobic interactions, thereby increasing the substrate's affinity for the binding pocket [32].

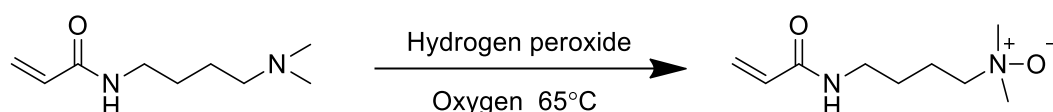
2. Experiment Methods

2.1 Materials

Following chemicals were used in this project: Dimethylaminopropylacrylamide (DMAPAA, GC, > 98%, TCI America), Hydrogen peroxide (50% wt. in H_2O, Sigma Aldrich), Diethylenetriaminepentaacetic acid (> 99%, Sigma Aldrich), Copper(I) bromide (99.9999%, Sigma, Aldrich), N,N,N′,N″,N″-Pentamethyldiethylenetriamine (PMDETA, 99%, Sigma Aldrich), Tris[2-(dimethylamino)ethyl]amine (Me_6TREN, 97%, Sigma Aldrich), Albumin bovine serum (BSA, analytical standard, Sigma Aldrich), 2-Iminothiolane hydrochloride (Traut's reagent, TLC, ≥ 98%, Sigma Aldrich), 3-(Maleimido)propionic acid N-hydroxysuccinimide ester (BMPS, HPLC, ≥ 98.5%, Sigma Aldrich), Human Plasma Fibrinogen (50-70%, Sigma Aldrich), Polypropylene (Sigma Aldrich), horseradish peroxidase-conjugated antifibrinogen, o-Phenylenediamine (≥ 98%, Sigma Aldrich), Hydrochloric acid (1N, Sigma Aldrich), 2-Bromo-2-methylpropionyl bromide (≥ 98%, Sigma Aldrich), N-Boc-ethylenediamine (≥ 98%, Sigma Aldrich), Triethylamine (≥ 99.5%, Sigma Aldrich), Methanol (anhydrous, ≥ 99.8%, Sigma Aldrich), Ethanol (ACS reagent, ≥ 99.5%), Dimethyl sulfoxide (DMSO, ACS reagent, ≥ 99.9%), Tetrahydrofuran (THF, anhydrous, ≥ 99.9%), Trifluoroacetic acid (TFA, 99%, TCI America), PBS buffer (pH=7.4).

2.2 Synthesis of TMAO Monomer

Synthesis and Purification of TMAO monomer were done by Priyesh Jain. 800 mg diethylenetriaminepentaacetic acid was added to 30 mL (deionized) DI water and mixed vigorously until the white powder was dissolved. Then Hydrogene Peroxide (50% solution, 2.87 g) was slowly added and reaction contents were heated to 60℃. Oxygen gas then was slowly purged into solution. Dimethylaminopropylacrylamide (14.4 g) in 10 mL DI water was added dropwise in 30 minutes. The reaction was carried out for 6 hours at 60°C. After completion of reaction, the reaction contents were cooled. Nuclear magnetic resonance (NMR) analysis of the product confirmed the formation of TMAO monomer. The pH of the final product was about 7.5 and solid content was about 32.9%. TMAO monomer was then precipitated by organic solvent. The monomer is colorless and viscous liquid. Synthesis procedure and NMR result are as below.



Scheme 1. Synthesis of TMAO monomer

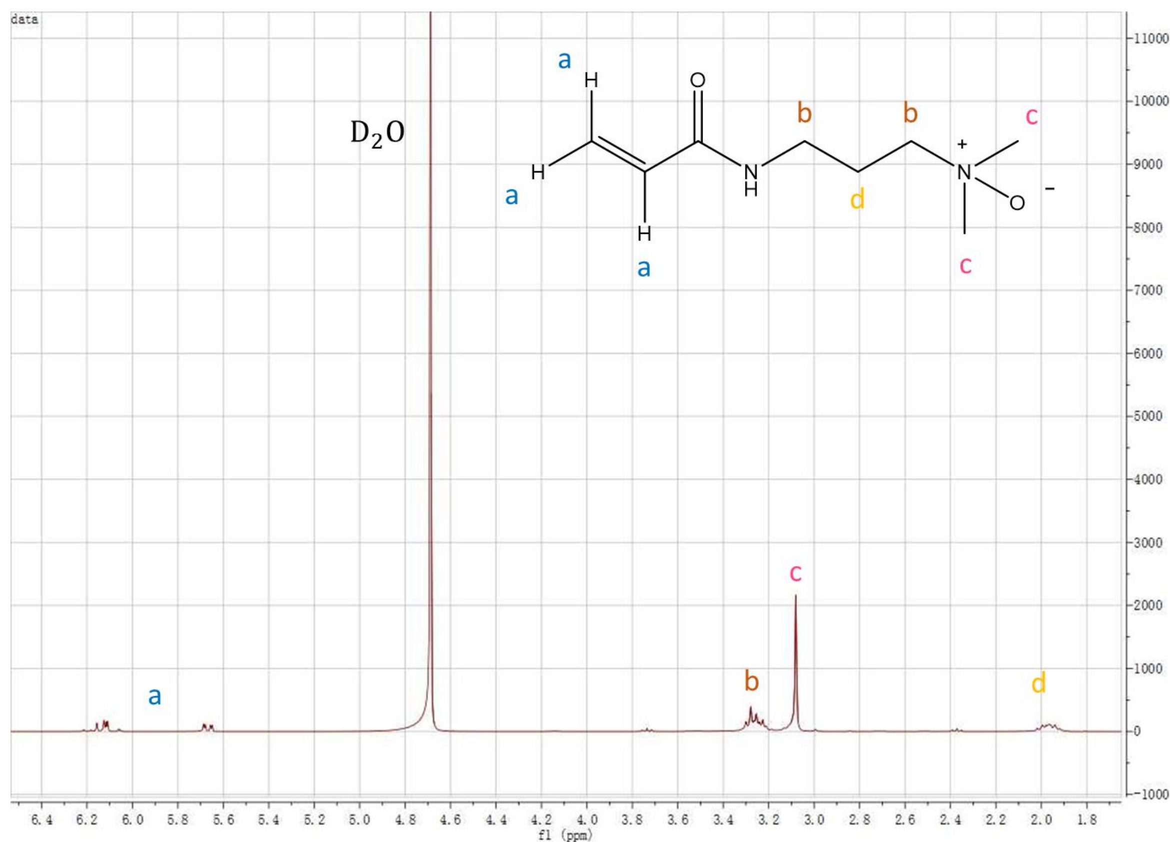
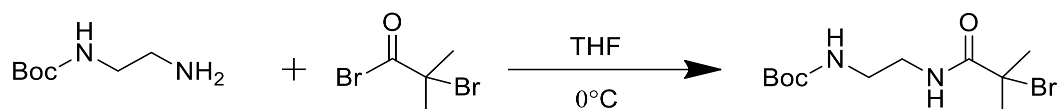


Figure 5. NMR spectrum of TMAO monomer

2.3 Living Radical Polymerization of TMAO Monomer

ATRP was applied to obtain TMAO polymer with well-controlled PDI. ATRP initiator was synthesized by dissolving *N*-Boc-ethylenediamine (0.4806g, 3mmol) and Triethylamine (0.334g, 3.3mmol) in 100mL THF. 2-Bromo-2-methylpropionyl bromide (0.7586g, 3.3mmol) was dissolved in 30 mL THF and then dropwise added into the mixture. The reaction was allowed at 0℃ for overnight. The product tert-butyl *N*-[2-(2-bromo-2-methylpropanamido)ethyl]carbamate (**1**) was purified by chromatographic column before use. Synthesis procedure and NMR result are as below.



Scheme 2. Synthesis of Boc protected ATRP initiator.

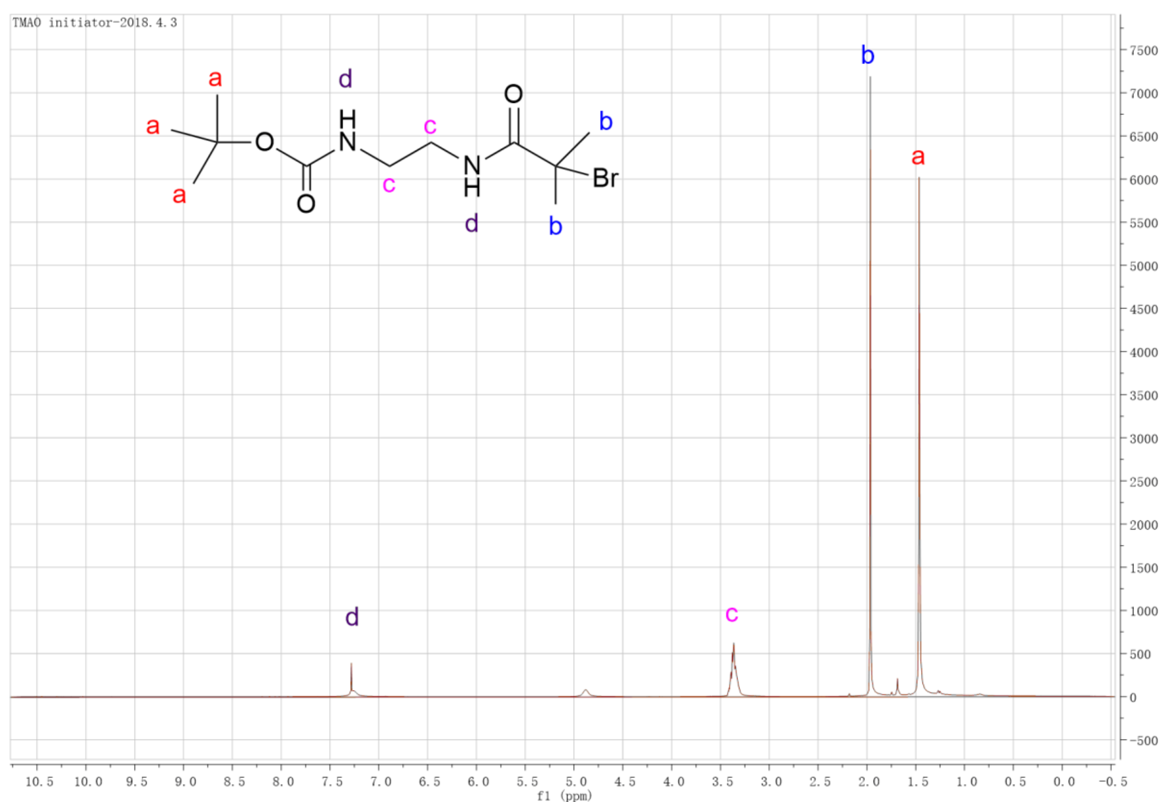
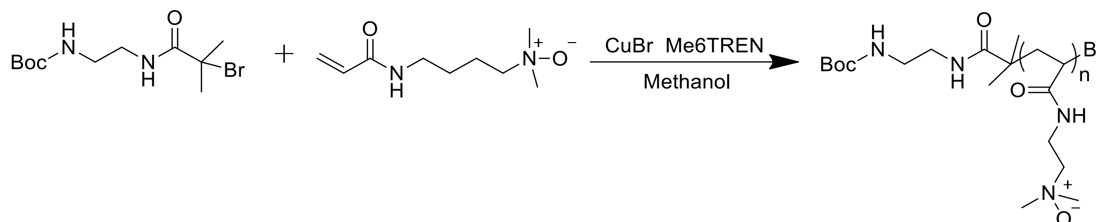


Figure 6. NMR spectrum of Boc protected ATRP initiator.

To carry out polymerization, **1** (30.7mg, 0.1mmol) and N-oxide monomer (1.72g, 10mmol) were placed into a Schlenk tube and deoxygenated via nitrogen-vacuum. 2mL deoxygenated mixed solvent (water and methanol) was added to dissolve all solids. The solution was then transferred via syringe to another Schlenk tube, containing Tris[2 – (dimethylamino)ethyl]amine (23mg, 0.1mmol), copper (I) bromide (14.3mg, 0.1mmol) and 2mL mixed solvent, previously degassed and filled with nitrogen. Reaction mixture was allowed to stir at room temperature for overnight.

The desired product, Boc protected N-oxide polymer was further purified via dialysis.



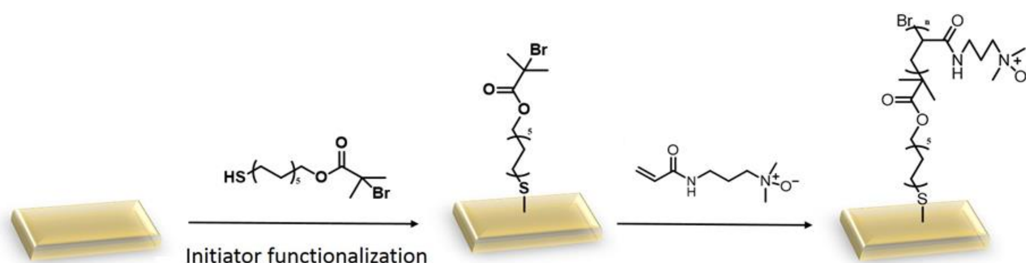
Scheme 3. Synthesis of TMAO polymer via ATRP

2.4 TMAO Polymer-Protein Conjugate

TMAO polymers obtained via above mentioned ATRP method were used to do protein conjugation. 500mg TMAO polymer was dissolved in 5mLTFA and stirred for 2hours at room temperature to remove the Boc protection group. Deprotected polymers were purified via dialysis. Then 80mg of deprotected polymer together with 0.72mg Traut's reagent were dissolved in 5mL PBS buffer and stirred for two hours at room temperature. Meanwhile, 3mg protein and 37.5μL BMPS solution (40mg/ml in DMSO) were dissolved in 5mL PBS, stirred for 1 hour at room temperature and washed by ultrafiltration (35K) to remove the unreacted BMPS. The washed protein was then mixed with polymer solution and reacted at 4℃ for overnight. Conjugated protein was purified by ultrafiltration (100K) for ten times and Gel Permeation Chromatography (GPC) was used for characterization.

2.5 Surface Coating and Method of SPR

TMAO coated surface was used as the characterization sample for SPR. SI-ATRP technology was applied to graft TMAO polymer brushes onto glass substrate coated with gold surface. Self-assembly monomer layer (SAM) was formed by soaking clean gold coated glass substrate in w-mercaptoundecyl bromoisobutyrate solution (0.2mmol/L in ethanol) for overnight. The initiator coated substrates, together with Copper(I) bromide (14.35mg, 0.1mmol) were then placed into a Schlenk tube and deoxygenated via pump-vacuum for ten cycles. TMAO monomer (1.72g, 10mmol), Me_6TREN(23mg, 0.1mmol), methanol (3.6mL) and H_2O(0.4mL) were added into Schlenk tube and deoxygenated via the same method. After fully deoxygenation, the mixed aqueous solution of TMAO monomer and Me_6TREN were transferred to the tube which held the substrate and copper bromide. The reaction mixture was placed at ambient temperature for overnight. The substrate was then taken out from mixture and washed with ethanol and water respectively for three times and air-dried before being used as SPR sample. The thickness of the coated surface was later characterized by Ellipsometer.



Scheme 4. Preparation of TMAO polymer brushes via SI-ATRP on gold surface

To implement SPR, a bare glass substrate was firstly loaded onto SPR sensor for primary cleaning. All flowing pipes were washed by flowing RBS, aqueous hydrochloric acid, DI water and PBS buffer. The glass substrate was then replaced with TMAO coated substrate for characterization. PBS was allowed to flow all testing pipes until there is no bubble on the surface of TMAO coated substrate. The instrument and software were set up following the SPR instructions. The characterization started with 10 minutes flow of PBS buffer, then followed by 10 minutes flow of 100% human serum and 10 minutes of PBS buffer. The instrument was cleaned by flowing RBS, DI water and air after experiment.

2.6 Preparation of TMAO hydrogel and Method of ELISA

TMAO hydrogel was fabricated by bulk photo-polymerization with a hydrogel aqueous solution containing TMAO monomer (0.67g DI water, 0.33g TMAO monomer), crosslinker N,N’-Methylenebis(acrylamide) (3.3mg) and photo-initiator 2-Hydroxy-2-methylpropiophenone (0.33mg). The hydrogel aqueous solution was placed between two glass slides separated by a 0.5 mm-thick polytetrafluoroethylene spacer, and was then photo-polymerized at room temperature for 30 mins. After

polymerization, hydrogels were removed from the casts and soaked in PBS for three days to remove unreacted chemicals and reach the fully hydrated hydrogel network. Phosphate buffered saline was refreshed every 12 hours.

Biopsy punches were used to punch the hydrated TMAO hydrogel sheet into 5 mm-diameter disks. Hydrogel disks were placed into a 24 well-plate and incubated with 1 mL of 1 mg/mL fibrinogen in PBS buffer for 1 hour, followed by 5 washes with pure PBS buffer. Hydrogel disks were then transferred to new wells and incubated with 1mL of horseradish peroxidase (HRP) conjugated anti-fibrinogen (1 μg/mL) in PBS buffer for 1 hour. All hydrogel disks were then transferred to new wells after 5 washes with pure PBS buffer. Next, 1mL 1 mg/mL o-phenylenediamine (OPD) 0.1 M citrate phosphate pH 5.0 solution, containing 0.03% hydrogen peroxide was added. After 15min incubation, the enzymatic reaction was stopped by adding an equal volume of 1 N HCl. Absorbance value at 492 nm was recorded by a plate reader, and was normalized to that of polypropylene (PP) sample. Average data were acquired from three specimens.

3. Results and Discussion

3.1 Synthesis of TMAO Monomer

Previous study has demonstrated zwitterionic material as a promising class of biomaterial. They show excellent performance to resist nonspecific protein adsorption, cell/bacterial adhesion and biofilm formation. Their ability to hold water and form a physical/ energetic barrier is the key mechanism to resist nonspecific protein adsorption. The hydrophilicity of zwitterionic materials arises from the pendant zwitterionic moieties in the side chain and these zwitterionic moieties are often derived from naturally occurring osmolytes. Among all osmolytes, trimethylamine-N-oxide ranks the first. Therefore, it is expected that TMAO monomer has strong hydrations similar to other zwitterionic monomers and is among the most hydrophilic materials known.

Our goal is to synthesize this TMAO monomer with N+–O- moieties. Compared with synthesis of other zwitterionic monomers, where multiple steps are often needed for synthesis, TMAO monomer can be easily prepared via single reaction step. Therefore, TMAO monomer can be easily synthesized in large quantity. Easy synthesis and low cost will promote a broader application of this promising novel material.

TMAO monomer is obtained in form of 33wt% aqueous solution right after harvesting from the synthesis reaction. This aqueous solution can be directly used to prepare hydrogel with different solid contents by diluting with DI water or buffer

solution. While for ATRP polymerization, an aqueous solution is usually not desirable as higher water content may result in less control of the PDI of polymer bruhses. In order to implement polymerization in different solvent, pure monomer is often needed. To obtain pure TMAO monomer without any solvent, the aqueous solution was dropwise added into acetone with volume ratio of 1:20 (monomer solution to acetone) for precipitation. The precipitant was then washed with excess ethyl ether for three times. Final pure product was viscous and colorless liquid. The pure TMAO monomer was used to do ATRP and SI-ATRP polymerization.

3.2 Living Radical Polymerization of TMAO

For biomedical applications, materials are typically used in form of polymers, such as regular polymers for conjugation, or crosslinked polymer networks for hydrogels. Therefore, for any newly developed material, it is crucial that the monomer can be polymerized. To show the simplicity of TMAO polymerization, we used photo-initiated free radical polymerization by mixing monomers with a minimal amount of photo-initiator 1173 and polymerizing them under 312nm UV for 10 mins. GPC result in **Fig. 7** indicates the success of polymerization.

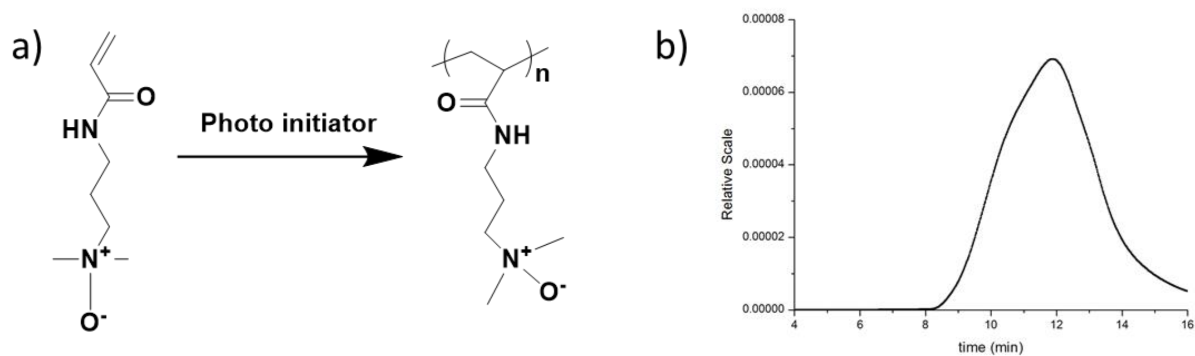


Figure 7. a) Scheme of TMAO polymerization via photo initiated free radical polymerization. b) GPC profile of TMAO polymer fabricated via photo initiated free radical polymerization.

Although TMAO polymers can be easily obtained via commonly used photo-initiated polymerization, it is clear that polymers prepared by free radical polymerization have very broad PDI, which is usually not desirable for many applications. Therefore, the precise control of TMAO polymer molecular weight with narrow PDI is needed. Among all controlled radical polymerization methods, ATRP is one of the most commonly used methods. Here we applied this technique for the controlled polymerization of TMAO monomer.

As to ATRP polymerization, the choice of solvent is very important as it has huge effect on polymerization behavior. Generally speaking, the higher the polarity of solvent, the worse the control of polymerization[33]. Therefore, aqueous solvent is often not desirable for ATRP. However, TMAO monomer itself is super-hydrophilic and thus a high polar solvent is required to dissolve TMAO monomer. To solve this conflict, we used a mixed solvent consisting of water (high polar component) and methanol (less polar component). Pure water was also used as TMAO monomer was initially obtained in form of an aqueous solution. By varying the ratio of water to

methanol, we found that the optimized solvent system was the mixed solvent of 90% methanol and 10% water. The PDI of TMAO polymer prepared under this solvent system was 1.076, which is desirably narrow for further use. While TMAO polymer prepared under pure water had a relatively high PDI of 1.455, this result is consistent with the theory that the higher polarity of solvent leads to less control of polymerization.

To demonstrate the stability of $N^{+}-O^{-}$ moiety during polymerization, we used NMR to characterize the chemical structure of TMAO polymer. According to **Fig. 9**, it is clear that the peak from zwitterion moiety was still there with correct integral value compared to theoretical calculation. This result proved that the zwitterion moiety from TMAO monomer is stable under ATRP polymerization conditions.

The well-controlled TMAO polymer with 9.193×10^{3} molecular weight was then used for protein conjugation. The optimized solvent system, 90% methanol and 10% water, was also applied for SI-ATRP to prepare well-controlled TMAO polymer brushes on gold surface.

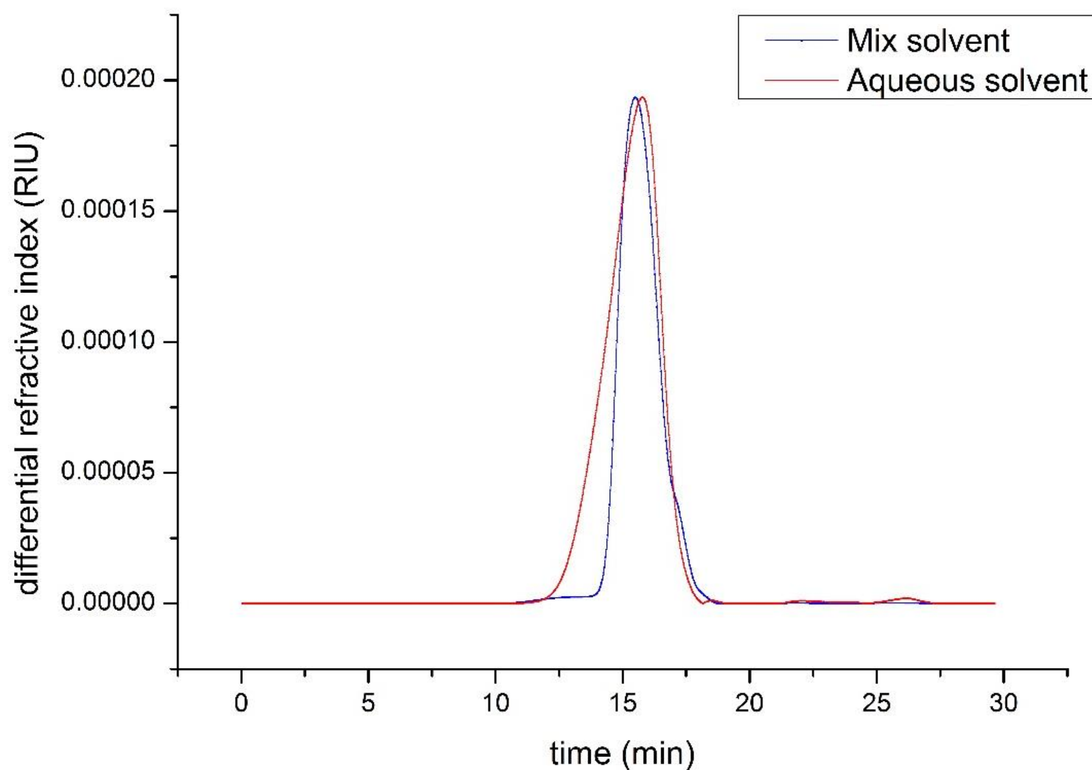


Figure 8. GPC spectrum of Boc protected TMAO polymer prepared via ATRP in aqueous solution and mix solvent (90% methanol and 10% water).

Table 1. Molecular weight and polydispersity of Boc protected TMAO polymer prepared under different conditions.

Solvent	Molecular Weight	Polydispersity
100% water	1.208×10^4	1.455
90% methanol & 10% water	9.193×10^3	1.076

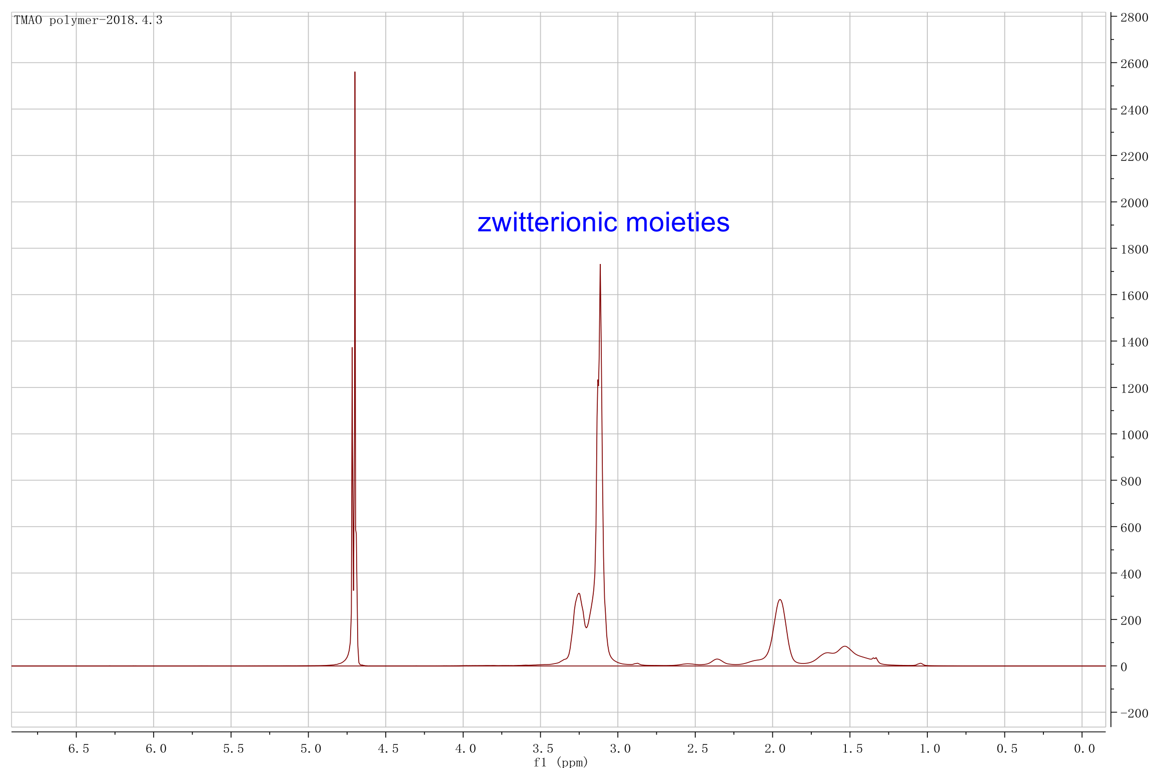
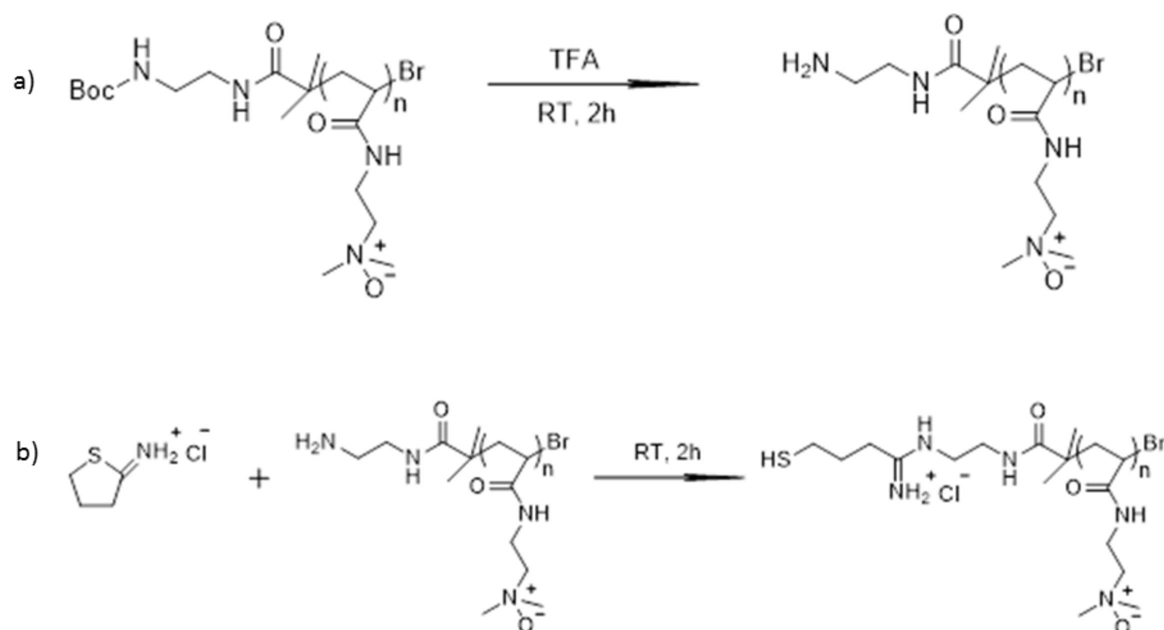


Figure 9. NMR spectrum of Boc protected TMAO polymer.

3.3 TMAO Conjugation of Different Proteins

Polymer conjugation is a commonly used technique to improve the performance of therapeutic proteins, such as improving the stability and prolonging the circulation time [34, 35, 36]. Although PEGylation is the most studied as well as industrially applied conjugation method, it has many issues, such as PEG triggers immune responses. To solve this PEGylation issue, the PI's group has developed zwitterionic polymer protein conjugates. Zwitterionic polymer protein conjugates outstand with better stability, longer protein circulation, and no material-triggered immune responses as zwitterionic materials are truly hydrophilic with very strong hydration. As TMAO is expected to be among the most hydrophilic zwitterionic materials, we believe that TMAO polymer conjugate can do a great job.

TMAO polymers obtained from bulk ATRP were used as starting materials for protein conjugation. The product directly obtained from ATRP was Boc groups protected. To release reactive amine moieties, the protecting groups were removed by dissolving polymers in TFA and stirring at room temperature for 2 hours. Purified amine terminated polymers were obtained by dialysis and freeze-dry. Amine terminated TMAO polymers were then mixed with Traut's reagent to induce reactive thiol groups into TMAO polymers. The mechanism of these procedures was shown in **Scheme 5.**



Scheme 5. a). Deprotection of Boc protecting group terminated TMAO polymer synthesized via ATRP. b). Thiol terminate TMAO polymer preparation via Traut's reagent reaction.

BSA and uricase were used as protein models for TMAO polymer conjugates. Both proteins have surface accessible amine groups from lysine. By mixing protein solutions with BMPS reagent, surface accessible amine groups can be modified into double bond, as **Fig. 10**. Then double bond of the modified protein reacts with thiol

terminated TMAO polymer via thiolene chemistry. The desired product, TMAO polymer-protein conjugates were purified by ultrafilter and characterized by GPC.

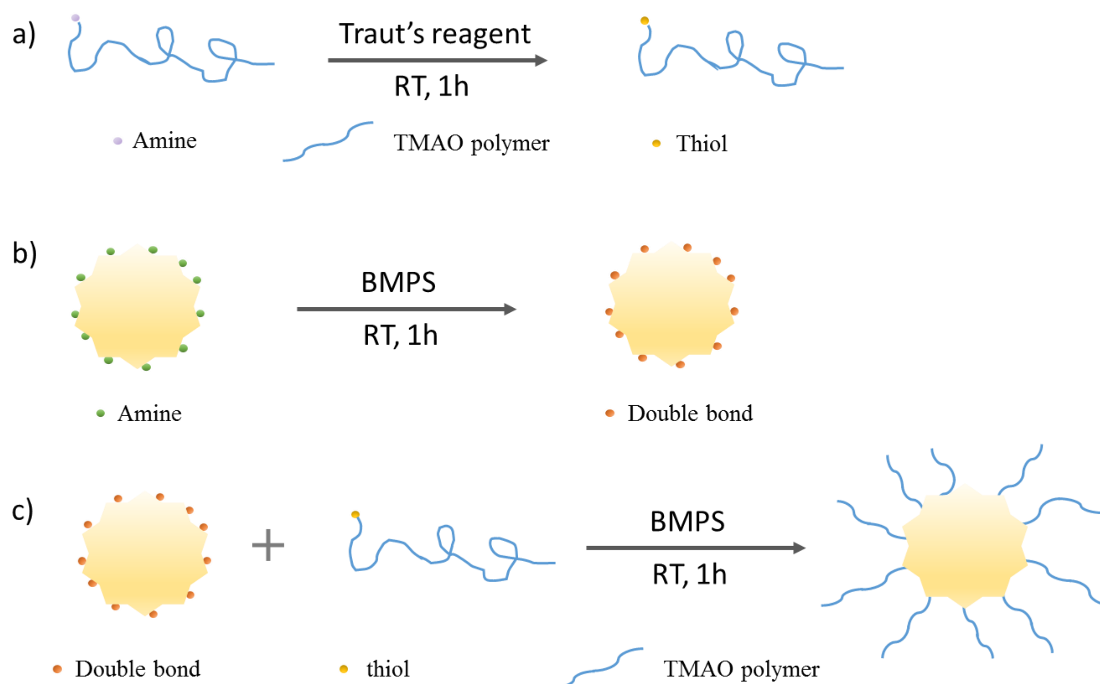


Figure 10. Scheme of TMAO polymer conjugate processes

To demonstrate the success of TMAO polymer conjugates, we measured the hydrodynamic sizes of both proteins before and after polymer conjugation. As conjugation goes on, TMAO polymers chemically attach to the surface of proteins, resulting in an increased hydrodynamic diameter. Therefore, a dramatic increase in hydrodynamic size suggests the occurrence of conjugation. According to GPC spectrum, **Fig. 11** & **Fig. 12**, both BSA and uricase shows dramatic increase in hydrodynamic size after conjugation with TMAO polymer. This result indicates that TMAO polymer had been successfully conjugated onto proteins. To test whether TMAO polymer conjugation has any effect on protein bioactivity, we measured

uricase bioactivity after conjugation with TMAO polymer. Results, **Fig. 13**, show that uricase activity only dropped approximate 10% after conjugation. This means TMAO polymer conjugation can protect protein without scarifying protein activity. *In vitro* results show that TMAO polymers work well for protein conjugation. Further study will focus on *in vivo* experiments. Protein circulation and material-triggered immune responses will be explored. PEGylated uricase will be used for comparison.

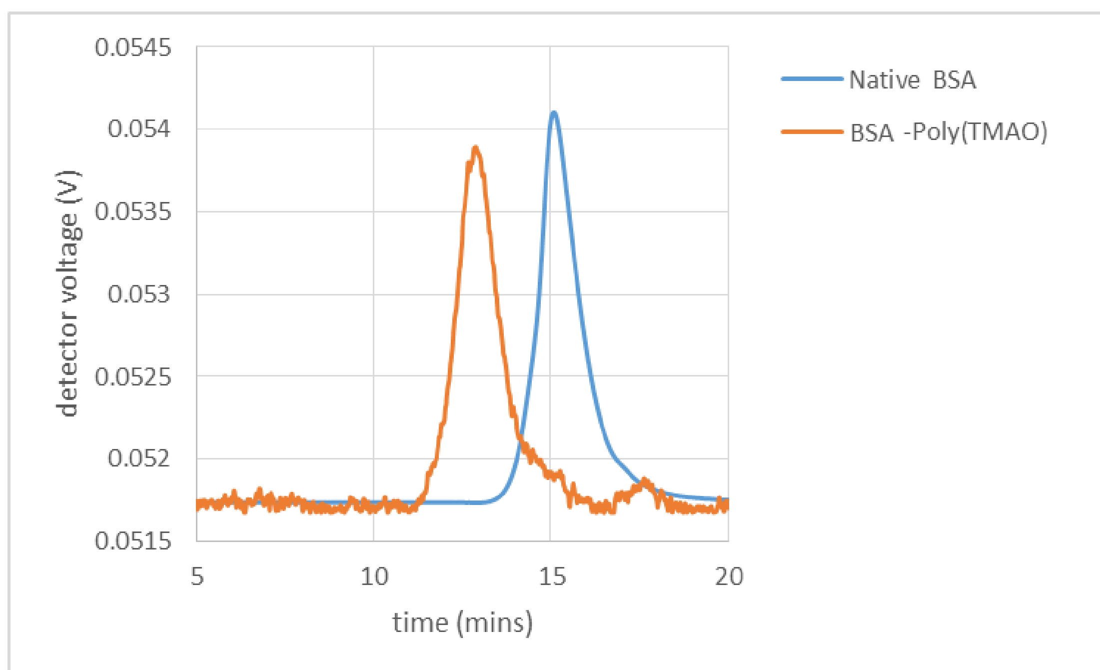


Figure 11. GPC spectrum of BSA protien before and after TMAO polymer conjugate.

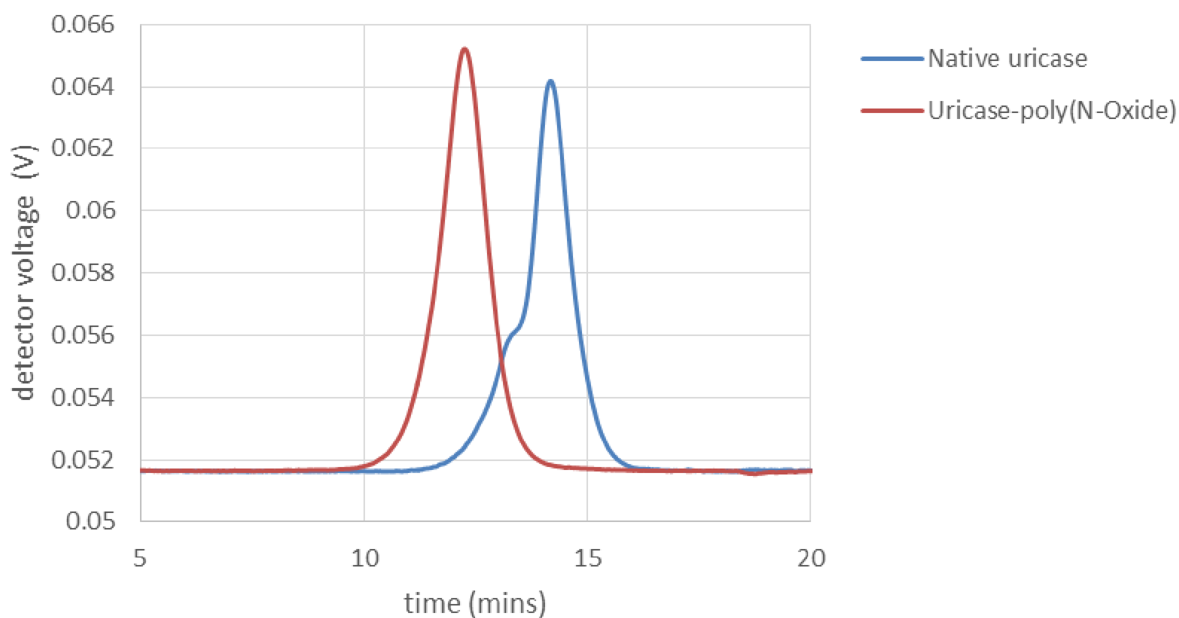


Figure 12. GPC spectrum of Uricase protien before and after TMAO polymer conjugate.

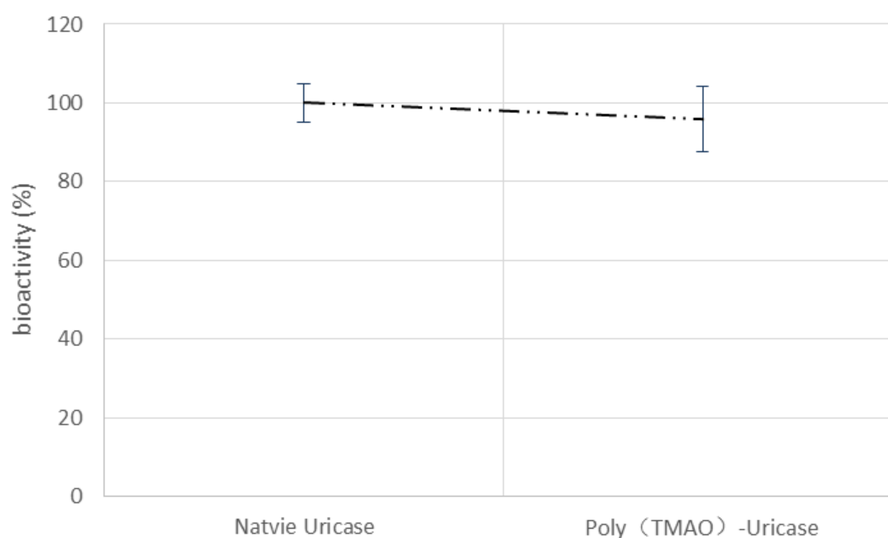


Figure 13. Relative bioactivity of Uricase before and after TMAO polymer conjugate.

3.4 Characterization of TMAO Surface Coating

Anti-biofouling is crucial for a wide range of applications, such as medical implants, biosensors, marine structures and industrial equipments. For biosensors, biofouling reduces sensitivity and specificity. [37]. While protein adsorption on biological implants reduces the performance of devices and may trigger side effects.

Surface coating with hydrophilic materials has been extensively studied and applied to reduce non-specific protein adsorption. However, many of these hydrophilic surfaces are often not enough to prevent undesirable protein adsorption and cell or bacteria adhesion within the required level. Even a small amount of protein on a surface might lead to the failure of biomedical devices. Therefore, surfaces with ultra-low fouling properties are needed for many applications. H. Vaisocherova *et al.* demonstrated that total nonspecific protein adsorption on the functionalized pCB was lower than $3ng/cm^2$ for 100% human blood serum [38], which is not achieved by any other materials including PEG. Since TMAO is a new member of zwitterionic monomers with very strong hydration, we believe that TMAO coated surface will do a great job. SI-ATRP technology was used to fabricate TMAO coated surfaces with desirable morphology and anti-fouling property.

Ellipsometer was used to measure the morphology of TMAO coated gold surface. The thickness of SAM was showed to be 2.2 ± 0.2nm and final thickness of coating was 10.8nm$\pm$0.8nm. Therefore, the approximate thickness of TMAO polymer brushes would be 8.6nm$\pm$0.8nm. This excellent surface uniformity (low thickness variation) demonstrates the well control of the PDI of polymer brushes on SI-ATRP.

The anti-fouling behavior of TMAO-coated gold surface was characterized by SPR. SPR is the most commonly used method to measure protein adsorption. The main advantage of this technique is its ability to measure binding affinities and association/dissociation kinetics in real time, in a label free environment with high sensitivity.

In this experiment, a higher refractive index means more adsorbed proteins onto TMAO polymer-coated surface. SPR experiment starts with 10mins flow of PBS buffer. The refractive index did not change during this 10mins as there was nothing attached to TMAO polymer brushes. Then 10mins flow of 100% human serum was used. The sensor signal dramatically increased to a stable level because the protein from serum interacted with TMAO polymer brushes and affected the surface refractive index. PBS buffer was injected again to wash away the unbind protein, after which detector signal simultaneously decreased.

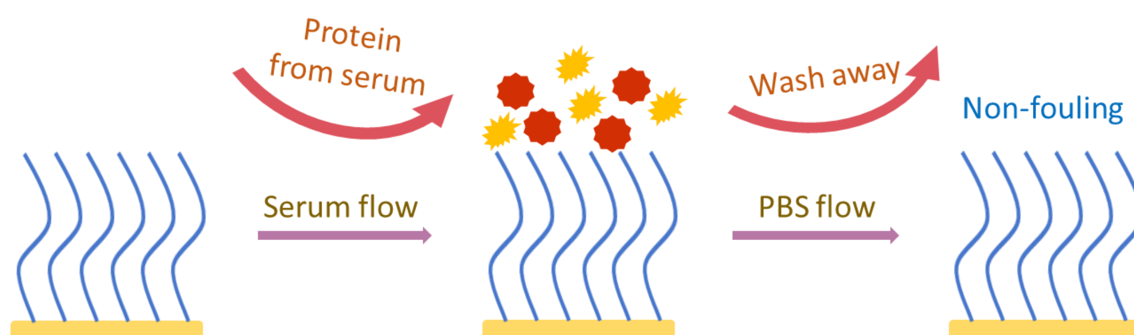


Figure 14. Scheme of SPR experiment implemented over non-fouling surface.

SPR detector wavelength shift was converted into protein adsorption by applying 1nm wavelength shift equals to 17ng/cm^2 protein adsorption. Protein adsorption profile throughout SPR process is shown as **Fig. 15**. According to the profile, the protein adsorption over TMAO polymer coated surface was 2.2ng/cm^2 after last 10mins PBS buffer wash. This low protein adsorption result demonstrates the excellent fouling-resistance property of TMAO polymer and its promising potential for surface coating.

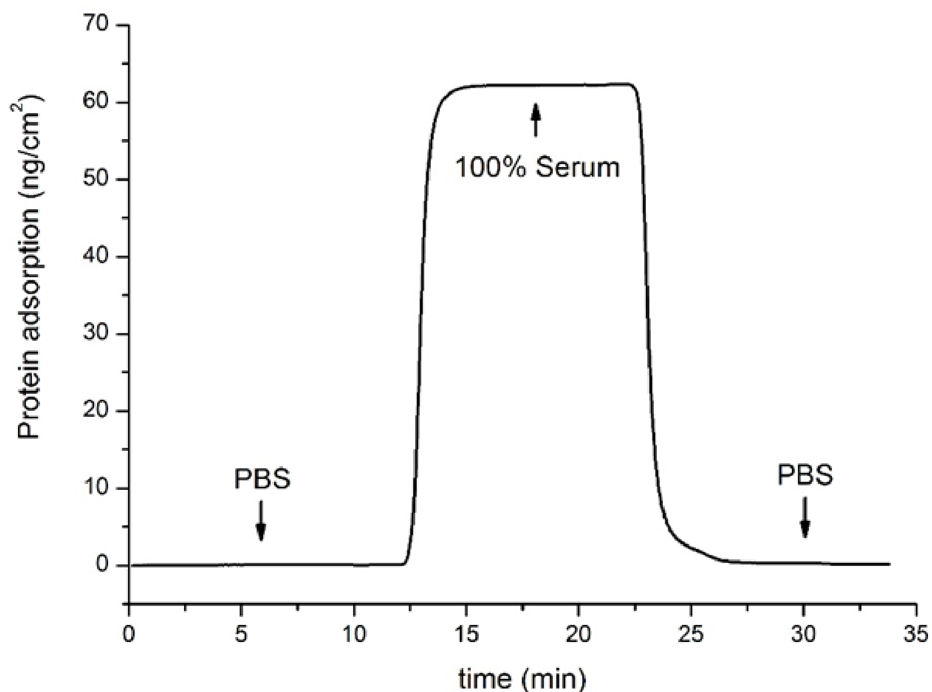


Figure 15. SPR sensorgram of protein detections from 100% blood serum flowing TMAO polymer grafted gold surface.

3.5 Protein Adsorption on TMAO Hydrogels

Hydrogels have long been used for biological and biomaterial applications because of their high water content, which imitates the living tissue environment, ensures high diffusive permeability, and provides biomimetic mechanical strengths [39,40,41]. Specific research interest has been given to PEG hydrogels because apart from typical advantages of hydrogels, they are also considered to be low biofouling. However, for long term application, PEG is susceptible to oxidation and gradually loses its functionality in biological media [2]. Besides, for applications in complex

media, PEG materials are often insufficient to meet the requirement. In the past years, the PI's group has demonstrated pCB based hydrogel as a promising material for biological applications [42]. Compared with PEG based hydrogels, pCB hydrogels outstand with better biostability, biocompatibility and ultra-low biofouling property. As TMAO is among the most hydrophilic zwitterions, TMAO based hydrogels should perform very well.

TMAO based hydrogels can be easily prepared via photo-initiation. Besides, other commonly used gelation methods, such as TEMED/APS initiation and AIBN initiation, were also successfully applied to TMAO hydrogels preparation. ELISA was used to characterize the fouling-resistance property of TMAO hydrogels with 1.5% MBAA crosslinker. PP substrate was used as a positive control.

According to **Fig. 16**, fibrinogen adsorption onto TMAO-based hydrogel is approximately 2.5% when compared with that onto PP substrate. This result demonstrates the strong surface hydration and excellent biofouling resistance properties of TMAO based hydrogels. Outstanding ultra-low fouling properties, together with simple preparation will make TMAO based hydrogels desirable for biomedical materials.

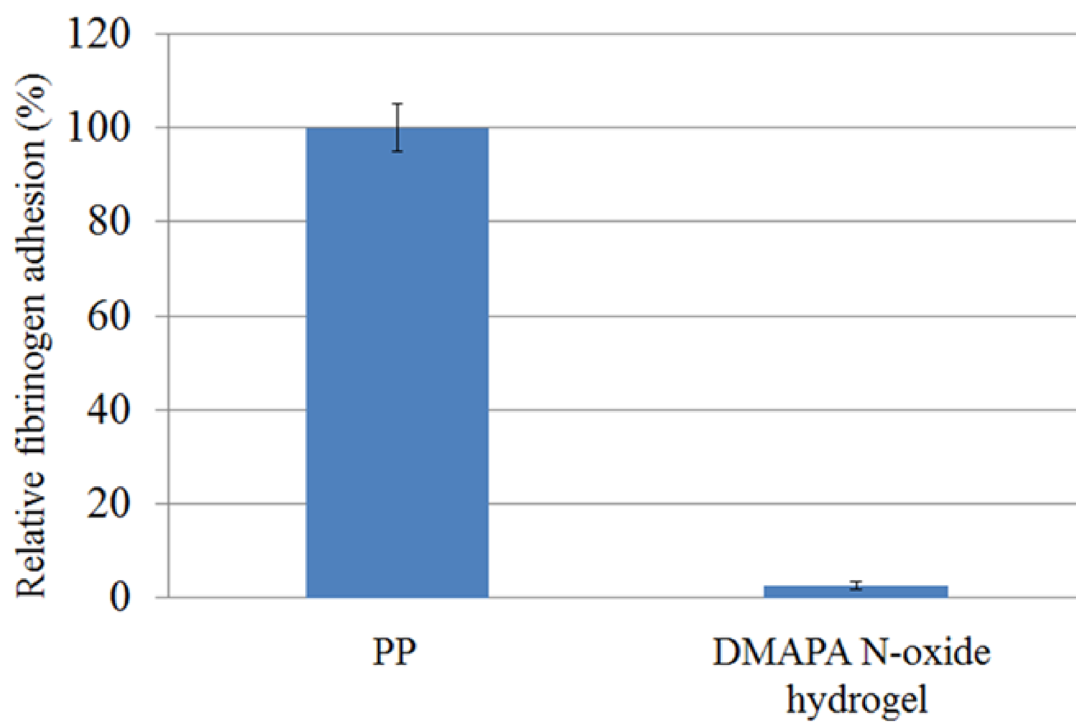


Figure 16. Relative fibrinogen adsorption of TMAO hydrogels via ELISA.

4. Conclusions

In summary, we successfully synthesized a new bioinspired zwitterionic monomer, TMAO, and developed a series of methods to prepare TAMO polymers, hydrogels, surface coatings and protein conjugates. More specifically, we applied ATRP polymerization and chemical conjugate technique to prepare TMAO polymer with well-controlled PDI for protein conjugates. Results show that TMAO polymer conjugation can be successfully applied to therapeutic proteins such as uricase without scarifying its bioactivity. We also used SI-ATRP method to prepare surface coatings with TMAO polymer brushes and studied their surface properties. SPR results show that TMAO polymer brushes effectively resist non-specific protein adsorption even in complex media (undiluted human serum here). In addition, we prepared TMAO polymer based hydrogels and demonstrated the excellent non-fouling property of these hydrogels. This work provides a fundamental understanding of this novel zwitterionic material and demonstrates several of its applications. Non-fouling TAMO polymers worth further exploration of its applications.

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