Chemo-enzymatic Synthesis and RAFT Polymerization of 6-*O*-Methacryloyl Mannose: A Suitable Glycopolymer for Binding to the Tetrameric Lectin Concanavalin A?

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Summary: The chemo-enzymatic synthesis of 6-*O*-methacryloyl mannose (MaM) glycomonomer was successfully performed for the first time. Subsequent aqueous RAFT polymerization of the monomer yielded well-defined, linear poly(6-*O*-methacryloyl mannose) (PMaM) glycopolymers without the need for protecting and deprotecting group chemistry. As well as investigating the RAFT polymerization kinetics of this monomer using various initial monomer to chain transfer agent concentration ratios, the protein binding ability of the generated glycopolymer was tested using concanavalin A, a known mannose-residue binding lectin.

Keywords: glycopolymer; protein binding; RAFT polymerization; reversible addition-fragmentation chain transfer polymerization

Introduction

Incorporation of carbohydrate moieties as either pendant or terminal groups to a polymer chain leads to the effective synthesis of glycopolymer materials.[1,2] These synthetic materials are generally water-soluble, highly polar and hydrophilic, biodegradable, and pharmacologically active.[3] These types of polymeric materials have garnered much interest of late for their potential in biomimetic applications, due to their similarity in functionality displayed with natural glycoconjugates. The research work of Kiessling in particular has studied the interactions of glycopolymers with protein and other cell surfaces.[4–7] Their research has shown that the architectural regularity of glycopolymers, in addition to their functionality, is paramount for biomimetic applications.[5] Controlled/living polymerization techniques provide a rather facile means to obtaining architecturally regular synthetic glycopolymers.

Several controlled polymerization techniques have recently been described in the literature for the synthesis of glycopolymers exhibiting narrow polydispersities and predetermined molecular weights. Living ionic,[8–13] ring opening,[14–16] and ring opening metathesis[4–7,17–18] polymerizations have all been effectively used; however, certain disadvantages exist with these techniques. Ionic polymerization processes are highly sensitive to the presence of impurities and acidic protons or electrophilic functionalities, which requires strenuous reagent purification and anhydrous conditions as well as prior protection of hydroxyl functionalities. Furthermore, ring opening metathesis polymerization is limited to strained monomers. In recent years, controlled radical polymerization (CRP) has addressed these disadvantages and become a viable alternative to synthesizing glycopolymers with control of molecular

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weight and polydispersity. This polymerization method allows for a wide range of polymerizable monomers and reaction conditions that are more tolerant of impurities and conditions, such as oxygen and water.

Of the various CRP techniques, the most interesting for glycopolymer synthesis are atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization. Unlike ring opening techniques that require strained monomers, such as norbornene, ATRP and RAFT techniques rely on the use of vinyl monomers to generate the desired polymer materials. Furthermore, both of these techniques are highly tolerant of aqueous medium,[19–20] provided care is taken when choosing the appropriate reaction conditions. One of the first controlled polymerization performed on unprotected glycomonomers in aqueous medium was reported by Narain and Armes.[21–22] They were able to polymerize two different methacrylate based glycomonomers using ATRP techniques in water and methanol solvent mixtures. Shortly thereafter, Lowe *et al.* reported on the aqueous synthesis of poly(2-methacryloxyethyl glucoside) *via* RAFT polymerization techniques using a water-soluble dithioester chain transfer agent.[23] Our research group has recently used this same water-soluble dithioester RAFT agent to synthesis several other glycopolymers derived from vinyl monomers.[24] In addition, the RAFT process was shown to be suitable to generate star-shaped glycopolymers.[25]

Building upon this prior research, this study investigates the chemo-enzymatic synthesis of a vinyl monomer derived from D-mannose. Controlled, aqueous RAFT polymerization of the synthesized sugar monomer (6-*O*-methacryloyl mannose) (MaM) was studied. In addition to the enzymatic monomer synthesis and controlled polymerization, protein binding studies were performed using concanavalin A (Con A) which has been shown to be a lectin particularly sensitive to mannose moieties as well as to be an activator in cellular signaling.[7]

Experimental Part

Materials

D-Mannose (Aldrich, 99% mixture of anomers), vinyl methacrylate (Aldrich, 98%), 4,4′-azobis(cyanopentanoic acid) (ACPA, Fluka, 98%), *N*,*N*-dimethyl acetamide (DMAc, Aldrich, 99.9% HPLC grade), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Aldrich) buffer solution (pH = 7.5), Concanavalin A (Con A, Aldrich), and hexane were all used without further purification. 2,2′-Azobisisobutyronitrile (AIBN, Aldrich, 98%) was recrystallized twice from methanol prior to use. Technical grade acetone, ethanol (absolute), and ethyl acetate were also used as received. 4-(Cyanopentanoic acid)-4-dithiobenzoate (CPADB) RAFT agent was synthesized according to literature methods.[20] Novozym 435® was kindly donated by Novozymes A/S.

Chemo-enzymatic Synthesis of 6-*O*-Methacryloyl Mannose (MaM)

Mannose substrate (7.2062 g, 0.04 mol), vinyl methacrylate (5.3822 g, 0.048 mol), *Candida Antarctica* lipase immobilized polymer (Novozym 435®, 4.03 g), and a few granules of BHT (to inhibit radical generation) were added to an Erlenmeyer flask containing 50 mL of acetone and sealed with a rubber septum. The flask was placed in a heated water stirring bath (50 °C and 150 rpm) and allowed to react for 5 days. Once finished, the yellow solution was filtered to remove the lipase enzyme from the monomer solution. Flash chromatography was performed (ethyl acetate:hexane: ethanol 7:2:1, Rf = 0.38) on the crude residue to separate the desired monomer from any side reaction products. The relevant fractions were combined and rotary evaporation performed to remove solvent, which resulted in a pale yellow oil. The residue was dissolved in water for freeze-drying to yield the final, white powder product (6.87 g, 74% yield). ^{1}H NMR (300 MHz, D_2O) δ: 1.93 (s, 3 H), 3.54–4.08 (m, 4 H), 4.29–4.51 (dd, 2 H), 4.89 and 5.14 (d, 1 H, anomeric), 5.71 (s, 1 H),

and 6.14 (s, 1H) ppm. ESI-MS: calculated for $C_{10}H_{15}O_7$, 247.21 and found 247.05 (M – H); calculated for $C_{12}H_{19}O_9$, 307.24 and found 307.02 ($M + CH_3COO^-$).

RAFT Homopolymerization of MaM Monomer

To a series of clean Schlenk tubes, 4 mL of a 0.4 M solution of MaM in water (0.3972 g, 1.6 mmol) and 0.118 mL of 59.8 mM ACPA in EtOH (1.973 mg, 7.04×10^{-3} mmol) were added. CPADB RAFT agent (0.12 M solution in EtOH) was combined with the monomer and initiator solutions so as to generate various concentration ratios of monomer to CPADB (100:1, 200:1, and 300:1). These reaction vials were then sealed with a rubber septum and subjected to at least three freeze-pump-thaw cycles to remove oxygen from the reaction solutions. After degassing, the vials were placed in a 70 °C oil bath and samples of the reaction solution were removed, *via* syringe, at regular timed intervals to monitor the reaction kinetics. Each sample was freeze-dried to remove solvent and a portion of the solids were analyzed by GPC to determine the molecular weight and monomer conversion. Polymer purification was performed by re-dissolving the sample in water followed by precipitating the polymer in methanol.

Protein Interaction with Glycopolymer Materials

Glycopolymer interaction with protein molecules was determined using turbidity measurements similar to those reported in the literature.[7] Concanavalin A (Con A) was dissolved in HEPES buffer solution (pH = 7.5) to afford a 30 μM solution. Glycopolymer samples were dissolved in HEPES buffer solution to afford a solution containing 1.5 mM *of mannose units*. Equal parts of each solution, 0.5 mL each, were combined and mixed vigorously for 5s before UV-Vis spectroscopy experiments to monitor the absorbance at 420 nm, which is indicative of cluster formation between mannose and Con A.

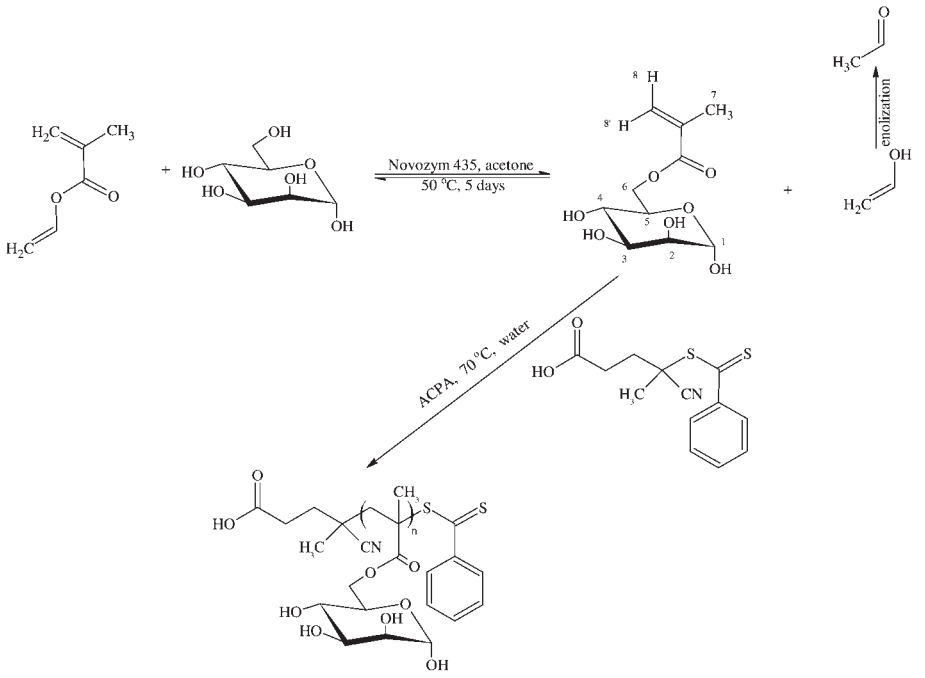
Characterization Methods

^{1}H NMR spectroscopy was conducted on a Bruker spectrometer (300 MHz) in either deuterium oxide or deuterated dimethyl sulfoxide (*d*-DMSO). UV-Vis spectroscopy was performed on a Varian 300 Scan UV-Vis spectrophotometer using 10-mm quartz glass cuvettes. Gel Permeation Chromatography (GPC) analysis of the subsequent glycopolymers were performed in *N*,*N*-dimethylacetamide (DMAc) (0.05%w/v LiBr, 0.05% BHT) at 40 °C (1 mL/min flow rate) using a Shimadzu modular system comprising a DGU-12A solvent degasser, a LC-10AT pump, a CTO-10A column oven and a RID-10A refractive index detector. The system was equipped with a 5.0 μm bead-size guard column (50×7.8mm) followed by four 300×7.8mm linear Phenomenex columns (10^5, 10^4, 10^3 and 500 Å). The calibration curve was generated with narrow polydispersity polystyrene standards ranging from 500 to 10^6 g/mol. ESI-MS analysis was performed with a Thermo-MAT high-pressure liquid chromatography system consisting of a solvent degasser, a quaternary pump, an autoinjector, and a dual-wavelength UV detector and equipped with a C8 Luna column (Phenomenex 150×4.6 mm, 100 Å pore size, 5μm particle size). The system was interfaced to a Thermo Finnigan LCQ Deca ion-trap mass spectrometer equipped with an atmospheric pressure-ionization source operated in nebulizer-assistend electrospray mode (ESI). The instrument was calibrated with caffeine (Aldrich), MRFA (tetrapeptide, Thermo Finnigan), Ultramark 1621 (Lancaster), and poly(propylene glycol) ($M_n = 2700$, Aldrich) in the mass range 195–3822 amu. Spectra were obtained in negative ion mode.

Results and Discussion

6-*O*-Methacryloyl Mannose (MaM) Synthesis and RAFT Polymerization Kinetics

Scheme 1 illustrates the chemo-enzymatic monomer synthesis and subsequent RAFT



Scheme 1.
Chemo-enzymatic synthesis and RAFT polymerization of 6-*O*-methacryloyl mannose.

polymerization reactions for MaM glycomonomer. *Candida antartica* lipase (CAL, Novozyme 435) has been shown to promote the transesterification of esters and sugar molecules in the presence of organic solvents.[26–28] However, this particular enzyme also catalyzes the breaking of ester bonds in the presence of aqueous media and thus accounting for the existence of a reaction equilibrium in the first step. By using vinyl methacrylate to generate the vinyl sugar monomer, the transesterification reaction yields an enol (ethylenol) as the by-product, which undergoes rapid rearrangement to form acetaldehyde. The enolization reaction allows for the transesterification reaction equilibrium to be shifted towards the glycomonomer product and resulting in a rather high vinyl sugar monomer yield. This is especially beneficial due to the relatively low solubility of mannose in acetone as observed by the large amount of sugar remaining in solution after the monomer synthesis. The excess mannose glycoside in the reaction solution is also consistent with both a slow reaction rate[22] and the selectivity of the lipase enzyme with various conjugated carboxylic acids.[29]

Thin liquid chromatography was used to check the purity of the reaction product. After filtering the system to remove the enzyme catalyst and unreacted mannose substrate, the reaction solution contains both the desired mono-substituted vinyl sugar as well as higher substituted side products, $R_f = 0.38$ and 0.7 respectively. Upon flash column chromatography to separate the high R_f side product, the mono-substituted vinyl monomer was obtained in a relatively high purity and characterized by both ^{1}H NMR and ESI-MS, Figure 1 and 2. The peaks are labeled according to the numbering system employed in Scheme 1. The ^{1}H NMR shows the difference in the vinyl protons as well as the anomeric protons from the sugar ring in roughly the correct proportions. ESI-MS analysis shows good agreement between the calculated molecular weight and the

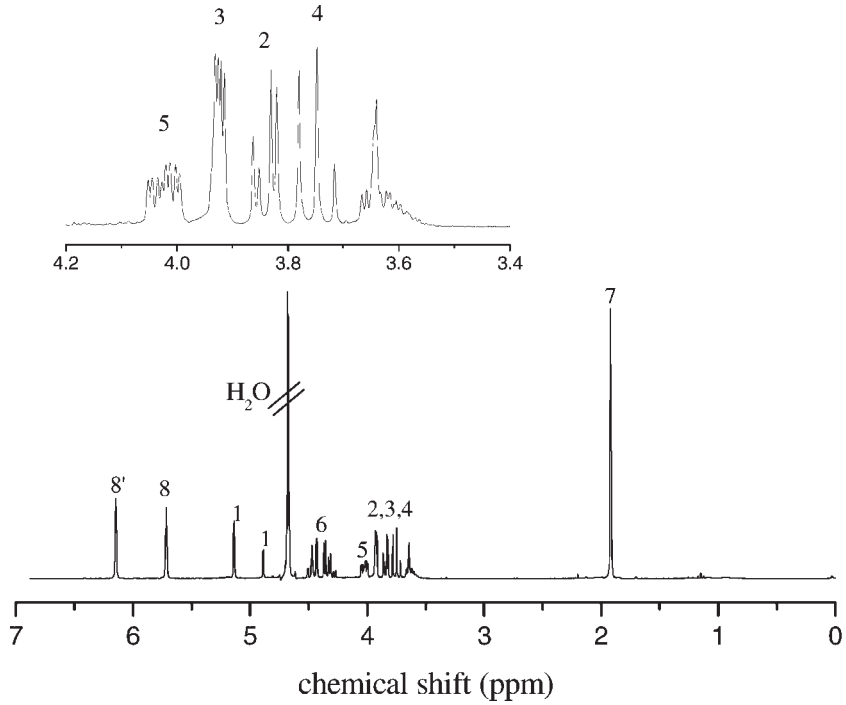


Figure 1.
^{1}H NMR of 6-*O*-methacryloyl mannose monomer. Peaks are labeled according to the numbering scheme depicted in the monomer synthesis reaction in Scheme 1.

experimentally obtained molecular weight for both the monomer minus hydrogen and the monomer plus acetate counter ion. The ESI-MS also showed two major higher molecular weight peaks which we attribute to monomer aggregates.

Once the monomer was successfully synthesized, RAFT polymerizations in

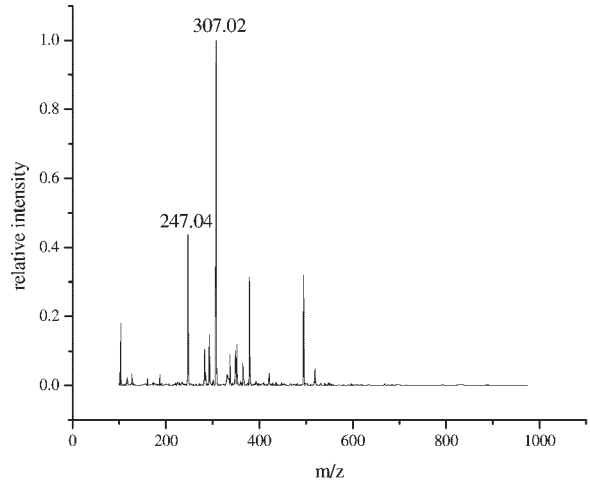


Figure 2.
Negative ion scan ESI-MS for 6-*O*-methacryloyl mannose monomer. The peak at 247.04 is indicative of M-proton (calc. 247.21), while 307.02 is indicative of M+counterion (calc. 307.24).

water-ethanol mixtures were performed to study the controlled polymerization kinetics. Unlike in the ATRP synthesis of glycopolymers,[21–22] RAFT polymerization of glycomonomers affords well-defined polymers without the use of protecting group chemistry. Recently, 4-cyanopentanoic acid dithiobenzoate (CPADB), a water soluble RAFT transfer agent, has been employed in the controlled, aqueous polymerization of glycomonomers.[23–24] Thus, this chain transfer agent was used for our polymerization systems as well as utilizing 4,4′-azobis(cyanopentanoic acid) (ACPA) as the water-soluble free radical initiator. RAFT polymerization kinetics were performed whereby the concentration of the monomer and the ACPA free radical initiator remained constant but the concentration of the CPADB varied so as to yield three different monomer to RAFT agent concentration ratios ($[M]_0$:$[CPADB]_0$ = 100:1, 200:1, and 300:1). Figure 3 shows the molecular weight as a function of percent conversion for each of the monomer to RAFT agent concentration ratios. As can be seen in the plot, the polymerizations exhibit a linear molecular weight increase as the polymerization progresses, which agree well with the theoretical molecular weights determined using Equation 1.

$$M_n = M_{monomer} * x * \frac{[M]_0}{[RAFT]_0} + M_{RAFT} \quad (1)$$

The molecular weight of the monomer and RAFT agent are represented by M_{monomer} and M_{RAFT}, respectively. The monomer conversion, x, was determined by dividing the peak area of the polymer by the sum of the polymer and monomer peak areas from the GPC traces.

Furthermore, the polydispersity for each of the polymerization reactions are below 1.14 and decreasing slightly during the course of the polymerization. This narrowing of the molecular weight during the course of the polymerization can also be seen in Figure 4. Monomodal GPC traces with a steadily decreasing peak width were observed for each kinetic run. At no time did the polymerizations yield a high molecular weight shoulder, indicative of bi-molecular termination. Also, the slope of the molecular weight evolution lines

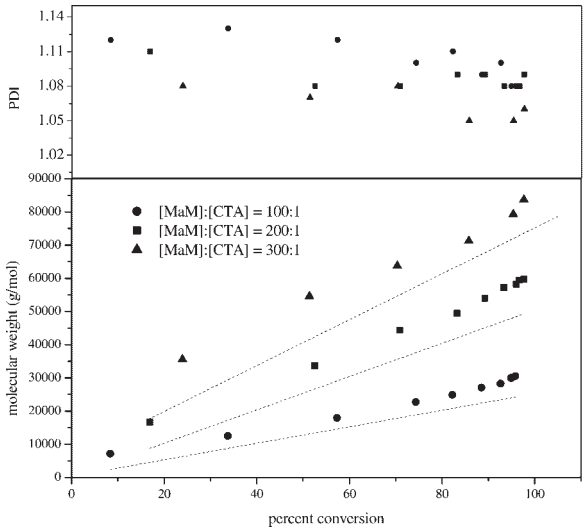


Figure 3.
Molecular weight evolution and polydispersity index as a function of percent conversion of 6-*O*-methacryloyl mannose monomer under RAFT polymerization conditions. Dotted line is representative of theoretical molecular weight.

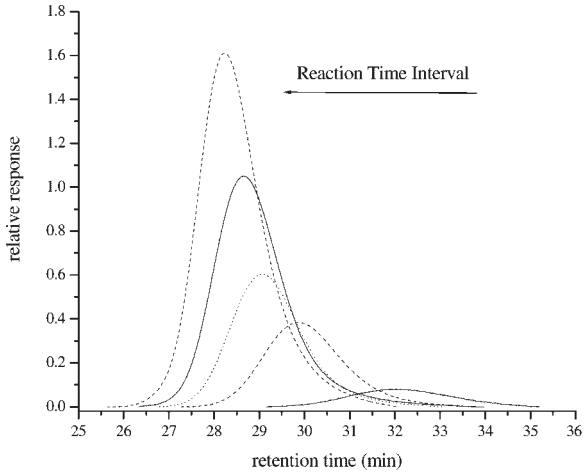


Figure 4.
Gel permeation chromatographs of PMaM evolution over the course of the kinetic run ([M]:[CTA] = 200:1).

increase as the monomer to RAFT agent ratio increases (Figure 3). In addition, the linear relationship between conversion and molecular weight is a further indication of the occurrence of a successful controlled radical polymerization. Typical for the RAFT process is also the delayed onset of the polymerization. An increasing inhibition time and increasing retardation with higher RAFT concentration is the result of the formation of a stable radical intermediate (Figure 5).

Protein Binding *via* Turbidimetric Assay

Glycopolymers are particularly interesting due to their functional similarity to naturally occurring glycoconjugates.[3] The binding of lectins to these glycoconjugates on cell surfaces tend to involve multiple attachment points.[30–31] Thus, by generating glycopolymers with specific protein binding properties, it could be possible to synthesize more effective drug delivery systems. Con A, a tetrameric lectin shown to have a binding affinity for mannose

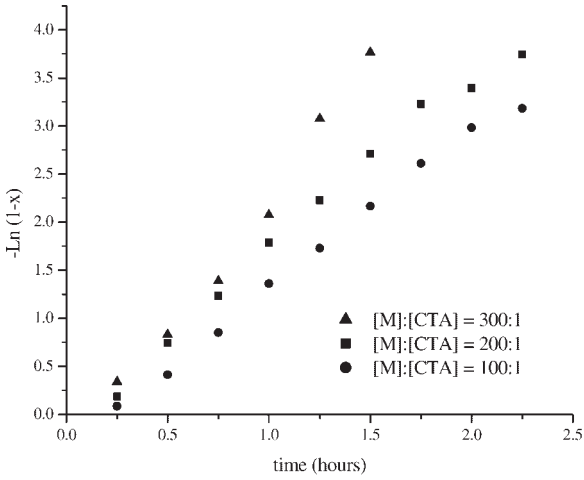


Figure 5.
First order kinetic plot for the RAFT polymerization of MaM monomer as a function of time.

moieties, has been shown to be an activator for cellular signaling events, such as cell adhesion, proliferation, and survival.[7] Several studies have been performed on the binding interactions between Con A and polymers[5–7,32] and dendrimers[29] containing mannopyranoside repeat units resulting. However, all of these polymers have linkages to the mannose residues through the C-1 carbons, as numerically depicted in Scheme 1. The monomer, and subsequent polymer, synthesized in this study is linked to the polymer backbone through the C-6 carbon. To test the binding ability of our PMaM polymer with Con A, a turbidimetric assay was performed.

The turbidity assay performed was similar to the one described by Cairo *et al.* in the literature.[7] Two HEPES buffer stock solutions (pH = 7.5) were made, one containing Con A and the other containing our synthesized PMaM sample. The PMaM solution was made so as to have a constant mannose residue concentration (1.5 mM of mannose units) regardless of the molecular weight of the glycopolymer sample being tested. Figure 6 shows the results for the turbidimetric assay with Con A in the HEPES buffer solution. As can be seen from the curves, the Con A lectin shows a strong absorbance at 280 nm, while the PMaM glycopolymer exhibits a rather broad absorbance at 310 nm. After mixing and waiting a few seconds to allow for cluster formation between the lectin and the mannose moieties, another spectrum was taken to monitor for an absorbance at 420 nm, which is indicative of this particular protein-carbohydrate interaction. Unfortunately, no absorbance was observed, even when the solutions were allowed to aggregate over an 8 h period.

Cairo *et al.* noted that the rate of cluster formation between Con A and mannose containing glycopolymers was dependent on the epitope density in the polymer.[7] They noted that the fastest rates, on the order of seconds and minutes, were for those glycopolymer samples containing the highest epitope densities. Since our samples are not copolymers with a Con A inactive glycomonomer, PMaM should respond similarly to the highest epitope density samples and register Con A cluster formation rates on the order of minutes. However, from Figure 6, it can clearly be deduced that PMaM does not interact with Con A. This would suggest that linking the mannose to the polymer backbone *via* the 6-carbon position of the mannopyranoside has altered the activity of the mannose and therefore the resulting glycopolymer. These findings fall in line with the computational work of Sekharudu *et al.*[32] and that of Goldstein[33] and Brewer[34] suggesting that the free hydroxyls at the 3-, 4-,

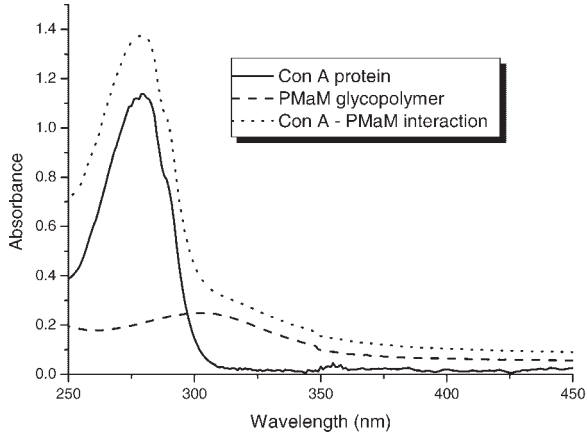


Figure 6.
UV-Vis spectra of PMaM glycopolymer and interaction with Con A protein – cluster formation between the mannose residues and lectin binding sites appears at roughly 420 nm.

and 6-carbon positions of mannose dictate the binding ability of Con A. Further investigations into this work for the development of an all mannose based glycopolymer with various Con A binding epitope density is currently being performed in our laboratory and will be the subject of future publications.

Conclusions

A simple route, using chemo-enzymatic synthesis, was used to generate 6-*O*-methacryloyl mannose glycomonomer. This method allows for the synthesis of glycomonomers in a single step without the need for tedious protection/deprotection chemistry. The successful, aqueous RAFT polymerization of this mannopyranoside monomer was performed to generate the desired glycopolymer. The protein binding ability of this glycopolymer using Con A lectin was studied using a turbidimetric assay. It was found that altering the 6-carbon position of the mannose residue to introduce methacryloyl functionality completely disrupted the protein-carbohydrate binding abilities.

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