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Glycopolymers Containing Hydrophobic Natural Compounds

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Résumé

Les glucides interagissent avec les lectines, des protéines liant les sucres, pour médier de nombreuses activités cellulaires telles que l'infection bactérienne, le transfert du signal cellulaire, l'adhésion cellulaire et la croissance. Les glycopolymères sont des polymères contenant des sucres. Ils peuvent se lier à des lectines ou à des surfaces contenant de la lectine et peuvent donc être utilisés pour la séparation et l'élimination des toxines et bactéries contenant des lectines et pour la reconnaissance des cellules cancéreuses. Dans ce travail, trois copolymères contenant du galactose ont été conçus et synthétisés en introduisant d'autres composés biologiques de nature hydrophobe tels que la bétuline et l'acide cholique pour donner des copolymères amphiphiles.

La bétuline est un composé naturel triterpénique ayant des activités anticancéreuses et antivirales. Son dérivé méthacrylate a été copolymérisé avec le glycomonomère par polymérisation RAFT pour préparer à la fois des copolymères statistiques et des copolymères séquencés. Leurs auto-assemblages forment des micelles. Les petites micelles formées par les copolymères statistiques ont facilité l'encapsulation du « Nile Red » et ont libéré davantage de ce composé hydrophobe. Ces glycopolymères interagissent avec une lectine se liant au galactose, RCA_{120}, et forment des agrégats. Les copolymères séquencés forment des agrégats plus gros à une vitesse plus rapide que les copolymères statistiques. Les glycopolymères à base de bétuline peuvent servir de supports biocompatibles pour la libération ciblée de médicaments.

Des glycopolymères séquencés portant des groupements latéraux de galactose, de dopamine et d'acide cholique (CA) ont été synthétisés par polymérisation RAFT. Ces copolymères se sont auto-assemblés en micelles dans un milieu aqueux. Les groupements de dopamine, situés au niveau du noyau micellaire, ont été auto-polymérisés dans une solution faiblement basique, stabilisant les micelles à la fois dans l'eau et dans les solvants organiques. Les micelles réticulées avaient une taille plus petite que les précurseurs avant la réticulation. L'introduction d'un plus grand nombre de groupes CA dans les copolymères a favorisé l'auto-assemblage pour former des agrégats plus grands, contrôlé le degré de réticulation des micelles et facilité l'encapsulation de composés hydrophobes. Les micelles réticulées principales présentent une libération lente mais soutenue du Nile Red et interagissent efficacement avec RCA_{120}, démontrant ainsi leur application potentielle pour la libération contrôlée de médicaments avec une propriété de ciblage.

L'oxydase de glucose (GOx) a une spécificité élevée pour le glucose, mais il peut également oxyder d'autres glucides. Cependant, il n'est jamais testé sur des glycopolymères synthétiques contenant des groupements de sucres. Les glycopolymères avec des groupements latéraux de galactose possédant des espaceurs PEG ont été synthétisés par une combinaison de polymérisations anionique et RAFT. L'oxydation enzymatique de ces glycopolymères par la GOx a été étudiée. Les sites actifs de l'enzyme sont devenus accessibles aux groupes pendants galactose attachés sur une chaîne de polymère via des espaceurs de PEG suffisamment longs. La reconnaissance des glycopolymères par la lectine peut également être facilitée par les espaceurs PEG. Les glycopolymères contenant des unités galactose oxydées ont montré une liaison réduite à la lectine RCA_{120}. C'est la première fois que de tels copolymères sont obtenus par conversion enzymatique.

Mots-clés: Glycopolymères, copolymères amphiphiles, micellisation, polymérisation RAFT, polymérisation anionique, oxydation enzymatique, galactose, glucose oxydase, acide cholique, bétuline.

Abstract

Carbohydrates interact with sugar-binding proteins, lectins, to mediate many cellular activities such as bacterial infection, cellular signal transfer, cell adhesion and cell growth. Glycopolymers are sugar-containing polymers. They can bind to lectins or lectin-containing surfaces and can thus be used in the separation and removal of lectin-bearing toxins and bacteria and in the recognition of cancer cells. In this work, three galactose-bearing copolymers were designed and synthesized by introducing biological compounds that are hydrophobic in nature, such as betulin and cholic acid to yield amphiphilic copolymers.

Betulin is a natural triterpene compound with anticancer and antiviral activities. Its methacrylate derivative was copolymerized with the glycomonomer by RAFT polymerization to prepare both random and block copolymers that self-assemble into micelles. The smaller micelles formed by the random copolymers facilitate the encapsulation of Nile Red and release more of this hydrophobic compound. These glycopolymers interact with a galactose-binding lectin, RCA_{120}, and form aggregates. The block copolymers form larger aggregates at a faster rate than the random copolymers. The betulin-based glycopolymers may serve as biocompatible carriers for targeted release of drugs.

Block glycopolymers bearing galactose, dopamine, and cholic acid (CA) pendants were synthesized by RAFT polymerization. These copolymers self-assemble into micelles in water. The dopamine moieties are located at the micellar core and self-polymerize in a weakly basic solution, stabilizing the micelles in both water and organic solvent. The crosslinked micelles are smaller in size than the precursors before crosslinking. Introducing more CA groups into the copolymers promote their self-assembly to form larger aggregates, control the degree of crosslinking of the micelles, and facilitate the encapsulation of hydrophobic compounds. The core-crosslinked micelles display a slow but sustained Nile Red release and interact effectively with RCA_{120}, demonstrating their potential application for the targeted release of drugs.

Glucose oxidase (GOx) has a high glucose specificity, but it can also oxidize other carbohydrates. However, it has never been tested on glycopolymers with pendant sugar groups. The glycopolymers with galactose pendants possessing PEG spacers were synthesized by a combination of anionic and RAFT polymerization. The enzymatic oxidation of these glycopolymers by GOx was studied. The active sites of enzyme become accessible to the galactose pendants attached on a polymer chain via PEG spacers that are sufficiently long.

Lectin recognition of the glycopolymers can also be facilitated by the PEG spacer. The glycopolymers containing oxidized galactose units show reduced binding to the lectin RCA_{120}. It is the first time such copolymers are obtained via enzymatic conversion.

Keywords: Glycopolymers, amphiphilic copolymers, micellization, RAFT polymerization, anionic polymerization, enzymatic oxidation, galactose, glucose oxidase, cholic acid, betulin.

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List of acronyms, abbreviations and symbols

[P]	Polymer concentration
Đ	Polydispersity
α-CD	α-Cyclodextrin
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) diammonium salt
$ABTS^{2-}$	2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonate) dianion
Ac	Acetyl
AC	Acryloyl cyclic carbonate
AIBN	2,2′-Azoisobutyronitrile
ATRP	Atom transfer radical polymerization
BSA	Bovine serum albumin
CA	Cholic acid
CL	ε-Caprolactone
CMC	Critical micelle concentration
ConA	Concanavalin A
CSACS	Centre for self-assembled chemical structures
CTA	*4*-Cyano-*4*-[(propylsulfanylthiocarbonyl)sulfanyl]pentanoic acid
DA	Dopamine
D_h	Hydrodynamic diameter
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
Dox	Doxorubicin

EC	Enzyme commission
E. coli	*Escherichia coli*
EO	Ethylene oxide
EO_nIpGa	IpGa with n units of ethylene oxide
FAD	Flavin adenine dinucleotide
FQRNT	Fonds de recherche du Québec - Nature et technologies
FRSQ	Fonds de recherche du Québec - Santé
Ga	Galactose
GDC	PMGA-*b*-P(DA-MCA)
GOx	Glucose oxidase
GRSTB	Groupe de recherche en sciences et technologies biomédicales
HIV	Human immunodeficiency virus
Ip	Isopropylidene
IpGa	*1,2:3,4*-Di-*O*-isopropylidene-*D*-galactopyranose
K_a	Binding constants
LCST	Lower critical solution temperature
MAA	Methacrylic acid
Mbet	Betulin-based methacrylate monomer
MCA	Methacrylate of the pegylated cholic acid (MCA)
MEO_nIpGa	Methacrylate of EO_nIpGa
MIpGa	*6-O*-Methacryloyl-*1,2:3,4-di-O*-isopropylidene-*D*-galactopyranose
MRI	Magnetic resonance imaging
MWCO	Molecular weight cut-off

Naph-K	Potassium naphthalene
NHS	*N*-hydroxysuccinimide
NMR	Nuclear magnetic resonance
NPA	*4*-Nitrophenyl acrylate
NPs	Nanoparticles
NR	Nile Red
NSERC	Natural Sciences and Engineering Research Council
P. aeruginosa	*Pseudomonas aeruginosa*
PB	Poly(*1,2*-butadiene)
PBA	Phenylboronic acid
PBS	Phosphate-buffered saline
PCL	Poly(*ε*-caprolactone)
PEG	Polyethylene glycol
PHPMA	Poly(*2*-hydroxypropyl)-methacrylamide
PLA	Polylactic acid
PMIpGa	Poly(*6-O*-Methacryloyl-*1,2:3,4-di-O*-isopropylidene-*D*-galactopyranose)
PNA	Peanut agglutinin
PNIPAm	Poly(*N*-isopropylacrylamide)
PPO	Poly(propylene oxide)
QCAM	Centre québécois sur les matériaux fonctionnels
RAFT	Reversible addition-fragmentation chain transfer
RCA_{120}	Ricinus communis (castor bean) agglutinin 120

ROP	Ring-opening polymerization
SEC	Size exclusion chromatography
SS	Disulfide bond
T_{cp}	Cloud point
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Tris	Tris(hydroxymethyl)aminomethane
UV-Vis	Ultraviolet-visible spectroscopy

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Chapter 1 Introduction

1.1 Amphiphilic copolymers and aggregation

The development of amphiphilic polymers is an important area of research in polymer chemistry.[1] They are often made by copolymerization of two or more types of monomers with different hydrophilicity or lipophilicity. Water tends to hydrate the hydrophilic portion of an amphiphile and exclude the hydrophobic portion.[2] Depending on the arrangement of repeat units, linear copolymers can be classified as random (or statistical), gradient or block copolymers (Figure 1.1).

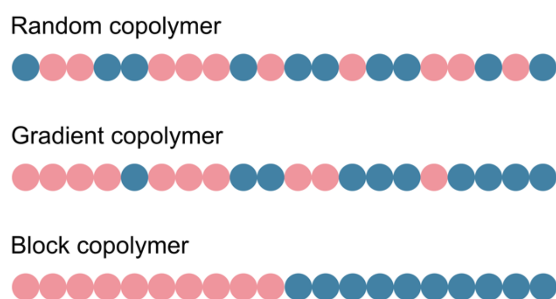

Figure 1.1 Monomers distribution along polymer chains as a function of position, illustrating random, gradient and block copolymers.

With the development of polymerization methods, recent advances in polymer chemistry allow the synthesis of well-defined copolymers with complex architectures. These methods include anionic and cationic polymerizations and the more recently developed living radical polymerization techniques including nitroxide-mediated radical polymerization (NMRP), atom transfer radical polymerization (ATRP), reversible addition-fragmentation chain transfer (RAFT) radical polymerization and ring-opening polymerization (ROP).[3]

Amphiphilic polymers can self-assemble spontaneously above a critical aggregation concentration in aqueous solutions.[4] The self-assembly occurs as an outcome of a delicate balance between attractive and repulsive interactions, such as hydrogen bonding, electrostatic and hydrophobic interactions.[5, 6]

Both theoretical[7-9] and experimental[10-12] research has been carried out on the self-assembly of amphiphilic copolymers, including the characterizations of the aggregates (critical aggregation concentration, aggregation number, size of aggregates) and the kinetics and

thermodynamics of aggregation. The aggregation process and the properties of the aggregates may be affected by the solvent quality, pH, temperature, and pressure, as well as by the repeat unit composition, molar mass, concentration and structure of the polymers. Techniques such as light and X-ray scattering and transmission and scanning electron microscopy have been used for the characterization of the aggregates.[13]

The micellization of block copolymers in water is better understood than that of random and gradient copolymers.[14-17] For block copolymers, the morphology of the aggregates depends on the relative volume ratio of the different blocks and on the packing parameter (p),

$$p = v/al \qquad (1)$$

where v is the hydrophobic volume, a is the interfacial area at the hydrophobic-hydrophilic interface and l is the chain length normal to the surface per molecule (Figure 1.2).[18, 19] This concept has been successfully used to predict and explain the formation of spherical micelles ($p \leq 1/3$), cylindrical micelles ($1/3 \leq p \leq 1/2$) and vesicles ($1/2 \leq p \leq 1$) depending on the copolymer volume fraction.

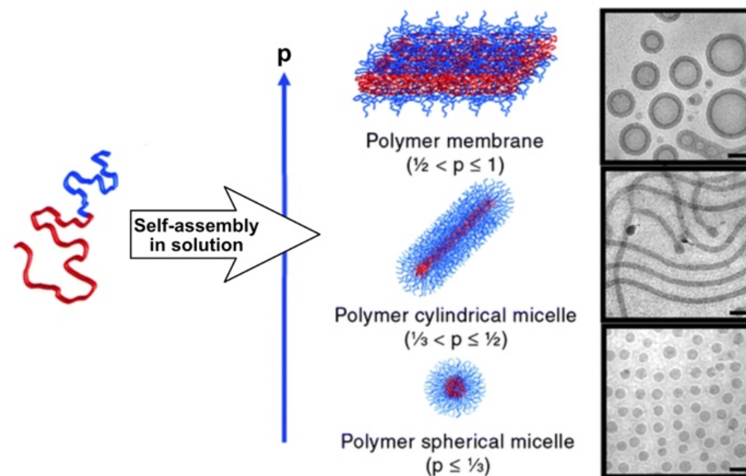

Figure 1.2 Schematic illustrating the self-assembly in solution of block copolymers in spherical and cylindrical micelles and vesicles and cryo-TEM micrographs of PB-*b*-PEG aggregates (scale bars = 100 nm).

1.2 Applications of amphiphilic copolymers

Amphiphilic copolymers have been incorporated into the surface of materials with potential for marine antibiofouling applications, without resorting to the addition of biocidal and toxic agents.[20] Amphiphiles can aggregate and co-assemble with other polymers and nanoparticles

to form hybrid materials[21-23] which may have potential applications in biosensing[24], food industry[25], and agrochemistry[26], and the removal of toxic compounds[27] and heavy metal ions[28].

Amphiphilic block copolymers comprising a biocompatible hydrophilic block and a bioactive hydrophobic block have been tested for use as drug carriers and for the incorporation and delivery of genes and enzymes.[29-31] The specific interactions of polypeptides and polysaccharides with target sites are important for the design of drug carriers for targeted release.[32]

1.3 Glycopolymers

Glycopolymers are synthetic polymers with pendant sugar groups. They can mimic the functions of carbohydrates in the binding and recognition of sugar-binding proteins, known as lectins. The first glycopolymers made by free radical polymerization were reported by Hrejsi et al. in 1978.[33] With the development of living/controlled radical polymerization methods, research on glycopolymers has progressed rapidly since the end of the 1990s. After the emergence of living/controlled radical polymerization methods, the fast increase in the number of publications on glycopolymers is shown in Figure 1.3. The design and synthesis of carbohydrate-containing copolymers and their potential applications are reviewed in Chapter 2.

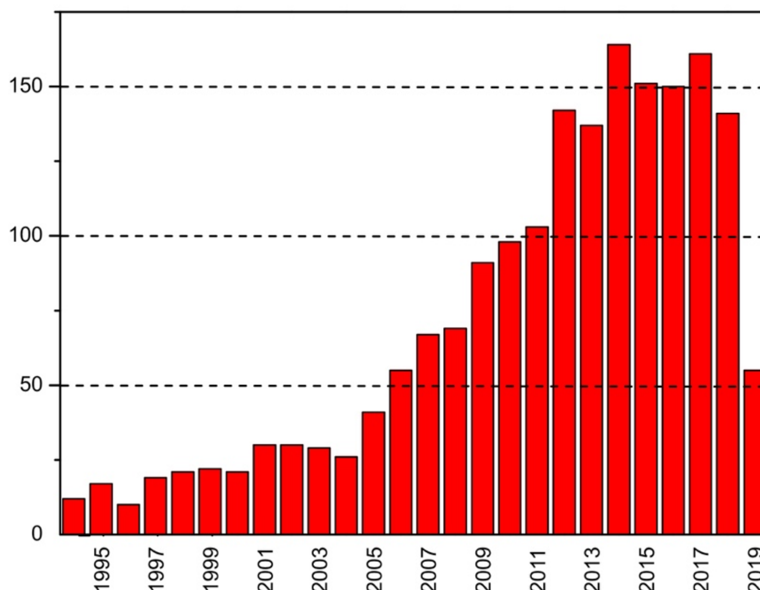

Figure 1.3 Publications per year on glycopolymers, based on a Web of Science search using the keyword “glycopolymers”. (April 2019)

1.4 Lectins

Lectins are a class of proteins that can reversibly bind to carbohydrates with high specificity. Hundreds of lectins have been identified and isolated from plants, animals or microorganisms. They are diverse in terms of structure and size and interact with different carbohydrates (Table 1.1).[34, 35] Concanavalin A (ConA, Figure 1.4), isolated from jack bean, has been widely studied since the 1970s.[35] ConA is a homo tetramer with four sugar binding sites. It has been revealed that the density of sugar residues and polymer composition play important roles in the amplification of sugar-ConA interaction by the "cluster effect".[36] Ricinus communis agglutinins (RCA) are the lectins produced by the beans of the castor tree. They are related with ricin, one of the most toxic lectins.[37] They can bind to galactose and its derivatives. Two RCAs with different numbers of sub-units have been widely studied.[38-40]

Table 1.1 Examples of carbohydrate recognition by lectins

Lectin	Carbohydrate
Concanavalin A (ConA)	mannose, glucosamine, glucose
Ricinus communis agglutinin 120, RCA_{120}	galactose, *N*-acetyl-*D*-galactosamine
Ricinus communis agglutinin 60, RCA_{60}	galactose, *N*-acetyl-*D*-galactosamine
Peanut agglutinin (PNA)	galactose
Sambucus nigra lectin	*N*-acetyl-*D*-galactosamine
Selectin	sialyl lewis^{X}
Galectin	galactose

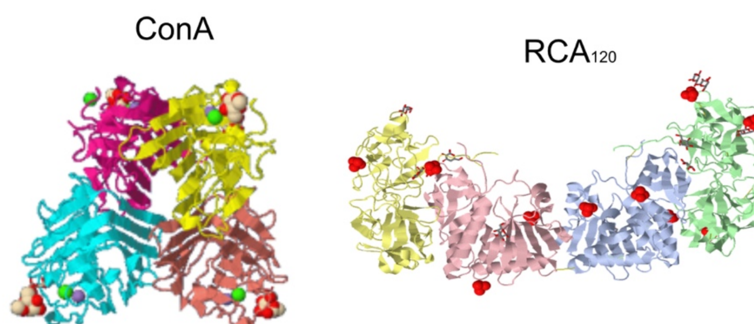

Figure 1.4 Structures of ConA and RCA_{120}.

Lectins have important roles to play in a variety of biological events. Some lectins are beneficial, such as CLEC11A which can facilitate bone growth[41], while some are toxic[42]. The interaction between lectins and sugars is the foundation of cell agglutination involving hemagglutination.[43] Lis and Sharon described the importance of recognition events involving lectins in the biological systems.[44] Ambrosi and coauthors highlighted lectins as tools for the glyco-code reader.[45] The 3D structures of the binding between lectins and carbohydrates have been studied.[46] The binding between oligosaccharides to their corresponding lectins is weak.[47] Their interactions are enhanced due to the "multivalency binding" of glycopolymers with a large number of sugar pendants along the polymer chain.[48] The choice of sugar, and molar mass and architectures of glycopolymers may all affect the lectin recognition and binding; their effects remain to be elucidated.

1.5 Critical solution temperatures

Polymers can manifest a change in solubility at certain critical temperatures, as shown in a typical phase diagram (Figure 1.5).[49] These include both the lower critical solution temperature (LCST) and upper critical solution temperature (UCST). Certain polymers are soluble in water below a critical temperature (T_c), due to hydrogen bonding between the polymer and water. Hydrogen bonds may be disrupted by raising the temperature, leading to the hydrophobic aggregation of the polymer and causing a phase separation. Poly(*N*-isopropylacrylamide) (PNIPAm, Figure 1.6) is one of the most-studied polymers possessing a LCST in water, first reported in the 1970s by Guillet.[50] More recently, PEG-based polymers have also been shown to exhibit varying LCST behaviors and attracted much interest due to their better biocompatibility.[51]

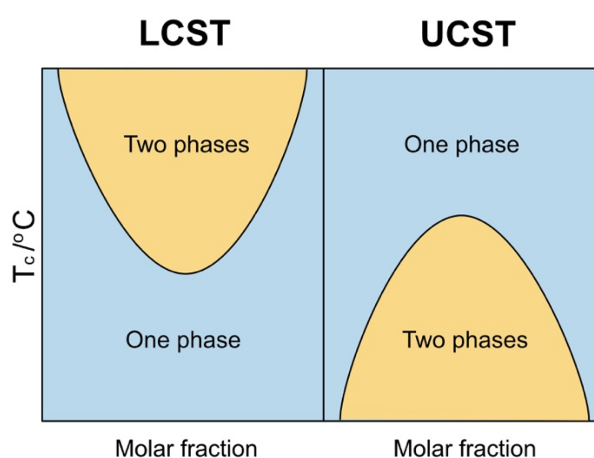

Figure 1.5 Typical phase diagrams of UCST and LCST polymers in aqueous solution.

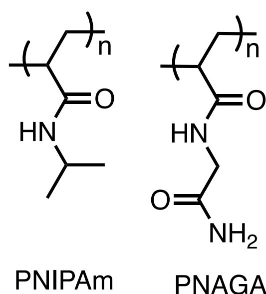

Figure 1.6 Structures of PNIPAm and PNAGA.

Similarly, if the intermolecular interactions are strong in a polymer (due to the formation of ionic bonds or hydrogen bonds), the polymer is insoluble in water and becomes soluble at the UCST, when the polymer-polymer interactions are disrupted in favor of the polymer-water interactions. A typical example of this kind of polymers is poly(*N*-acryloyl glycinamide) (PNAGA, Figure 1.6).

1.6 Objectives and the scope of this thesis

Certain natural biocompounds that are hydrophobic in nature can be incorporated into the primarily hydrophilic glycopolymers, making them amphiphilic with special properties and functions. It is also of interest to convert glycopolymers into copolymers by green chemistry methods using enzymes.

The main objective of this thesis is to design and synthesize new amphiphilic glycopolymers bearing natural compounds, to study their micellization and lectin binding. To this end, galactose-containing copolymers have been successfully synthesized. Betulin-containing monomers were copolymerized with glycomonomers by RAFT polymerization to yield both random and block copolymers bearing betulin pendants. Their binding properties with lectin have been studied and compared. To stabilize the micelles formed by the copolymers bearing galactose and cholic acid residues, dopamine was incorporated into the hydrophobic core to crosslink the micelles by self-polymerization. Finally, glycopolymers bearing PEGylated galactose pendants were synthesized by a combination of anionic and RAFT polymerizations. The enzymatic oxidation of glycopolymer by glucose oxidase yielded random sugar-containing copolymers with oxidized sugar residues.

This thesis consists of six chapters, including this general introduction and a general conclusion at the end. All the work presented has been performed by the author of this thesis under the supervision of Professor Julian Zhu. In certain cases, other co-workers in our group

participated or helped and characterization of the polymers and are listed as co-authors in the publications. Dr. Yongguang Jia was a postdoctoral fellow in our group. He helped in the synthesis of glycopolymer bearing betulin pendants by RAFT polymerization, who is a coauthor of the first research work. Alexander Cunningham helped in the anionic polymerization of PEGylated glycopolymers, who is a coauthor of the third research work.

Chapter 2 reviews the synthesis, properties and selected applications of carbohydrate-containing random and block copolymers. It provides the background and up-to-date report on the progress in this area. (Z. Ma and X. X. Zhu, Copolymers containing carbohydrates and other biomolecules: Design, synthesis and applications. *Journal of Materials Chemistry B*, **2019**, 24, 1361-1378.)

Chapter 3 describes the copolymerization of glycomonomers and betulin-bearing monomers by RAFT polymerization to yield both random and block copolymers. The copolymers can form micelles into which Nile Red can be incorporated. They can interact with galactose-binding lectins. The block copolymers tend to form larger clusters at a faster rate than the random copolymers. This paper has been published in *Biomacromolecules*. (Z. Ma, Y-G. Jia, and X. X. Zhu, Glycopolymers bearing galactose and betulin: Synthesis, encapsulation, and lectin recognition. *Biomacromolecules*, **2017**, 18, 3812-3818.)

Chapter 4 describes the synthesis of block glycopolymers bearing galactose, dopamine, and cholic acid pendants by RAFT polymerization. The dopamine moieties located in the hydrophobic core can self-polymerize in a weakly basic solution, stabilizing the micelles in both water and DMSO. The cholic acid comonomers were added to control the crosslinking density, which affects the loading and release of hydrophobic compounds. The core crosslinked micelles displayed a slow but sustained release of encapsulated compounds and interact effectively with lectin. This paper has been published in *Molecular Pharmaceutics* (Z. Ma and X. X. Zhu, Core-crosslinked micelles made of glycopolymers bearing dopamine and cholic acid pendants. *Molecular Pharmaceutics,* **2018**, 15, 2348-2354.)

Chapter 5 presents the enzymatic oxidation of glycopolymers bearing PEGylated galactose as pendant groups by glucose oxidase (GOx). The active sites of GOx become accessible for the galactose pendants on glycopolymers with PEG spacers that are sufficiently long. Lectin recognition of the glycopolymers was also influenced by the PEG spacer. The glycopolymers containing oxidized galactose units showed reduced binding to the lectin RCA_{120}. This paper has been submitted for publication. (Z. Ma, A. Cunningham and X. X. Zhu, Enzymatic

conversion of galactose-polymer into copolymers containing galactonic acid by glucose oxidase. To be resubmitted for publication)

Chapter 6 is a general conclusion of the work carried out in this thesis project with suggestions for future work.

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Chapter 2
Copolymers containing carbohydrates and other biomolecules: Design, synthesis and applications*

Abstract

Glycopolymers are synthetic polymers containing pendant carbohydrate groups. Other biological compounds can be incorporated into the glycopolymers to prepare both random and block copolymers aimed for bio-related applications: boronic acid can be introduced as a functional group to obtain crosslinked hydrogels; cholesterol and bile acids can be introduced to render the polymers more hydrophobic for the formation of micelles. Sugar-containing block copolymers with biocompatible blocks such as polypeptides, poly(ethylene glycol), poly(lactic acid) and poly(ε-caprolactone) were also prepared for potential applications as drug carriers. These glycopolymers interact with lectins or lectin-containing surfaces as natural carbohydrate mimics. This review highlights the recent progress in the synthesis of random or block glycopolymers. Examples of the bio-related applications of the glycopolymers in the separation and removal of toxins and bacteria, tumor cell recognition and glucose-responsive insulin delivery are presented and discussed.

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*Z. Ma and X. X. Zhu, "Copolymers containing carbohydrates and other biomolecules: Design, synthesis and applications". *J. Mater. Chem. B*. **2019**, 7, 1361-1378

2.1 Introduction

Polyesters and polyoxiranes are widely used in a variety of applications. Poly(ethylene glycol) (PEG) is by far the most popular polyester used as the biocompatible hydrophilic block.[1] However, it has shortcomings such as the lack of targeting ability without functionalization,[2] shielding of the functional groups in the shell,[3] and allergic reactions of certain population.[4] Synthetic polymers with pendant sugar groups are emerging as alternatives in biomedical and pharmaceutical uses in drug delivery[5] and in the filtration of bacteria[6, 7] from water through the interactions with sugar-recognizing surface groups due to cell recognition between the sugar and membrane receptors known as lectins. This specific recognition gives glycopolymers an advantage to serve as the shell of polymeric micelles by improving their targeting capability. These applications require controls over the polymer architecture and distribution of sugar units. Obtaining well-defined copolymers of sugar-containing monomers with other monomers is a challenge due to the low solubility of glycomonomers in common organic solvents, limited availability of monomers, and difficulties in achieving high yields.[8, 9]

The definition of glycopolymers has not yet been clearly established. In a broad sense, glycopolymers include functionalized natural polysaccharides. Polysaccharides, known as glycans, are natural macromolecules that are composed of long chains of sugar linked together by glycosidic bonds with different structures ranging from linear to branched. They are stable, non-toxic, biodegradable and abundant in nature.[10] Along with their ubiquity, polysaccharides display a wide range of biological functions from acting as an energy source (starch) to providing structural materials (cellulose and chitosan). To enhance their bioavailability or to endow polysaccharides with special bioactivities, numerous polysaccharide derivatives have been obtained through chemical and enzymatic modifications.[11-13] The development of hybrid materials based on natural polysaccharides has been reviewed by Garcia-Valdez *et al*.[14] and Meng and Edgar.[15] In a narrower sense, glycopolymers refer to synthetic polymers containing sugar moieties that act as specific biological functional groups, capable of mimicking the structural and functional features of polysaccharides, thanks to the variations in branching, anomeric status, linkage positions and specific substitutions.[16] In this review, glycopolymers refer to synthetic polymers with sugar pendants, including linear and branched polymers as well as dendrimers.

Glycopolymers can be prepared by either the polymerization of glycomonomers (sugar-containing monomers) or the post-modification of precursors with sugar-bearing reagents. The

latter is simple and convenient, making use of thiol-ene, azide-alkyne, and amidation chemistry, *etc*., as reviewed previously.[17] With the development of new polymerization techniques, the synthesis of glycopolymer has witnessed rapid progress. Linear copolymers or glycodendrimers have been made and their synthesis and properties have been reviewed by Ladmiral *et al*.[18] and Okada.[19]

The inclusion of other natural biological compounds besides carbohydrates introduces interesting biological and chemical properties, making the copolymers suitable in various applications. In this paper, we review the recent research progress on both random and block copolymers containing sugar moieties and other biocompounds, including their synthesis, properties and potential applications.

2.2 Design and synthesis of glycopolymers

2.2.1 Glycomonomers

Since carbohydrates possess various functional groups, sugar chemistry always requires tedious multi-step protection and deprotection reactions. Sugars in their pyranose forms are the most stable, therefore the glycomonomers are commonly synthesized from the sugar in their pyranose form as a recourse to the protecting chemistry. Acetyl (Ac) and isopropylidene protection are widely used, and Figure 2.1 shows a selection of protected glycomonomers.

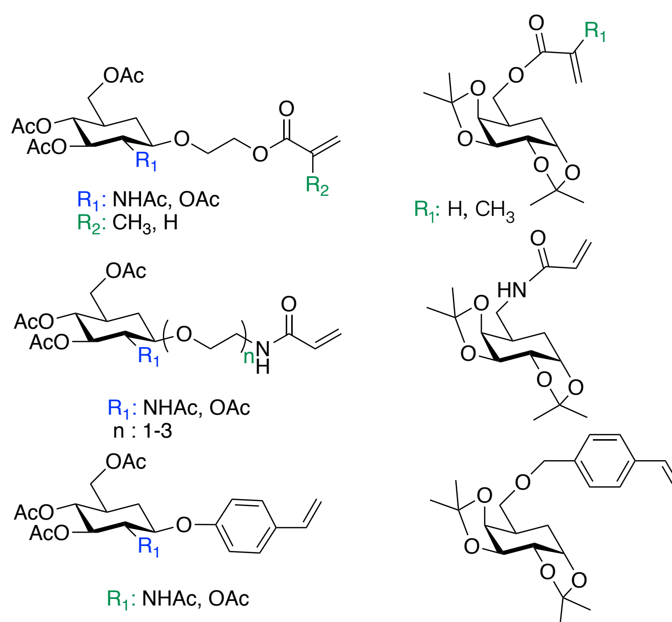
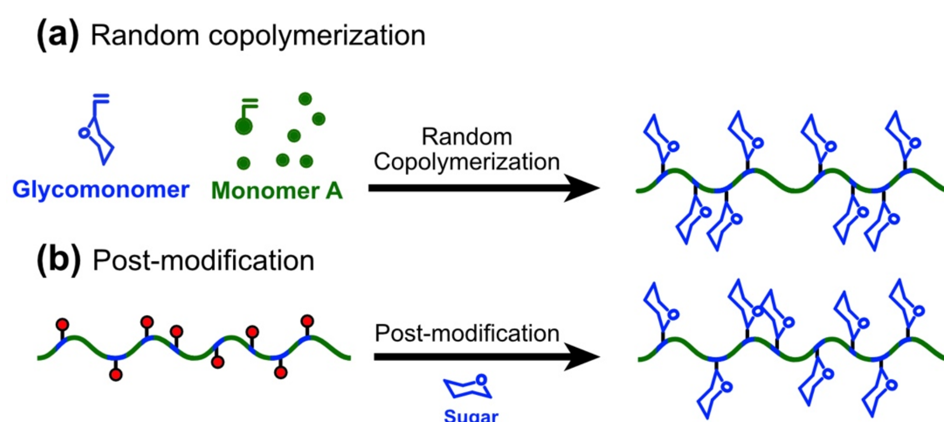


Figure 2.1 Chemical structures of representative glycomonomers with protecting groups.

Glycopolymers may be obtained by polymerization of the protected monomers and then the deprotection of the resulting copolymers, known as the post-deprotection technique. However, the disadvantage of post-deprotection is the difficulty in the quantitative removal of protecting groups on sugar residues. Incomplete deprotection of sugar moieties may introduce hydrophobicity and reduce their biological activity. Glycopolymers may also be synthesized by polymerization of deprotected monomers, which led to better-defined glycopolymers.[20] They have higher molar mass with relative lower polydispersity.[21] Glycomonomers were also made by other methods, including active ester transformation[22] and click reactions.[23, 24]

2.2.2 Random copolymers

Scheme 2.1 Representative syntheses of sugar-containing random copolymers using (a) random copolymerization and (b) post-modification of precursor random copolymers.



Random or statistical copolymers can be made by free radical polymerization (Scheme 2.1a). Glycopolymers with pendant phenylboronic acid groups were synthesized. Such polymers self-assembled to form nanoparticles (NPs) with a narrow size distribution using the nanoprecipitation method.[25] Insulin was loaded into such NPs with a loading capacity of *ca.* 10% and its release was correlated with the glucose concentration, suggesting their potential use as a self-regulated insulin delivery system. Well-defined cationic glycopolymer-based gene carriers in both random and block structures were synthesized by RAFT polymerization.[26] The random copolymers showed lower toxicity and higher gene expression than the corresponding diblock copolymers. Increasing the number of sugar residues in the copolymers resulted in a decreased transfection efficiency. Thermo-responsive glycopolymers were synthesized by RAFT polymerization. The cloud point (T_{cp}) is strongly affected by the polymer structure and composition.[27] The T_{cp} changed more drastically with the glycomonomer content in the random

copolymers, but only slightly in the block copolymers. The glycopolymers that can bind to lectins are useful for carbohydrate-protein or carbohydrate-cell interaction studies. *4*-Acrylamidophenyl α-mannoside, *4*-acrylamidophenyl *N*-acetyl-β-glucosamine and acrylamide were copolymerized via RAFT polymerization.[28] The thiol-terminal functionalized copolymers were prepared and grafted to gold NPs (Figure 2.2), which aggregated on the addition of saccharide-binding proteins (lectins).

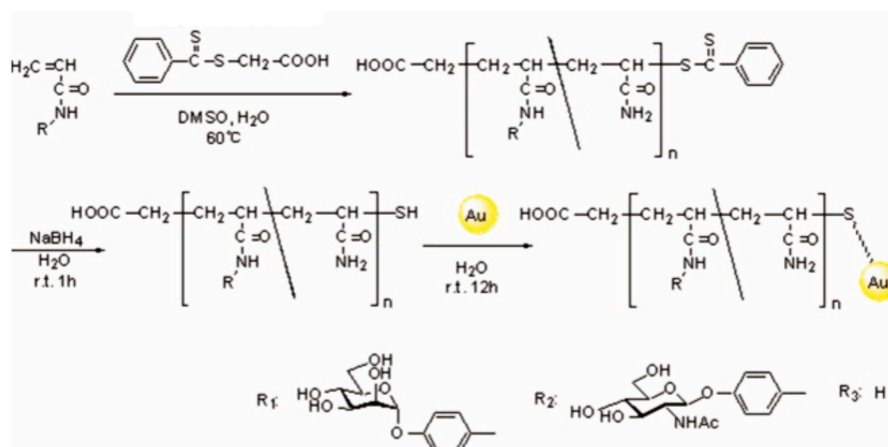


Figure 2.2 Preparation of glycopolymer-grafted gold NPs.

A series of glycopolymers based on polynorbornene[29] and polycyclooctene[30] backbone (Figure 2.3) were synthesized by ring-opening polymerization (ROP). These glycopolymers were found to self-assemble into NPs, micelles, vesicles, or tubular aggregates because of the hydrophobicity of the polymer backbone. The appropriate choice of polymer backbone for optimal cellular activation is dependent on the scaffold structure as well as the pendant sugars.

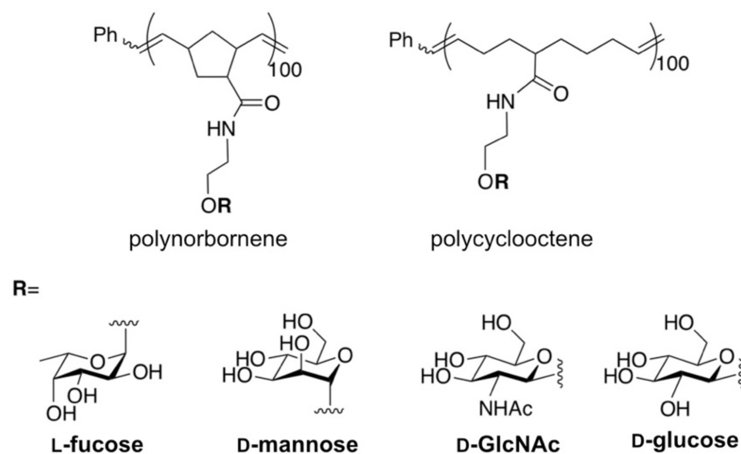


Figure 2.3 Structures of glycopolymers based on polynorbornene and polycyclooctene backbones synthesized by ROP.

Polypeptides are synthetic polymers that mimic natural proteins and have been widely used in various bioapplications such as drug/gene carriers and in tissue engineering.[31, 32] Copolymers with polypeptide segments formed various aggregate structures depending on their molecular design. ROP allowed the synthesis of random glycopolypeptides as well, such as those made of *6*-deoxy-*6*-azido-glyco-*N*-carboxyanhydride by Shaikh *et al*.[33] The azide functionality allowed for the easy attachment of bioactive groups or fluorescent probes by a click reaction. The charged glycopolypeptides showed a helical conformation in water.

The preparation of random copolymers by post-modification is simpler and more convenient (Scheme 2.1b). Random glycopolymers bearing *T*-antigens were synthesized via amidation of *N*-hydroxysuccinimide (NHS)-functionalized polymers with aminated carbohydrate ligand, demonstrating hetero-bifunctional antigenicity toward peanut agglutinin (PNA, a lectin) and streptavidin.[34] The carbohydrate ligands had different spacer lengths, and their binding to PNA decreased for glycopolymers bearing shorter spacers, due to making the sugar less accessible to the PNA binding sites. Cerrada *et al*. used a commercially available ethylene-vinyl alcohol copolymer modified with *4*-nitrophenyl chloroformate followed by reaction with different amino-saccharides to obtain random glycopolymers (Figure 2.4).[35] The glycopolymers were water-soluble and showed specific interaction to either Concanavalin A (Con A, a lectin) or Ricinus communis agglutinin (RCA, a lectin) depending on the attached sugars. Moog *et al*. obtained four different highly specific selectin-binding glycopolymers based on the sugar-containing poly(*2*-hydroxypropyl methacrylamide) random copolymers by a similar method using poly(pentafluorophenyl methacrylate)s.[36] These glycopolymers containing either the sialyl-Lewis^{X} (a tetrasaccharide composed of a fucose and an *N*-acetyllactosamine) or the individual carbohydrates fucose, galactose, and sialic acids strongly bound to different types of selectins. Selectin are cell adhesion proteins considered to be a type of lectin. Glycopolymers with 3 types of monosaccharides can achieve comparable binding affinities *in vitro* and can also significantly inhibit the migration of macrophages. Similar random glycopolymers with tyramine-*O*-sulfate groups were synthesized by the same group.[37] The sulfated counterparts induced a decrease in infiltrating and residing in macrophages, increased T helper cells, and aggravated immune-mediated liver injury.

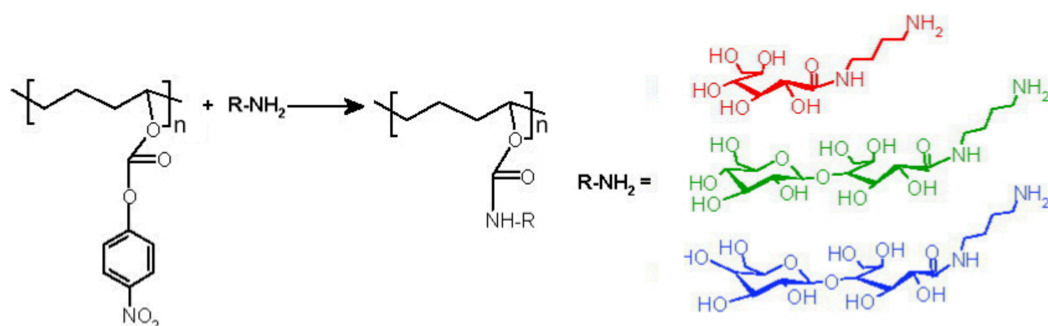


Figure 2.4 Preparation of glycopolymer by amidation of the *4*-nitrophenyl-functionalized precursor polymers with amino sugar ligands.

Sugar-containing polypeptides synthesized by post-modification of functionalized polypeptides were also reported. Random glycopolypeptides bearing *L*-glutamate and glucosylated *L*-/*DL*-allyl- or *DL*-propargylglycine were synthesized by ROP and thiol-ene reaction (Figure 2.5).[38] All samples adopted a random-coil conformation in neutral and basic media and an α-helical conformation in acidic media; the helical content depended on the number and configuration of allyl-/propargylglycine units. Turbidity assays provided evidence for the selective binding of the polymers to Con A. Similar pH- and thermo-responsive random glycopolypeptides were synthesized by the same group.[39] The formation of the helical structure was strongly affected by pH- and temperature changes in the media. Amphiphilic random glycopolypeptides were synthesized by a combination of ROP and click chemistry.[40] These copolymers formed clusters with diameters between 250 and 300 nm in water and had both hydrophobic and hydrophilic domains as evidenced by the incorporation of hydrophobic and hydrophilic dyes. The spherical clusters containing 10 and 20% mannose were confirmed to be surface bioactive and were found to interact with Con A.

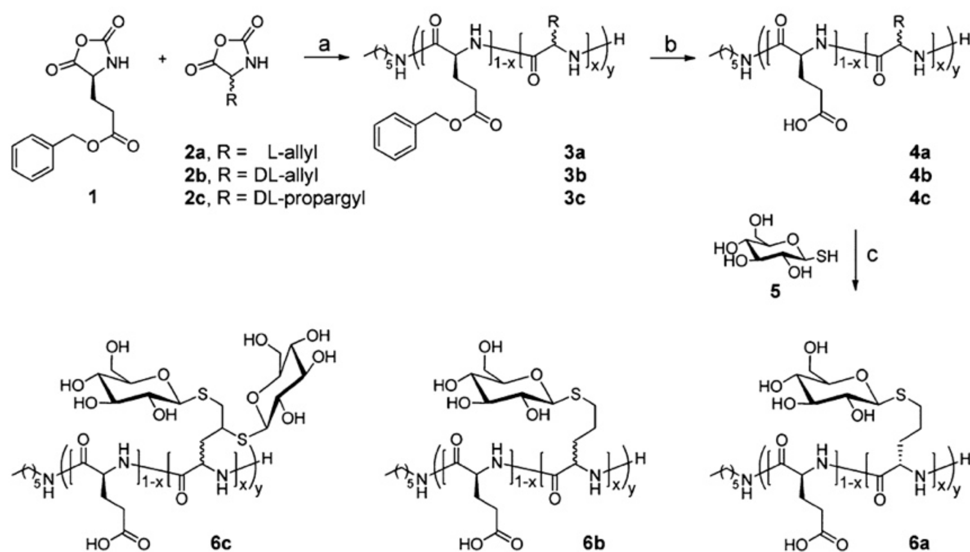
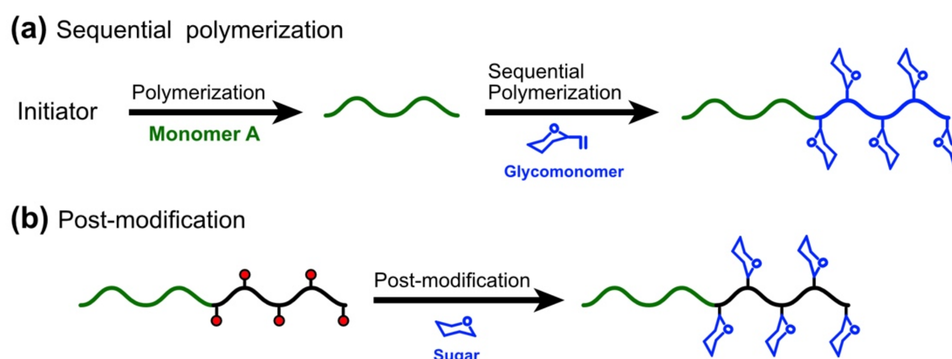


Figure 2.5 Preparation of glycopolymer by thiol-ene reaction of allyl-containing poly-peptides with *1*-thio-*β*-D-glucopyranose.

2.2.3 Block copolymers

Block copolymers have properties characteristic of the corresponding homopolymer blocks as well as new properties due to the connectedness of the blocks. As shown in Scheme 2.2, the block copolymers can also be synthesized either by the sequential addition of two or more polymerizable monomers during polymerization, or by the post-modification of precursor-functionalized block copolymers. The latter method has been accomplished though different chemical means and can be expanded to the preparation tri- and multi-block copolymers as well, but well-defined block polymers are hard to obtain because of incomplete reactions caused by steric hindrance, as will be indicated partially hydrophobic in nature. Multi-block copolymers can be prepared by living/controlled sequential polymerization.

Scheme 2.2 Representative synthesis of diblock copolymers using (a) the living/controlled sequential copolymerization and (b) the post-modification of functionalized block copolymer precursors.



Poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) are among the most used polymers to prepare biodegradable and biocompatible materials since they can be hydrolyzed and metabolized in the body. A hydrophilic glycopolymer block was attached on a PLA derivative bearing a NHS terminal functional group through active ester chemistry.[41] Such block copolymers were assembled into NPs through a nano-precipitation method, which exhibited good affinity to Con A.

The first example of block copolymers was prepared by a combination of ROP of CL and ATRP of methacrylate isopropylidene-protected galactose.[42] Then biomimetic star-shaped PCL-*b*-glycopolymers were synthesized by the same method.[43] The degree of crystallization of the PCL segment within the copolymers decreased with increasing glycopolymer length. The glyco-aggregates changed from spherical to worm-like, then to vesicles with decreasing weight fraction of the hydrophilic sugar block. PCL-based triblock copolymers with polypseudorotaxane and glycopolymer segments were synthesized by a similar procedure using polypseudorotaxane made by the inclusion complexation of PCL with α-cyclodextrin (α-CD) as a macroinitiator.[44] The self-assembled aggregates formed by these triblock copolymers have a hydrophilic glycopolymer shell and a polypseudorotaxane core, which changed from spherical micelles to vesicles with decreasing weight fraction of the glycopolymer segments.

Block copolymers can be prepared by a combination of sequential polymerization and post-modification chemistry. Sequential ROP of acryloyl cyclic carbonate (AC) and ε-CL were used to afford biodegradable PAC-*b*-PCL block copolymers, then thiolated sugars were attached though a thiol-ene reaction to yield glycopolymer-*b*-PCL with different sugar functionalities

(Figure 2.6).[45] Glyco-micelles with tailored sugar functionalities showed high doxorubicin (Dox) loading capacity, and high uptake by the asialoglycoprotein receptor of HepG2 liver cancer cells, becoming promising candidates for liver cancer chemotherapy. PCL-*b*-glycopolypeptides with disulfide linkages were synthesized from a diblock copolymer with an azidoethyl functionality segment onto which galactosyl and lactosyl groups were conjugated via a click reaction.[46] These reduction-sensitive block glycopolymers showed specific recognition with the lectin from Triticum Vulgaris (wheat) and significant potential as theragnostic nanocarriers for magnetic resonance imaging (MRI) and chemotherapy. PCL-*b*-glycopolymer blocks were synthesized via a combination of ROP, RAFT polymerization, and reactive ester-amine reaction. They self-assembled into micelles to encapsulate Dox and to conjugate with Con A.[47] The Dox-loaded micelles exhibited mucoadhesive property and enhanced in vitro cytotoxicity against UMUC3 human urothelial carcinoma cells, useful in intravesical therapy of non-muscle invasive bladder cancer.

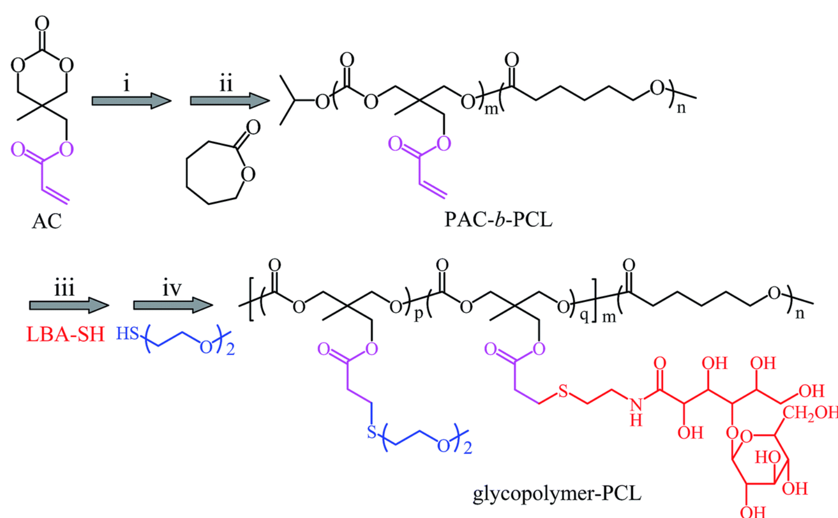


Figure 2.6 Synthesis of block glycopolymers by ROP of AC and ε-CL, followed by post-polymerization modification with thiolated lactobionic acid (LBA) and 2-(2-methoxyethoxy) ethanethiol through the thiol-ene reaction.

PEG is a widely used nonionic polymer for bio-related uses, due to its non-toxicity, non-immunogenicity, resistance to protein absorption and high aqueous solubility. A wide range of sugar-based block copolymers were prepared using macroinitiators based on PEG, poly(propylene oxide) (PPO) or PCL and by sequential addition of glycomonomers or other methacrylic monomers via ATRP.[48] PEG-*b*-poly(*2*-acryloxyethyl-galactose) were synthesized successfully by ATRP, then boronated with poly(*3*-methacrylamido phenylboronic acid) to form crosslinked nanoparticles by the complexation of phenylboronic acid and *cis*-diol-

containing compound.[49] The aggregation and precipitation of the NPs were observed at pH 5 and 6, while the particle size increased significantly when the pH was raised to 7. The NPs also showed clear sensitivity to glucose, galactose, mannose, and sucrose in a weakly basic solution. PEG-*b*-poly(*2*-acryloxyethyl galactose)-*b*-poly(acrylic acid) was prepared by sequential ATRP, in order to develop sugar-decorated crosslinked micelles with cystamine cores for targeted delivery and release of Dox into liver tumor cells.[50]

Block copolymers can be achieved by the post-modification chemistry of block copolymer precursors with PEG segments. Anionic ring-opening copolymerization of ethylene oxide with glycidyl propargyl ether afforded a block copolymer precursor, and then copper(I)-catalyzed azide-alkyne cycloaddition with mannopyranosyl azide led to PEG-based glycopolymers which bound to Con A due to their multivalent character.[51] Double hydrophilic block copolymers containing a PEG segment and a glycopolymer segment were synthesized by RAFT polymerization and the azide-alkyne cycloaddition reaction (Figure 2.7).[52] These block copolymers self-assembled into spherical micelles in aqueous solutions via hydrogen bonding and coordination with Ca^{2+}.

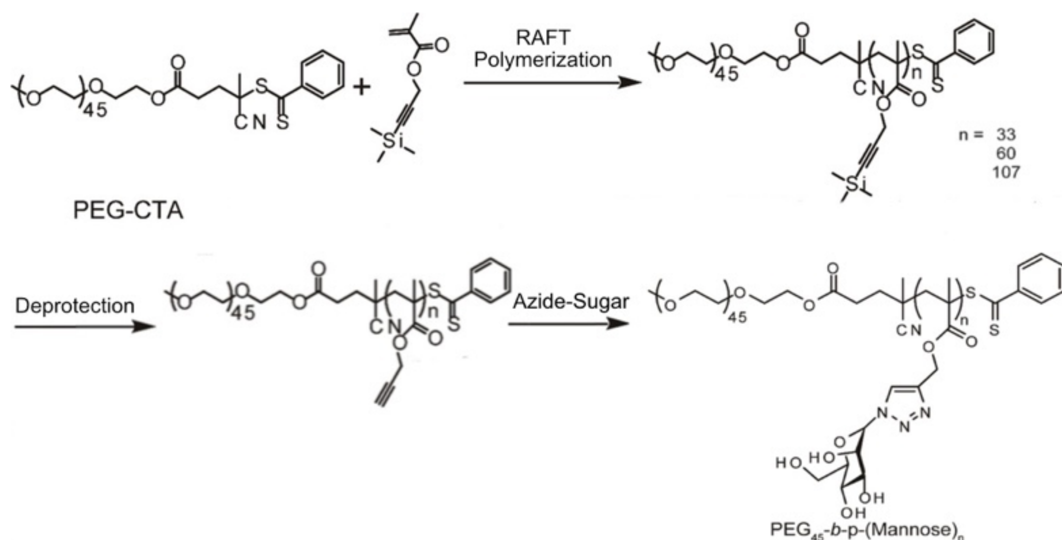


Figure 2.7 Synthesis of block copolymers containing a PEG segment and a glycopolymer segment via RAFT polymerization and the azide-alkyne cycloaddition reaction.

Polymers with oligo(ethylene glycol) side-chains showed promising potential in biomedical fields because of their adjustable thermo-responsiveness, nontoxicity, and biocompatibility.[53-55] Thermoresponsive double hydrophilic diblock copolymers poly(*2*-(*2'*-methoxyethoxy)ethyl methacrylate-*co*-oligo(ethylene glycol) methacrylate)-*b*-poly(*6*-*O*-methacryloyl-*D*-galacto pyranose) with various compositions and molecular weights were obtained by sequential

RAFT polymerization.[56] These block copolymers were thermo-responsive and underwent micellization to encapsulate hydrophobic compounds above their T_{cp}. The micelles dissociated into unimers below their T_{cp} and the entrapped compounds were released. Double hydrophilic block glycopolymers composed of a thermo-responsive oligo(ethylene glycol)-based block and a galactose-functionalized block (Figure 2.8A) were synthesized by RAFT polymerization.[57] These block copolymers showed a lower critical solution temperature (LCST)-type phase transition, depending on their concentration and structure (Figure 2.8B). Various morphologies of the aggregates were observed including spheres, vesicles, and ellipsoids, and showed hepatoma-targeting properties.

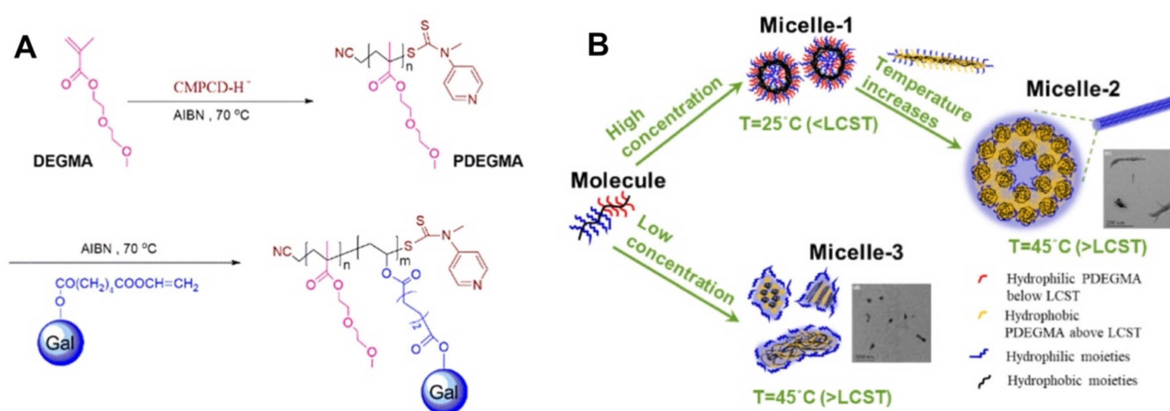
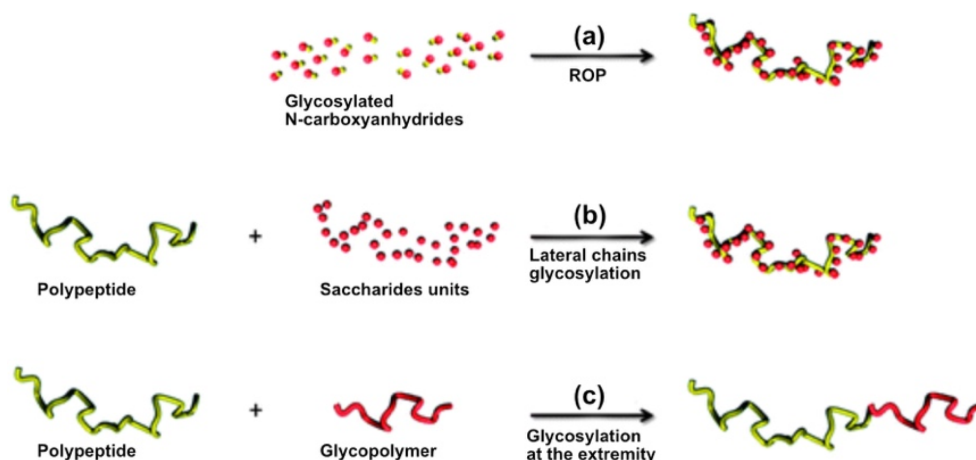


Figure 2.8 (A) Synthesis of thermo-responsive block copolymers containing oligo(ethylene glycol) side-chains via sequential RAFT polymerization and (B) their self-assembled micelles: micelle-1 at [P] = 0.20 mg mL^{-1} and 25 °C; micelle-2 at [P] = 0.20 mg mL^{-1} and 45 °C; micelle-3 at [P] = 0.02 mg mL^{-1} and 45 °C. The insets sketch the possible structures of the micelles.

Lecommandoux *et al*. summarized the methods to prepare synthetic block glyco-polypeptides (Scheme 2.3): (a) ROP of glycosylated monomers, (b) glycosylation of the synthetic polypeptides, (c) glycosylation at the end of the polypeptides.[58] They reported the sequential ROP of benzyl-*L*-glutamate and propargylglycine *N*-carboxy anhydrides to afford block polypeptides with different segments, then the glycosylated block was obtained by click reaction using azide-functionalized galactose.[59] Depending on the block polypeptides composition and the self-assembly protocol, the morphology of the structures formed ranged from worm-like micelles to vesicles. The same method was used to prepare iminosugar-based block glycopolypeptides[60] and the "tree-like" amphiphilic glycopolypeptides by attaching oligo-saccharides.[61] Wang and colleagues prepared a series of diblock glycopolypeptide

analogues with a redox-responsive disulfide linkage by a similar method.[62] The block copolymers formed micelles with α-helical polypeptides in the cores.

Scheme 2.3 Preparation of synthetic glycopolypeptides by (a) ROP of glycosylated monomers, (b) glycosylation of synthetic polypeptides, (c) glycosylation at the extremity of polypeptides.



Multi-block glycopolypeptides were also synthesized by ROP (Figure 2.9).[63] The statistical, diblock, and tetra-block glycopolypeptides adopted a random coil conformation, while the octa-block glycopolypeptide was mostly α-helical. All glycopolypeptides were biologically active and bound to lectins, where the binding affinity to RCA_{120} depended on the polymer composition and polymer structure. Glycopolymer-polypeptide triblock copolymers were synthesized by ROP of *L*-alanine *N*-carboxy anhydride monomers using the glycopolymer as a macroinitiator made by ATRP.[64] Peptide bioconjugates may improve stabilities and pharmacokinetics, reduce immunogenicity, and target the peptide/protein delivery to specific sites in the body. Glycopolymer-peptide bioconjugates were constructed by grafting reduced glutathione onto acrylate-functional block glycopolymers via thiol-ene chemistry.[65] Glyco-micelles formed in aqueous solutions and demonstrated pH-responsive property and specific interaction with Con A. Cationic block and statistical copolymers with galactose and (*L*-)-lysine moieties were prepared by RAFT polymerization.[66] They both showed a high DNA binding affinity, but the cytotoxicity and gene transfection efficacy strongly depended on the polymer architectures and the galactose content. The statistical copolymer cationic glycopolymer showed lower toxicity, faster cellular uptake, and higher efficiency and serum-compatibility as a gene carrier.

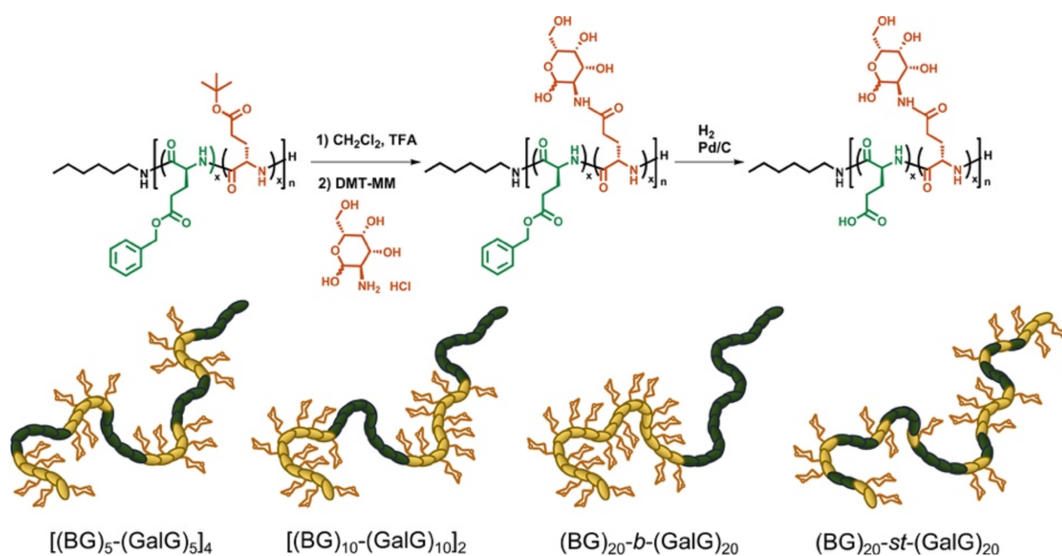


Figure 2.9 Preparation of multi-block glycopolypeptides by a block-sequence-controlled ROP.

Cholesterol is a useful compound in membrane regulation, cellular signal transduction, steroidogenesis, and bile acid synthesis.[67] Amphiphilic copolymers containing cholesterol may be useful in drug/gene delivery, tissue engineering, organ regeneration, and clinical diagnostics and biomedical imaging.[68] Diblock copolymers with a cholesterol-containing hydrophobic block and a galactose-based hydrophilic block prepared by sequential RAFT polymerization[69] self-assembled into aggregates with various morphologies from spherical NPs to nanofibers depending on the hydrophobic/hydrophilic block balance. The morphology-variable aggregates showed different lectin recognition and bovine serum albumin (BSA) adsorption. pH-responsive diblock copolymers with similar structure were synthesized by copolymerization of methacrylic acid (MAA) and a cholesterol-containing monomer using the glycopolymer block as a macro-RAFT initiator (Figure 2.10).[70] The micelles formed were stable and efficiently encapsulated Dox due to electrostatic interactions. The copolymer with higher MAA content released the drug faster, strongly depending on the pH of the buffer solution.

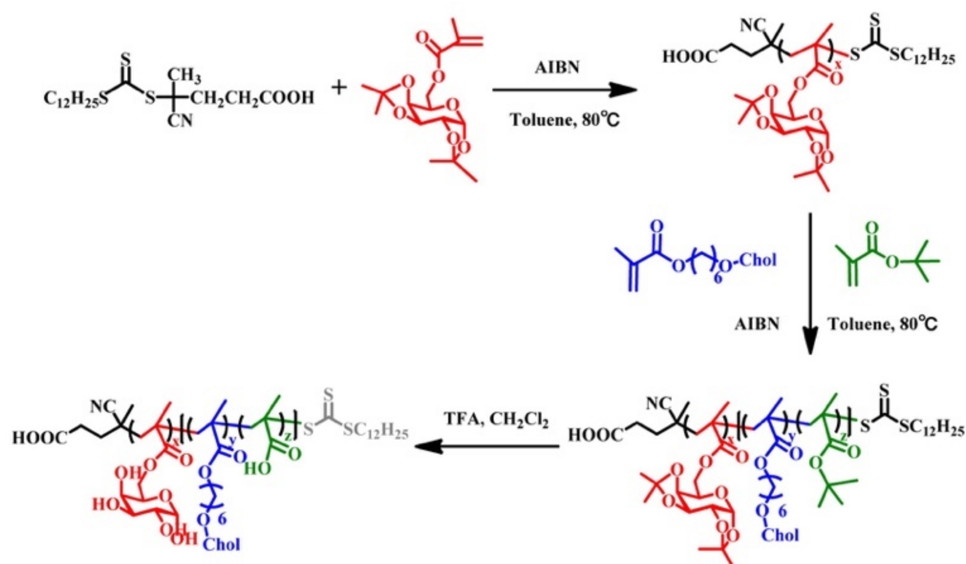


Figure 2.10 Preparation of pH-responsive block copolymers with a cholesterol-containing hydrophobic block and a galactose-based hydrophilic block by sequential RAFT polymerization.

Bile acids are natural steroidal compounds serving as surfactants in the digestive tracts of humans and most animals. They are biosynthesized from cholesterol in the liver and stored in the gallbladder. The modification of the hydroxyl and carboxylic groups of bile acids can give rise to a diverse range of new materials, as reviewed previously.[71, 72] Three diblock copolymers consisting of glucosamine and cholic acid (CA) pendants with different hydrophilic and hydrophobic chain lengths were synthesized by RAFT polymerization (Figure 2.11).[73] They self-assembled into micelles which were optimized as a drug delivery vehicle by changing the hydrophobic or hydrophilic chain length to improve the drug loading content of the micelles.

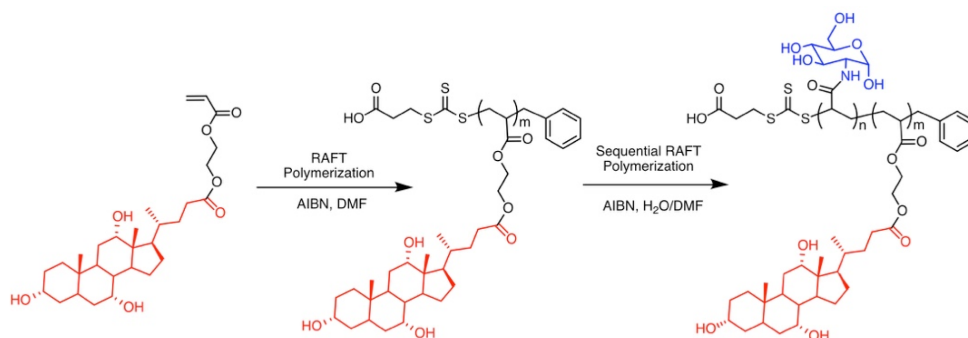


Figure 2.11 Synthesis of block glycopolymer consisting a cholic acid hydrophobic block and a glucosamine hydrophilic block by RAFT polymerization.

Betulin, a pentacyclic triterpene, is one of the major components of birch bark.[74] Betulin has three functional positions, namely a secondary and a primary OH group and an alkene

moiety, providing the possibility of chemical modifications to yield derivatives to study the structure-activity relationship. Betulin was shown to elicit a broad range of biological and pharmacological properties, including anti-bacterial, anti-viral, anti-fungal, and anti-cancer activities.[75] It was conjugated to methacrylate and then copolymerized with a galactose-bearing comonomer by RAFT polymerization to yield both random and block copolymers (Figure 2.12).[76] The sugar corona of glyco-micelles showed an important effect on the self-assembly and lectin binding. The absence of such extended sugar blocks on the random copolymer-based micelles led to the formation of smaller clusters at a slower rate. The block copolymers facilitated the formation of clusters, since the hydrophilic galactose moieties of the micelles were more exposed to bind to the lectin.

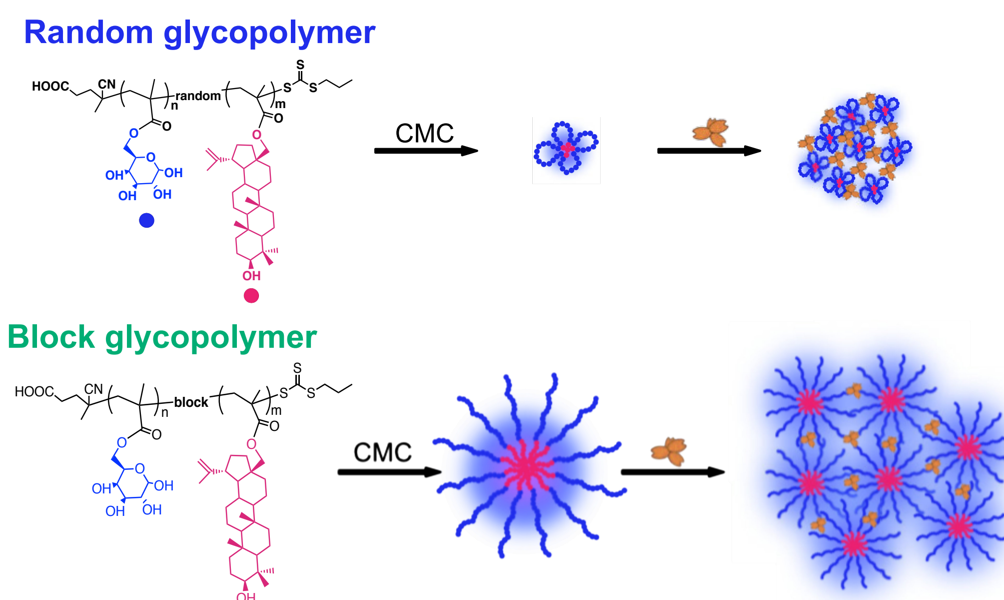


Figure 2.12 Self-assembly and lectin binding of random and block glycopolymers containing betulin.

Catechols are aromatic derivatives with two vicinal (*ortho*-) hydroxyl groups occurring in nature ubiquitously. Dopamine (DA) and other analogues can undergo self-polymerization under mild basic conditions (Tris-HCl buffer; pH = 8.5)[77] and form crosslinked structures that can be controlled by the self-polymerization time[78] or pH-dependent ligand-metal coordination,[79] making them suitable in the preparation of stable micelles. Block glycopolymers bearing a galactose-containing hydrophilic segment and a hydrophobic random segment with DA and CA pendants were synthesized by RAFT polymerization.[80] The dopamine moieties self-polymerized in a weakly basic solution, stabilizing the glyco-micelles in both water and organic solvents. Introducing CA groups into the copolymers promoted their

self-assembly into larger aggregates, controlled the crosslinking of the stabilized micelles, and facilitated the encapsulation of hydrophobic compounds. A bioadhesive random glycopolymer was synthesized by thermally-initiated free radical polymerization, including a glycopolymer segment, a catechol segment, and a crosslinking azide segment.[81] Bulk adhesion properties of the terpolymer were enhanced by crosslinking via strain-promoted azide-alkyne cycloaddition.

Porphyrins are a family of organic heterocyclic macrocycles with photophysical properties well-suited for clinical phototherapy and cancer imaging, but limited by poor water solubility, bioavailability, biocompatibility and skin phototoxicity for their wider bioapplications.[82] Glycopolymer modification of porphyrin may enhance its aqueous solubility, reduce its cytotoxicity and enable its specific binding to lectin-type of receptors on certain malignant cell surface. With this on mind, star-shaped porphyrin-cored PCL-*b*-glycopolymers were synthesized by a combination of ROP and ATRP.[83] The star-shaped block copolymers showed Con A affinity and formed different aggregates from spherical to worm-like micelles depending on the PCL to glycopolymer weight ratio. Star-shaped porphyrin-cored PLA-*b*-glycopolymers were prepared by the same method (Figure 2.13), which showed low cytotoxicity and targeted photodynamic therapy property.[84] Porphyrin-glycopolymer conjugates were also synthesized by thiol-ene click reaction with thiol-terminated glycopolymer and allyl-containing protoporphyrin IX.[85]

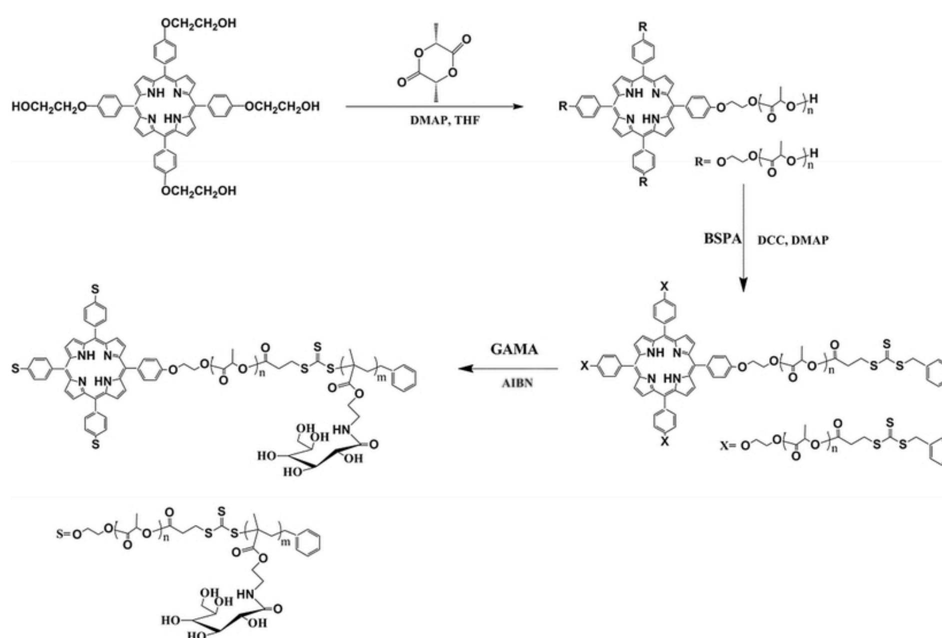


Figure 2.13 Synthesis of star-shaped copolymers consisting a porphyrin core and four PLA-*b*-glycopolymer arms.

2.3 Some properties and applications

2.3.1 Lectin binding

Since the 1960s, many types of saccharides including mono-, oligo-, and polysaccharides were found to bind specifically to certain lectins.[86, 87] Lectins (lect- means “chosen” (from Latin) + -ins) are carbohydrate-binding proteins. Hundreds of lectins have been evaluated and isolated from organisms such as animals, plants and microorganisms, as discussed in depth by Ting and colleagues.[88] These diverse lectins with various structures and sizes showed certain biological properties such as trafficking and clearance of glycoproteins, cell adhesion and immune defense. The factors influencing these mechanisms are still not well understood. Many preliminary glycopolymer-lectin binding experiments have been evaluated, especially with plant lectins Con A, RCA and PNA, by UV-visible spectroscopy,[89] isothermal titration calorimetry[90, 91] or surface plasmon resonance.[90]

Most oligosaccharides bind to their corresponding lectins weakly and seldom show association constants beyond 10^{-6} M^{-1},[18] which is not sufficient to effectively mediate the in vivo biology process. Their bindings are significantly enhanced due to the “multivalency effect” of glycopolymers with a large number of sugar residues along the polymer backbone (Figure 2.14a). Multivalency binding results in the formation of a protective polymer layer around the target with steric stabilization of the complex. In the early days, the studies on the binding between glycopolymers and lectin showed that glycopolymers with higher degrees of polymerization showed larger binding affinities, because of the multiple binding to one lectin (Figure 2.14b).[92, 93] A good match between the ligand and the binding site is a determining parameter to achieve good binding, as Whitesides elaborated in a review.[94] Optimal binding can be achieved by matching the distance between the sugar residues and that between the binding sites of a lectin.

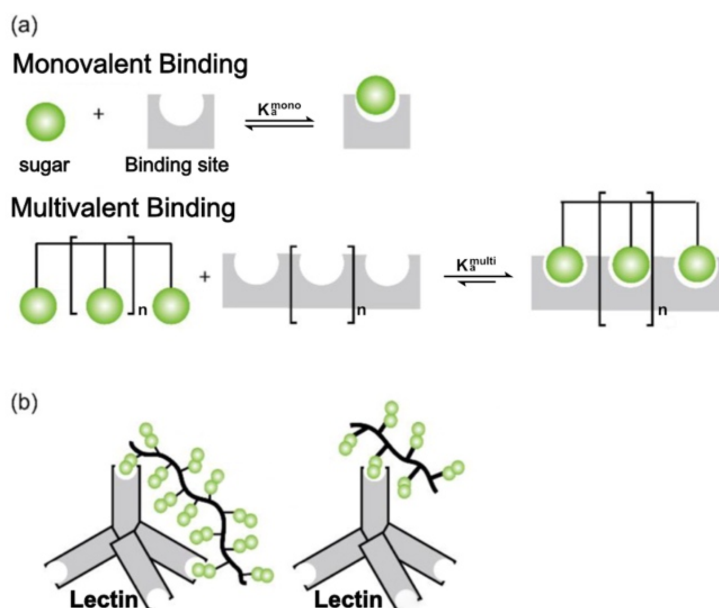


Figure 2.14 Illustration of the concept of the multivalent effect of glycopolymers: (a) comparison of monovalent and multivalent binding, including the definition of cooperativity; (b) multivalent binding of glycopolymers with different degrees of polymerization to a tetrameric lectin.

The arrangement of sugars in glycopolymers contributes to their lectin recognition. Glycopolypeptides with defined block sequences were prepared by sequential addition of two *N*-carboxy anhydride derivatives, followed by post-modification.[63] For glycopolypeptides with comparable compositions and molar masses, the octa-block glycopolypeptide favored interaction with RCA_{120} while the tetra-block showed the strongest binding to Galectin-3, suggesting that lectins are sensitive to the glyco-code and the precise control of sugar residues in polypeptides. The homo-glycopolymer and di- and tri-block copolymers with different sugar arrangements and molar masses were prepared by RAFT polymerization and click chemistry.[95] The binding constants (K_a) of the glycopolymer with ca. 100 repeating units of sugar residues were 5 times higher than that with 30 or 50 units (Figure 2.15). The tri-block copolymers with similar monomer repeating units containing a block with oligo-PEG side chain and two glyco-blocks at the end of the polymer also showed strong molecular recognition capability. The authors shown that multipoint binding was achieved, when the theoretical distance between the molecular chain terminals was close to the distance between the binding sites.

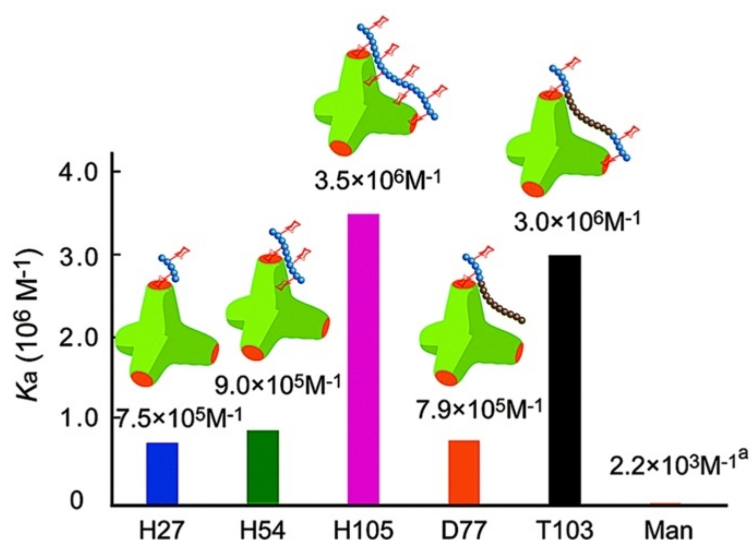


Figure 2.15 Arrangement of sugars in copolymers contributes to their lectin recognition and the binding constants of homo-(H) glycopolymer, di-(D), and tri-(T) block copolymers with different repeating units.

The multivalency binding amplifies the glycopolymer interactions with lectin-containing pathogens and viruses, which promotes the development of glyco-nanomedicine. Most lectins are toxic and pathogenic, such as ricin, AB5 and cholera toxin, which are normally inhibited by glyco-proteins or antibodies in humans. By the accumulation of weak interactions, the binding between glycopolymers and toxin is similar to antibody binding, which may be useful in the development of glyco-therapeutics. Yoshida *et al*. synthesized active anti-HIV (human immunodeficiency virus) polymethacrylates having pendant sulfated oligosaccharides and analyzed the relationship between the polymethacrylates structures and their biological activities.[96] The capture, detection and removal of pathogens and viruses by glycopolymers have been widely studied and summarized in a review by Miura *et al*.[97]

2.3.2 Bacterial affinity

The adhesion of bacterial pathogens to host cells is a prerequisite for a majority of infectious diseases. It is governed by interactions between the lectin-containing surface of the bacteria and corresponding saccharides displayed at the cell periphery. To enhance the adhesion and infection of cells, bacteria like *Escherichia coli* (*E. coli*) possess hundreds of hair-like organelles with adhesive proteins on their cell surface.

Yan *et al*. reported that bacteria exhibit a poor tendency to self-aggregate in aqueous solutions, while clusters of bacteria were observed when they were incubated with glycopolymers or glyco-nanoparticles (Figure 2.16).[7] The glyco-NPs efficiently agglutinated

E. coli due to the strong interaction of pendant *n*-heptyl-*α*-*D*-mannose groups to the membrane of the bacteria. Such glycopolymers also efficiently prevented and disrupted invasive *E. coli* adhesion *in vitro* at very low concentrations (Figure 2.16).[98] Glycopolymers were 10^6 times more potent than monosaccharides for their capacity to disrupt the binding of adherent *E. coli* to T84 intestinal epithelial cells, supporting their potential applications in the anti-adhesive treatment of *E. coli*-induced inflammatory diseases.

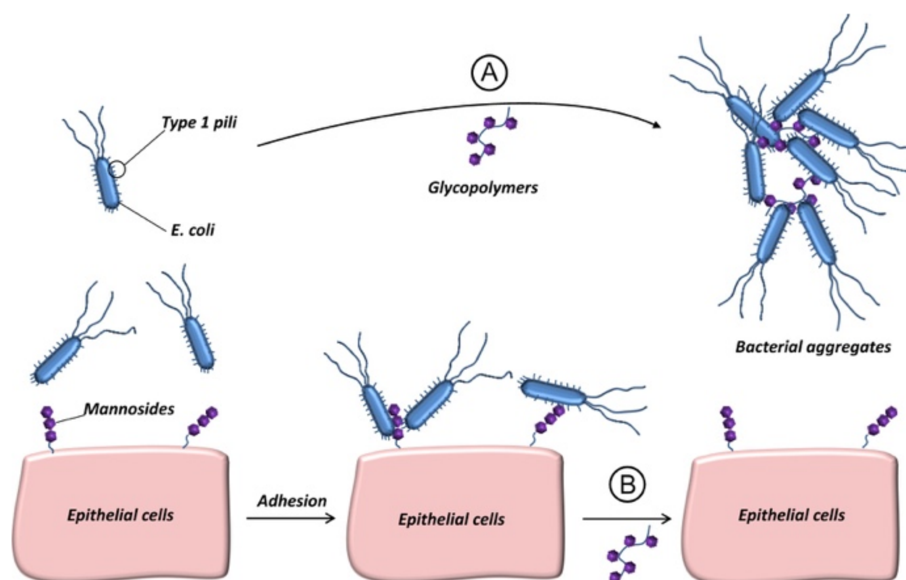


Figure 2.16 Illustration of glycopolymers anti-adhesive action. (A) Sequestration of free bacteria in the lumen of the gut, (B) Disruption of established *E. coli* and cell interactions.

Copolymer-gold NP composites with glycopolymers and quaternized polymers were proposed for use in specific *E. coli* killing (Figure 2.17).[99] The charged polymers not only endowed the nanocomposite with bactericidal capacity but also generated a charged protective layer on the surface of gold, which ensured the nanoparticle stability under physiological conditions. The specific affinity of the glycopolymer for the pili of *E. coli* effectively improved the bacterial target specificity of the anti-bacterial material, while the binding between the nanocomposites and the bacteria was strengthened by the multivalent effect of the glycopolymer.

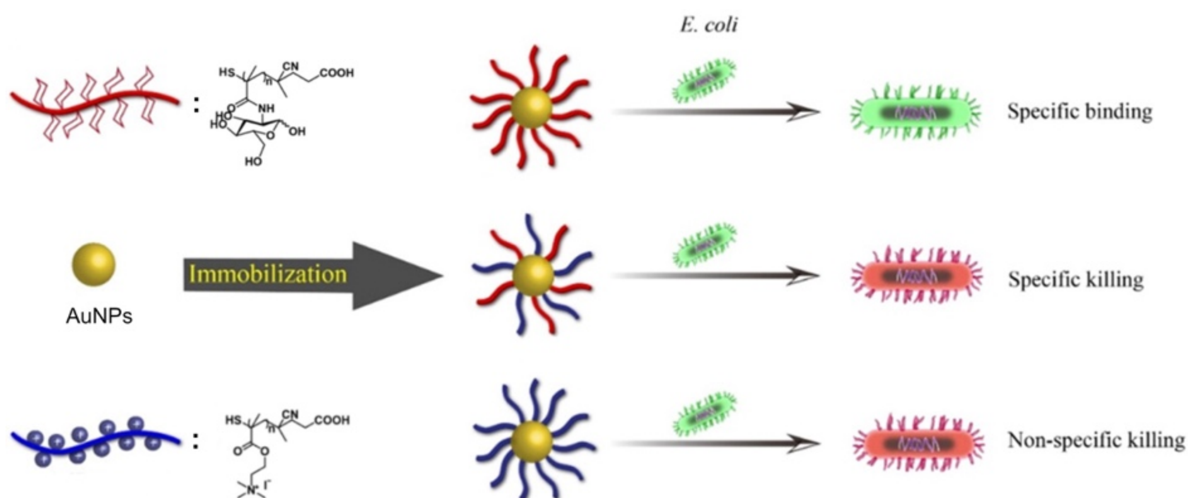


Figure 2.17 Bacterial binding and killing properties of the nanocomposites may be controlled by the different polymers. The nanoparticles with only glycopolymer exhibited specific binding to *E. coli*, the ones with charged polymer could kill *E. coli* in a nonspecific way, and the ones with both polymers could recognize *E. coli* and kill it in a specific way.

Glycopolypeptides bearing *L*-arginine, vancomycin, and colistin pendants can act against multiple targets, leading to a broad-spectrum antimicrobial activity favorably combining specific and nonspecific perturbations of the bacterial membrane.[100] Such glycopolypeptides were stable under physiological conditions and effective against both *Gram*-positive and *Gram*-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*) at low concentrations. A thermo-responsive surface was developed with block copolymers consisting of poly(*N*-isopropylacrylamide) (PNIPAm) block and a glyco-block to study the *P.* aeruginosa infection, which is believed to be mediated by lectin-glycopolymer interactions.[101] Although *P. aeruginosa* adhered to the PNIPAm-modified surfaces at temperatures above their LCST due to a hydrophobic interaction, the bacterium-substratum bond stiffness is stronger between *P. aeruginosa* and a glycopolymer-based PNIPAm surface, suggesting the lectin-carbohydrate interaction plays a significant role in bacterial infections. Four-armed star copolymers with glyco-segments and antimicrobial polypeptides were developed for anti-infective agents.[6] These copolymers with mannose-based segments enhanced bactericidal efficacy against both *Gram*-negative and *Gram*-positive bacteria, due to their specific affinity for bacterial surfaces.

2.3.3 Cancer cell affinity

Cancer is a leading cause of death in the world, and the vast number of cancer patients are treated with chemotherapy and radiation therapy, which typically have only partial effectiveness and lead to serious side effects.[102] Thanks to the enforced specific binding between the ligand and over-expressed receptors on the diseased cell surfaces (e.g., HER2, folate receptor, CD44, *etc*.), there has been a rapid development and validation of targeted drug carriers. The interaction between receptors and carbohydrates provides the foundation of cell agglutination, and glycopolymers have shown great promise.

A glycopolymer-based targeted drug carrier was first reported in 2013.[50] The galactose-decorated crosslinked micelles with an ionic core were loaded with Dox. They efficiently delivered and released Dox into the nucleus of HepG2 cells (human liver carcinoma cells), and the intensity of fluorescence from Dox observed in HepG2 cells was stronger than that incubated with the micelles without galactose ligands, while little fluorescence was observed in normal cells (NIH3T3).

By sequential polymerization of NIPAm and fructose, mannose, glucose, or galactose glycomonomers, a family of glycopolymers with different sugars attached was obtained.[103] Cellular uptake tests indicated an enhanced uptake of these glycopolymers into MDA-MB-231 (human breast cancer cells) in comparison to L929 cells, with the fructosylated glycopolymer showing the most pronounced uptake. Amphiphilic diblock PCL-*b*-glycopolypeptides bearing disulfide bonds were synthesized, then Dox and superparamagnetic iron oxide NPs were simultaneously encapsulated in the hydrophobic core of the micelles.[46] The fluorescence images and flow cytometry studies revealed that Dox could be efficiently transported into HepG2 tumor cells by the glyco-micelles. The micelles with superparamagnetic iron oxide enabled MRI contrast enhancement. Glycopolymer-*b*-PCL block copolymer micelles with tailored lactose functionalities were developed and investigated for hepatoma-targeted Dox delivery.[45] Flow cytometric tests indicated that HepG2 liver cancer cells (asialoglycoprotein receptor overexpressed) incubated with Dox-loaded glyco-micelles had a 6 to 17-fold higher Dox level, depending on sugar densities on the polymer backbone, as compared to those treated with the corresponding Dox-loaded non-glyco micelles under the same conditions.

Lecommandoux *et al*. reported the synthesis of block glycopolypeptides, and found that the polysaccharide shells favored specific targeting to the CD44 receptor over-expressing cancer cells.[104-106] Chen and coworkers prepared glycopolymers decorated with nano-phthalocyanines by an electrostatic self-assembly strategy.[107] The *in vitro* and *in vivo* photodynamic therapy

experiments indicated that the photo-active nanocomposites inhibited the growth of tumor cells.

2.3.4 Insulin delivery

Relying on the reversible covalent reaction between boronic acid and *cis*-diol-containing compounds, numerous macro- or meso-porous materials, NPs, and stimuli-responsive materials with boronate affinity were developed for the selective isolation of *cis*-diol-containing compounds, such as nucleosides, nucleotides, nucleic acids, catechols, saccharides and glycoproteins (Figure 2.18).[108, 109] Phenylboronic acid (PBA) and its derivatives existed in an equilibrium between an uncharged hydrophobic form and a charged hydrophilic form with the reported pK_a in the range of 8.2 - 8.8.[110] The complexation of the uncharged PBA with *cis*-diols is unstable due to its susceptibility to hydrolysis, while the charged PBA can form a stable complex, providing a possibility for designing glucose- and pH-responsive polymeric carriers for self-regulated delivery of insulin.

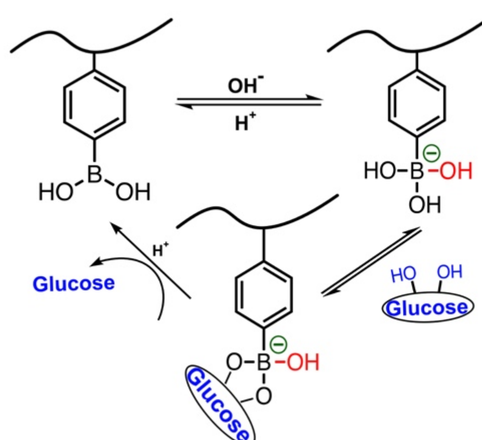


Figure 2.18 Equilibria between different forms of PBA and its interaction with glucose.

The Shi group reported the fabrication of glucose-responsive micellar complexes by the self-assembly of a PBA-containing block copolymer and a glycopolymer based on the complexation between PBA and the sugar residues (Figure 2.19).[111, 112] The micelles were stable under physiological condition (PBS 7.4) but dissociated in the presence of glucose due to the replacement of glycosyl groups by free glucose. The glucose-triggered on-off release of insulin was clearly achieved, providing an effective strategy for self-regulated insulin delivery in response to the physiological glucose level.

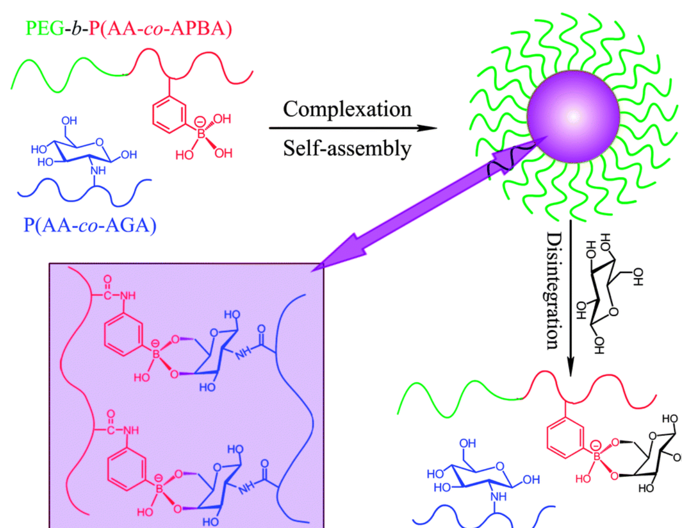


Figure 2.19 Schematic illustration for the formation and glucose-responsive disintegration of the complex micelles made by the complexation of PBA-containing copolymer and glycopolymer.

Li and coworkers reported the synthesis of PBA-containing amphiphilic glycopolymers in random or block form by RAFT polymerization to explore the effect of polymer structure on their aggregates and their glucose-response property.[113, 114] Both copolymers with good cytocompatibility self-assembled into spherical NPs with narrow size distribution in aqueous solution. As glucose-sensitive carriers, insulin was encapsulated into both copolymers, and the release presented a glucose-dependence pattern. For random copolymer NPs, insulin had a faster release rate and more cumulative amounts than that from the block ones.[115] The synthesis and application aspects of glucose-responsive polymeric carriers based on phenylboronic acid were reviewed by Ma and Shi.[110]

2.3.5 Glyco-NPs

Glyco-NPs can be prepared through covalent crosslinking or by non-covalent hydrogen bonding, electrostatic and hydrophobic interactions. Hydrophilic and ionic comonomers, such as MAA and acrylate acid (AA) may be added to the glycopolymers to induce specific interactions to prepare particles or aggregates. MAA or AA was copolymerized with glycomonomers to yield pH-sensitive materials. Copolymers of *2*-methacrylamido glucopyranose and MAA were synthesized by RAFT polymerization and then used as templates to prepare glycopolymer-functionalized Ag NPs through microwave irradiation.[116] These NPs interacted with K562 cells and inhibited the cell viability. Polymeric colloids based on glycopolymers were prepared by copolymerizing MAA and methacryloyl derivative of

glucofuranose using ethylene glycol dimethacrylate as a crosslinker. The sizes and the surface characteristics of the particles depended on the MAA content. Crosslinked polymers were synthesized by copolymerization of MAA, poly(ethylene glycol)monomethyl ether methacrylate, and glucose-*6*-acrylate-*1,2,3,4*-tetraacetate monomer using cubane as a crosslinking agent.[117] Insulin was entrapped and its release increased at higher contents of MAA of the copolymers. Similar crosslinked copolymers bearing ibuprofen pendants were synthesized by the same group.[118] By changing the MAA content, these hydrogels showed controlled release of ibuprofen by hydrolysis the ester bond between the drug and polymer backbone. Glycosylated nanogels were prepared by electrostatic interactions between glycopolymers with MAA and quaternary ammonium chitosan (Figure 2.20).[119] These nanogels were stable in 10 mM HEPES buffer solution for 7 days and possessed a specific binding capability to Con A. They exhibited a much higher affinity and cytotoxicity towards K562 cancer cells than with normal cells. Other glyco-nanocarriers and their applications are summarized by Kang *et al.*[5] and Yilmaz and Becer.[120]

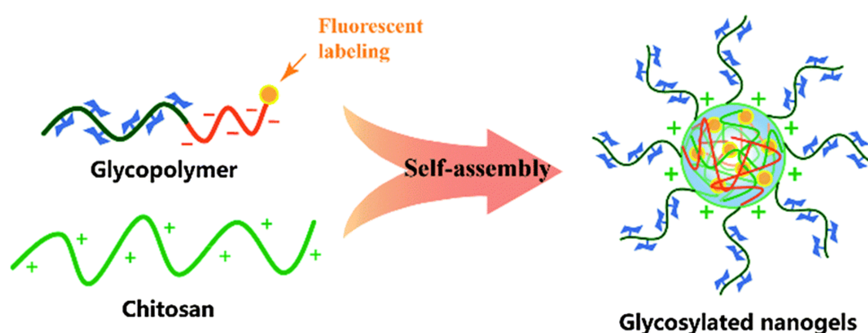


Figure 2.20 Schematic illustration of the preparation of nanogels from glycopolymers with MAA and quaternary ammonium chitosan.

2.3.6 Gels and cell culture

Sugar-based hydrogels may be expected to replace agar for cell culture due to their biocompatibility and bioavailability. Akaike's group cultured hepatocytes with lactose-substituted polystyrene *in vitro* and showed the saccharide structure and density induced specific hepatocytes response.[121, 122] Molecularly imprinted hydrogels were prepared as chiral stationary phases for the resolution of isomers in polar organic eluents.[123] The synthesis of crosslinked glyco-resins and their use as chromatography supports to purify or isolate proteins were summarized by Okada.[19]

2.4 Conclusion

The preparation of glycomonomers normally needs protection and deprotection reactions. Glycopolymers may be synthesized by polymerizing the glycomonomers with protecting groups and then deprotection after polymerization, or by polymerizing the already deprotected glycomonomers. Glycopolymers may also be obtained by post-modification of a polymer containing appropriate functional groups through thiol-ene, click and amidation chemistry. Random copolymers are easier to prepare by copolymerization with functional monomers than block copolymers. Block copolymers can easily aggregate in the form of micelles, ellipsoids, worm-like aggregates and vesicles. Glycopolymers have the ability to mimic functions of natural carbohydrates to interact with lectins and lectin-containing surfaces by multivalent bonding. The binding between glycopolymers and lectin-bearing pathogens, viruses and bacteria promotes the development of new glyco-medicine. Glycopolymers can specifically bind to some diseased cells, especially tumor cells with over-expressed lectin receptors on the membrane. There has been a rapid development of glyco-carriers for targeted delivery in the past decade. Glucose-responsive micellar delivery systems may be developed by the complexation of boronic acid and sugar for self-regulated insulin delivery. The sugar-based hydrogels are biodegradable and biocompatible, making them suitable for cell culture. Because of their hydrophilicity and bioavailability, glycopolymers are promising alternatives for PEG in a variety of bio-related applications.

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Chapter 3

Glycopolymers bearing galactose and betulin: Synthesis, encapsulation, and lectin recognition*

Abstract

Betulin is a natural triterpene compound with anti-carcinogen and antiviral activities. It is conjugated with methacrylate and then copolymerized with a galactose-bearing comonomer by RAFT polymerization to yield both random and block copolymers. These glycopolymers are designed to possess similar molecular weights and monomer compositions for easy comparison. They self-assembled into micelles as shown by dynamic light scattering (DLS) and transmission electron microscopy. The smaller micelles formed by the random copolymers facilitated the encapsulation of Nile Red and released more of this hydrophobic model compound (46% in 4 days versus 32% released from the block copolymers). These glycopolymers interacted with lectins, such as RCA_{120}, as studied by turbidity assay and DLS. The block copolymers formed larger aggregates and clustered faster than the random copolymers. The betulin-based glycopolymers may serve as biocompatible multifunctional biomaterials and carriers for use in targeted release of drugs.

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3.1 Introduction

Amphiphilic polymers consisting of hydrophilic and hydrophobic segments can spontaneously self-assemble in aqueous solutions to form micelles with sizes ranging from tens to hundreds of nanometers and may serve as effective drug carriers.[1, 2] These micelles have a rather narrow size distribution and show a unique core-shell structure in water.[3] The hydrophobic core of the micelles creates a microenvironment for the accommodation of lipophilic molecules, while the hydrophilic shell protects the encapsulated drug from fast degradation and elimination in the body.[4-6]

Glycopolymers with sugar as the hydrophilic segment have gained considerable popularity due to the cell recognition between the sugar and membrane receptors known as lectins. These polymers can modulate their bio-distribution and induce specific cellular uptake by lectin-mediated endocytosis.[7-11] The choice of sugar,[12] molecular weight[13, 14] and composition[15] of the polymers can determine the lectin recognition and binding affinity. Random and block copolymers can affect their self-assembling behaviors in water.[16, 17] Although random copolymers exhibited good glycopolymer-lectin interactions,[18-20] block copolymers have advantages of more regular structures and easier self-assembly. [21-23] Liu et al. synthesized block copolymers with glucose, and found that the sugar block could extend outward from the micelle corona and readily encounter and interact with lectins.[24] Nagasaki et al. made block copolymers with different sugar blocks, which showed promise as active targeting drug vehicles.[25] However, the relationship between the glycopolymer-lectin interaction and polymer structure remains to be elucidated.

Betulin is a triterpene found in large quantities in the bark of white birch, almost insoluble in aqueous buffers,[26, 27] and has been found to possess anticarcinogen, anticancer and antiviral activities.[28-30] Betulin and betulinic acid were incorporated in prodrugs for release tests.[31-33] It may be used as an interesting building block for amphiphilic copolymers with biological activities and with the possibility of further functionalization. Due to the natural abundance, easy availability and pharmaceutical application of betulin, we would like to test its use in the preparation of copolymers with comonomers based on carbohydrates. This represents the first attempt to introduce betulin to glycopolymers and to test the potential applications of such polymers.

To explore the effect of glycopolymers structure on their drug delivery and lectin recognition, we designed and prepared a series of random and block copolymers by RAFT

polymerization and studied their self-assembly behavior. The micelles formed by these glycopolymers were loaded with a model hydrophobic molecule to study its release from the micelles. The interaction between the glycopolymers and a lectin was studied to demonstrate their potential use as a targeted drug release device.

3.2 Experimental section

3.2.1 Materials

All reagents were purchased from Aldrich and used without further purification unless otherwise stated. *6*-*O*-Methacryloyl-*1,2:3,4*-di-*O*-isopropylidene-*D*-galactopyranose (MIpGa) monomer was synthesized according to the literature.[34] *4*-Cyano-*4*-[(propylsulfanyl thiocarbonyl)sulfanyl] pentanoic acid was used as the chain transfer agent (CTA).[35] The initiator *2,2'*-azoisobutyronitrile (AIBN) was recrystallized twice from methanol. Milli-Q water was used for the experiments, and *1,4*-dioxane was purified by treatment with sodium metal and redistillation.

3.2.2 Synthesis of betulin-based methacrylate monomer (MBet)

Betulin (2.12 g, 4.8 mmol) and *4*-(dimethylamino) pyridine (0.4 g) were dissolved in CH_2Cl_2 (35 mL). Methacrylic anhydride (0.75 mL, 5.0 mmol, in 9 mL of CH_2Cl_2) was added dropwise. The resulting mixture was stirred at room temperature for 24 h, and then washed with 5% aqueous NaOH, water and dried with $MgSO_4$. The organic phases were concentrated and purified by column chromatography on silica gel (EA/hexane = 15/85 as eluent), affording a white solid (1.59 g, yield: 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 3H), 0.82 (s, 3H), 0.97 (s, 3H), 0.98 (s, 3H), 1.4 (s, 3H), 1.69 (s, 3H), 1.96 (s, 3H), 2.41 (ddd, *J*= 6.0, 11.2, 11.2 Hz, 1H), 3.18 (dd, *J*= 5.2, 11.6 Hz, 1H), 3.92 (d, *J*= 11.2 Hz, 1H), 4.33 (dd, *J*= 11.2 Hz, 1H), 4.59 (br s, 1H), 4.69 (d, *J*= 1.6 Hz, 1H), 5.55 (t, *J*=1.6 Hz, 1H), 6.11 (br s, 1H). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 14.9, 15.5, 16.20, 16.25, 18.4, 18.5, 19.3, 21.0, 25.4, 27.3, 27.6, 28.1, 29.4, 29.8, 30.1, 34.4, 34.8, 37.3, 37.8, 38.9, 39.0, 41.0, 42.9, 46.8, 47.9, 49.0, 50.6, 55.5, 63.1, 76.8, 110.0, 125.4, 136.7, 150.3, 168.0.

3.2.3 Polymerization

The random copolymerization was performed with a [MIpGa]: [MBet]: [CTA]: [AIBN] ratio of 150: 7.5: 1: 0.3. The solution was purged with argon for 30 min and then stirred at 75°C overnight. The polymerization was quenched by cooling in an ice bath. The resultant

copolymer was precipitated, filtered, washed with methanol, and dried under the vacuum. By changing the feed ratio of MIpGa to MBet, two random copolymers, P(MIpGa-*r*-MBet3%) and P(MIpGa-*r*-MBet6%), were obtained, where 3 and 6% represent the molar percentages of the MBet in the mixture of the comonomers.

The block copolymers were prepared by the sequential RAFT polymerization, where PMIpGa was synthesized first. Briefly, MIpGa, CTA and AIBN ([MIpGa]: [CTA]: [AIBN] = 150: 1: 0.3) were dissolved in 1,4-dioxane, and the mixture was purged with argon for 30 min and transferred to an oil bath. The polymerization proceeded at 75°C and was quenched after 10 h. The polymers were purified by precipitating with methanol three times and dried under vacuum. By adjusting the ratio of PMIpGa to MBet, two block copolymers, P(MIpGa-*b*-MBet3%) and P(MIpGa-*b*-MBet6%), were obtained by using the same conditions.

3.2.4 Characterization

^{1}H and ^{13}C NMR spectra of the polymers in $CDCl_3$ or DMSO-d_6 were recorded on a Bruker AV400 spectrometer. Size exclusion chromatography (SEC) was performed on a Breeze system from Waters equipped with a 717 plus auto-sampler, a 1525 Binary HPLC pump, and a 2410 refractive index detector. The THF eluent was filtered using 0.2 μm nylon Millipore filters (flow rate: 1 mL/min). Polystyrene standards (2500-608000 g/mol) were used for calibration.

Transmission electron microscopy (TEM) was performed on a FEI Tecnai 12 TEM equipped with a Gatan 792 Bioscan 1k × 1k wide-angle multi-scan CCD camera at 80 kV. The samples were prepared by placing a drop of polymer solution (0.1 g/L in water) on carbon-coated copper grids (300 mesh, Carbon Type B, Ted Pella, Inc.). The solution was frozen, followed by the removal of water through the freeze-drying. Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer NanoZS instrument (Malvern CGS-2 apparatus) equipped with a He-Ne laser at a wavelength of 633 nm and a scattering angle of 173°. The suspensions (concentration of 1.0 g/L) were prepared and filtered through 0.45 μm Millipore filters.

Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). The amounts of pyrene were chosen to obtain a saturated concentration of 6×10^{-7} M in the final suspension. The emission spectra were recorded in the range of 350-500 nm with a fixed emission at 335 nm. The slit widths for excitation and emission were 5.0 and 2.5 nm, respectively. The intensity ratios of the third over the first emission bands (I_3/I_1)

were recorded and plotted against the polymer concentration. The critical micelle concentration (CMC) is taken as the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low polymer concentrations.

3.2.5 Loading and release of Nile Red (NR)

The experimental conditions of the NR loading and release are based on a previous report.[16] NR is selected as a model hydrophobic compound for these studies at room temperature. The amount of NR loaded is expressed as the weight ratio (mg of NR per gram of polymer).

3.2.6 Lectin recognition

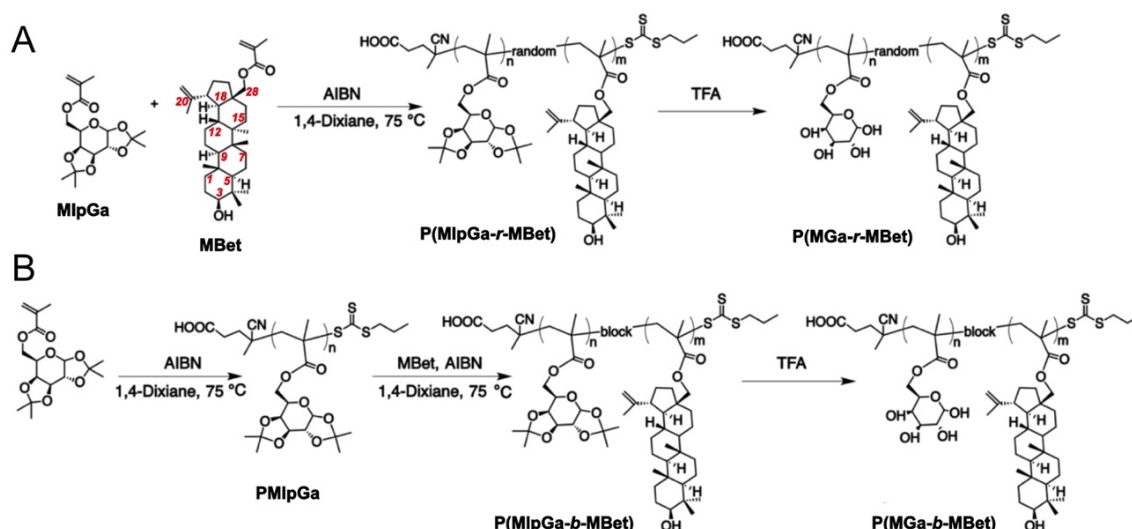
Lectin recognition of the random and block copolymer-based micelles in aqueous solution was studied by UV-Vis spectroscopy and DLS. The RCA_{120} solution was added to the glycopolymer solutions to form the final mixtures containing 0.75 mg mL^{-1} glycopolymer and 0.25 mg mL^{-1} RCA_{120}. The turbidity changes at $\lambda = 420$ nm were recorded on a Cary 500 UV-Vis spectrophotometer. Three independent runs were performed for each sample. In a typical DLS measurement, the polymeric micelles were filtered through a 0.45 mm Millipore filter into clean scintillation vials, followed by the addition of filtered RCA_{120}. The D_h was recorded per 3 minutes for 30 min.

3.3 Results and discussion

3.3.1 Synthesis of glycopolymers

The synthesis of the polymers is illustrated in Scheme 3.1. The molecular structures of the monomers are confirmed by ^1H NMR spectra (Figure 3.S1). RAFT polymerization method was used to prepare the random and block copolymers containing two different molar fractions (3 and 6 mol%) of the betulin monomer. The copolymers are identified as P(MIpGa-*r*-MBet3%) and P(MIpGa-*r*-MBet6%), and as P(MIpGa-*b*-MBet3%) and P(MIpGa-*b*-MBet6%), respectively. The exact molar percentages of the MBet in the final copolymers are listed in Table 3.1. The molecular weight of the copolymers ranged from 22 to 25 kDa, with relatively narrow dispersity (1.17-1.23).

Scheme 3.1 Synthesis of (A) the random and (B) block copolymers.



The typical ^{1}H NMR spectra for the homo- and copolymers are shown in Figure 3.1. The protons of the isopropylidene (Ip) protecting groups are distinctly visible at δ = 1.2-1.6 ppm. For PMIpGa, the featured proton resonance signals at δ = 5.53 and 3.35 ppm (insert) may be assigned to the proton attached to the anomeric carbon in the sugar ring and the methylene group adjacent to the trithiol group of the CTA, indicating the successful RAFT polymerization. Although of the most characteristic peaks of betulin are overlapped or undetectable, the ^{1}H signals at δ = 3.21 (insert) and 0.82 ppm may be assigned to the proton attached to the triterpene adjacent to the secondary alcohol (C3) and the proton of the methyl group attached to the triterpene, demonstrating the presence of betulin in the copolymers. However, it is hard to estimate the fraction of betulin via the integral ratio of the NMR signals, due to its low fraction in the copolymers. To do so, the secondary alcohol of betulin in the polymer was reacted with benzenesulfonyl chloride, which shifted the C3 proton signals from 3.21 ppm down field to the range of 4.20-4.60 ppm, overlapping with the proton attached to the anomeric carbon in the galactose. At the same time, new peaks showed up at 7.40-8.05 ppm for the proton in the benzene ring (Figure 3.S2). The integrations of these peaks are compared with the integral of the sugar proton at 5.53 ppm to estimate the fraction of betulin. Table 3.1 lists the final compositions determined by NMR and the other characteristics of the glycopolymers. The small deviation of the final composition from the feed ratios may be caused by minor differences in reactivity of methacrylate comonomers derived from betulin and galactose during the block and random copolymerization. Solubility differences of the monomers and the propagating polymer chains may also be attributed to the deviation. The SEC traces (Figure

3.S3) show narrow and mono-disperse peaks, indicating the successful synthesis of the random and block copolymers with narrow molecular weight distributions. The final glycopolymers were obtained after deprotection of isopropylidene group in TFA/water (8/2, v/v). The complete disappearance of isopropylidene proton resonance signals at 1.2-1.6 ppm indicated its successful removal, but the ester bonds and triterpene remained intact (Figure 3.S4), as reported.[34, 36]

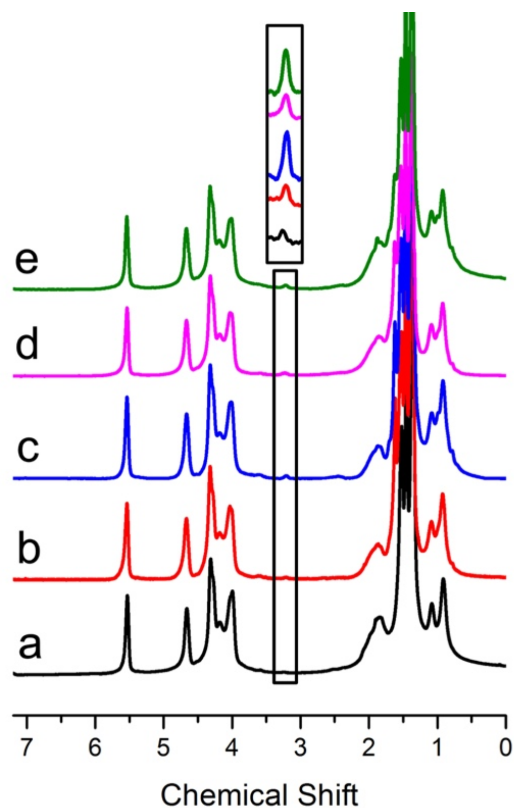


Figure 3.1 ^{1}H NMR spectra of (a) PMIpGa, (b) P(MIpGa-*r*-MBet3%), (c) P(MIpGa-*r*-MBet6%), (d) P(MIpGa-*b*-MBet3%), (e) P(MIpGa-*b*-MBet6%) in $CDCl_3$.

Table 3.1 Composition, micellization characteristics and release profiles of the glycopolymers

Glycopolymers[a]	MBet (mol%)[b]	M_n (g/mol)[c]	$Đ$[c]	CMC (mg/L)[d]	Micellar diameter (nm)[e]	NR loading capacity (mg/g)[f]	NR cumulative release (%)[g]
P(MIpGa-*r*-MBet3%)	2.7	24,000	1.21	35.5	-	-	-
P(MIpGa-*r*-MBet6%)	6.3	24,600	1.19	10.0	16	25.5	45.9
PMIpGa	-	22,500	1.17	-	-	-	-
P(MIpGa-*b*-MBe3%)	3.1	24,400	1.19	28.7	-	-	-
P(MIpGa-*b*-MBet6%)	5.6	25,000	1.23	5.5	59	14.4	32.5

[a] The approximate molar percentage of betulin comonomer in the copolymer. [b] Molar percentage calculated from ^{1}H NMR spectra. [c] Determined by SEC. [d] Determined by pyrene fluorescence. [e] Determined by DLS at 25 °C in water. [f] Determined by UV-vis absorbance. [g] At the release point of 108 h. We do not indicate the micellar sizes for glycopolymers with 3% of betulin, due to their multiple peaks in the DLS results.

3.3.2 Self-Assembly of random and block copolymers

Both random and block copolymers formed micelles in aqueous media and their CMCs were measured by fluorescence spectroscopy by the use of pyrene as a probe of the hydrophobic environment of the micelles (Table 3.1).[16, 37] All block and random copolymers made show a CMC under ambient conditions, serving as an indication for the formation of micelles. For both random and block copolymers, a higher betulin content lowers the CMC values due to the increased hydrophobicity of the polymer. The block copolymers have a lower CMC than the random copolymers. The 3% of the hydrophobic betulin contents in both random and block copolymers are low so that these copolymers may be too hydrophilic to form stable micelles which shows multiple peaks in DLS results (Figure 3.S5). With higher contents of betulin (ca. 6 mol%), both P(MGa-*r*-MBet6%) and P(MGa-*b*-MBet6%) showed unimodal distributions of the micelles formed in water (Figure 3.3A), indicating that the higher amount of betulin can facilitate the formation of more stable and uniform micelles. Therefore, they are selected for the following NR encapsulation and release experiments. The random copolymer formed smaller aggregates (16 nm) with a broader size distribution than the block copolymer (59 nm). This is further confirmed by the TEM images (Figure 3.2) showing mono-disperse spherical micelles formed by both random and block copolymers. They are dehydrated during the

preparation of the TEM samples and are smaller in size than the average D_h values measured by DLS. The D_h of the block copolymer was 59 nm (Figure 3.3A), larger than that of the random copolymers, indicating a more complex structure, better and more accurately termed as "micellar aggregates". These results are consistent with our previous study comparing random and block copolymers,[17] where the micellar size of block copolymers is larger than that of random ones, probably due to the different core-shell structures of the copolymers.

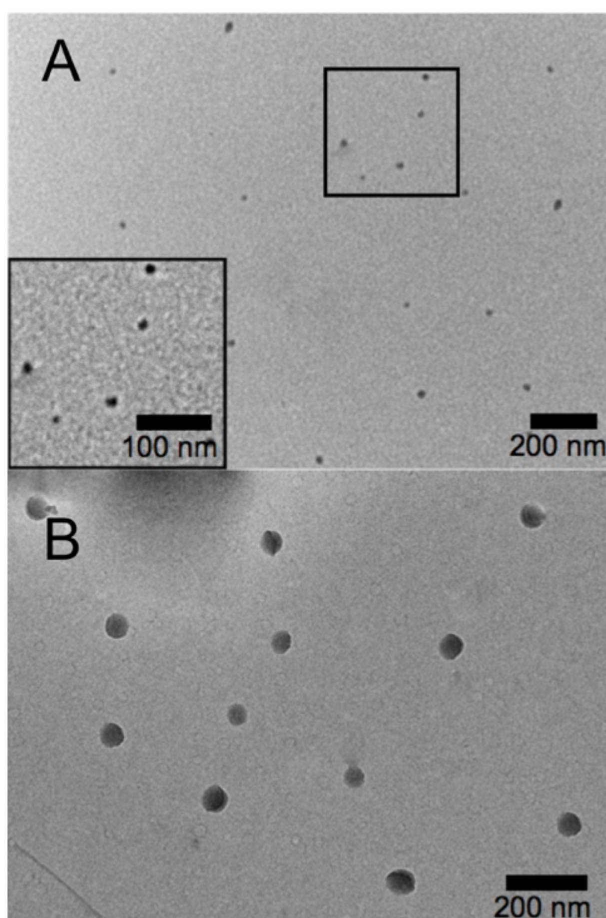


Figure 3.2 TEM images of (A) P(MGa-*r*-MBet6%) and (B) P(MGa-*b*-MBet6%).

3.3.3 Loading and release of NR

NR is an uncharged hydrophobic molecule (water solubility ca. 80 μg/L)[38] and was used as a model compound to study the loading and release behavior of the glycopolymers at room temperature only. Once loaded with NR, the aggregates of P(MGa-*b*-MBet6%) are enlarged from 59 to 65 nm for the D_h value accompanied by a broader size distribution (Figure 3.3A). In the case of the random copolymer, the aggregates actually shrank in size likely to be denser though smaller. In fact, the random copolymer has a higher loading capacity than the block copolymer (25.5 vs. 14.4 mg/g), showing a more efficient encapsulation of NR. This may be

caused by the relatively thinner hydrophilic shell with the random distributed comonomers. This result is similar as a previous study for other polymers, which may be attributed to different structures of the micellar aggregates formed by the block and random copolymers.[17] Figure 3.3B shows the release profiles of NR-loaded micelles formed by P(MGa-*r*-MBet6%) and P(MGa-*b*-MBet6%) at room temperature, respectively. The micellar aggregates of the random copolymer showed a faster and higher cumulative release (45.9% NR released) at 108 h, versus 32.5% released by the block copolymer. This serves as another indication that the higher stability of the aggregates formed by the block copolymer, which slows down the release of NR. A similar behavior was observed in a previous study on other types of random and block copolymers.[27] Depending on the required loading and release efficiency in real time applications, one may choose a random or a block copolymer.

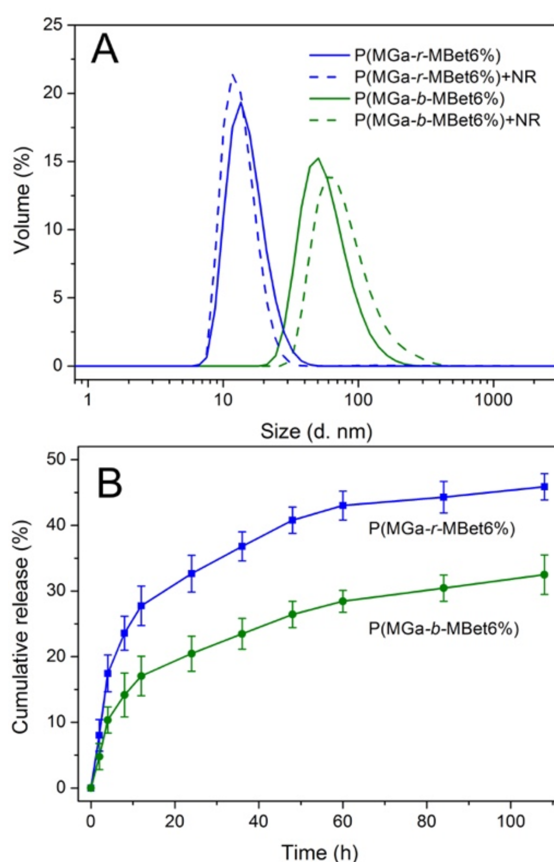


Figure 3.3 (A) Volume-average size distribution of the micelles formed by glycopolymers (1.0 g/L) before and after loading of NR, and (B) NR release profiles from the micelles in aqueous solutions (pH = 7.4, 25 °C, [p] = 1.0 g/L).

3.3.4 Lectin recognition of glycopolymers

To study the binding of the galactose moieties with lectin, the micelles formed by two types of copolymers were mixed with RCA_{120}, a multivalent lectin that specifically binds galactose. The lectin agglomeration capability was studied using UV-Vis spectroscopy and DLS for both glycopolymers-based micelle solutions upon the addition of RCA_{120}, and Con A served as a control. Figure 3.4A shows the transmittance change at 420 nm of the polymer suspensions incubated with the two lectins as a function of time. The solutions of both random and block copolymers rapidly became turbid upon the addition of the RCA_{120}, P(MGa-*b*-MBet6%) displayed a slightly faster clustering rate than P(MGa-*r*-MBet6%). The solution of galactose-based homopolymer showed a transmittance decrease in the presence of the RCA_{120} and reached a plateau quickly after 5 min. No obvious change in transmittance was found with the solutions of the copolymers upon the addition of Con A (Figure 3.4A dashes on the top), indicating the specific binding between the glycopolymers and RCA_{120}. After the addition of RCA_{120} into the clear polymer solutions, the binding between lectin and the sugar units of the copolymers caused aggregation, leading to a turbid solution (Figure 3.4A inset).

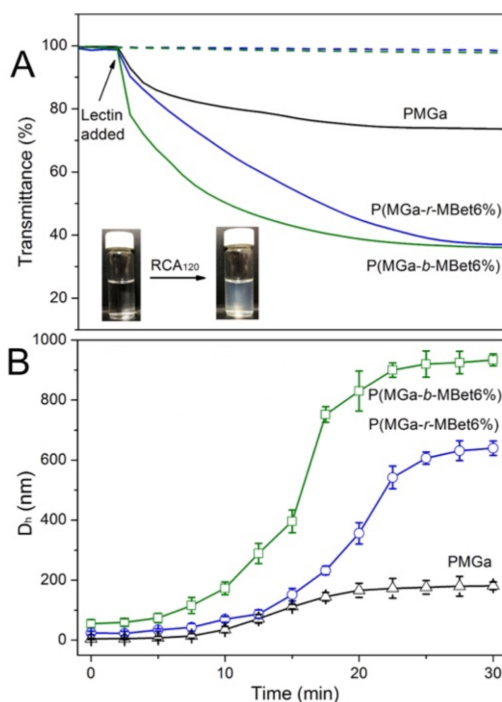
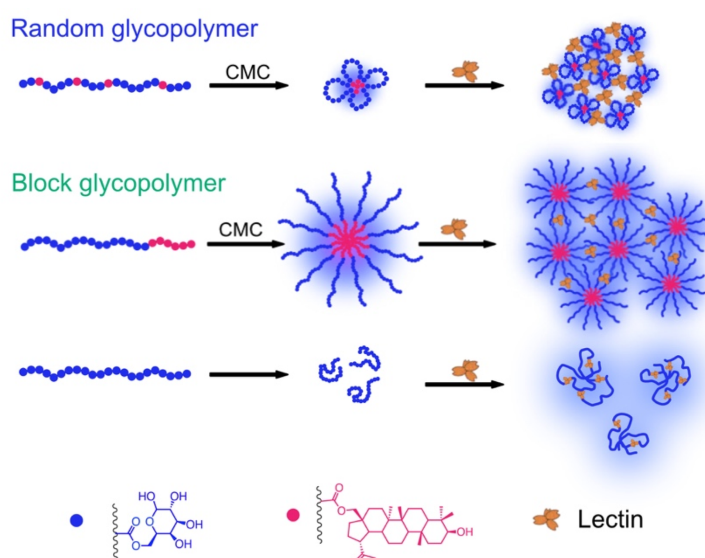


Figure 3.4 (A) Transmittance variation (λ = 420 nm) of the micelle solution (0.5 mg mL^{-1}) as a function of time upon the addition of lectins RCA_{120} (solid) and Con A (dots) at 0.5 mg mL^{-1}. The insert shows the turbidity change of a solution of P(MGa-*b*-MBet6%) upon the addition of the lectin RCA_{120}. (B) Variation of the hydrodynamic diameter of lectin-glycopolymer clusters as a function of time.

The aggregates increase in size with time upon the addition of the lectin as shown by the light scattering experiments (Figure 3.4B). RCA_{120} is known to have two identical binding sites.[39] Therefore, inter-micellar crosslinking may occur to increase the hydrodynamic diameter of the aggregates to about 600 and 900 nm for P(MGa-*r*-MBet6%) and P(MGa-*b*-MBet6%), respectively, after 30 min. Interestingly, the D_h of PMGa increased to 150 nm within the first 20 min, and remained at this value afterwards, which is consistent with the turbidity results.

Based on these results, a mechanism for the self-assembly of these glycopolymers and their interaction with lectin is proposed, as shown in Scheme 3.2. The copolymers self-assemble in an aqueous solution to form primary micelles at a concentration above their CMC. The size of micelles formed by the block copolymer is ca. 4 times larger than those formed by the random copolymer. Lectins, specifically RCA_{120}, may interact with the galactose units on the shell of glycopolymer micelles and link them through divalent binding sites to form larger aggregates. The micelles formed by the random copolymer aggregate at a slower rate since the galactose moieties in the polymer are randomly distributed along the polymer chain and may be less exposed to bind to the lectin. The galactose units of the block copolymers are grouped together and may bind to the lectin more easily. The homopolymer PMGa is more soluble in water and can easily and quickly bind to lectin, but does not lead to large aggregates that cause marked changes in turbidity (Figure 3.4). The block copolymers could form larger aggregates than the homopolymer. Once bound to RCA_{120}, they formed larger clusters due to the additional hydrophobic interactions, leading to a larger turbidity change.

Scheme 3.2 Illustration of self-assembly and lectin recognition of glycopolymers.



3.4 Conclusion

Betulin is a biocompound existing in abundance in birch barks with pharmaceutical implications and it is an interesting building block in the preparation of polymeric biomaterials. In this study, we used the RAFT polymerization method to incorporate betulin into a series of glycopolymers based on galactose. Both the random and block copolymers self-assembled into micellar aggregates that can incorporate hydrophobic molecules, such as NR, into their micellar cores. The smaller micelles formed by the random copolymers make the encapsulation of NR easier. The release studies showed a higher cumulative release after 4 days from micelles formed by the random copolymer. The structure of glycopolymers also affects their lectin recognition behavior. For example, the block copolymers interact with a lectin (RCA_{120}) to form larger clusters at a faster rate than the random copolymers. The hydrophilic corona of micelles has an important effect on the self-assembly and lectin binding by the polymers. The absence of such extended sugar blocks on the random copolymer-based micelles leads to the formation of smaller clusters at a slower rate. The block copolymers facilitate the formation of clusters, since the hydrophilic galactose moieties of the micelles are more exposed to bind to lectin. The homo-glycopolymers, however, are more soluble in water than the copolymers and can easily and quickly bind to lectin, leading to the formation of relatively smaller aggregates. These are the first examples of glycopolymers that contain betulin and they showed promise for use in targeted release of drugs. Issues regarding the cytotoxicity, targeting effect, improvement of drug bioavailability and especially the medicinal effects of the betulin moieties are subjects of further studies.

3.5 Supporting information

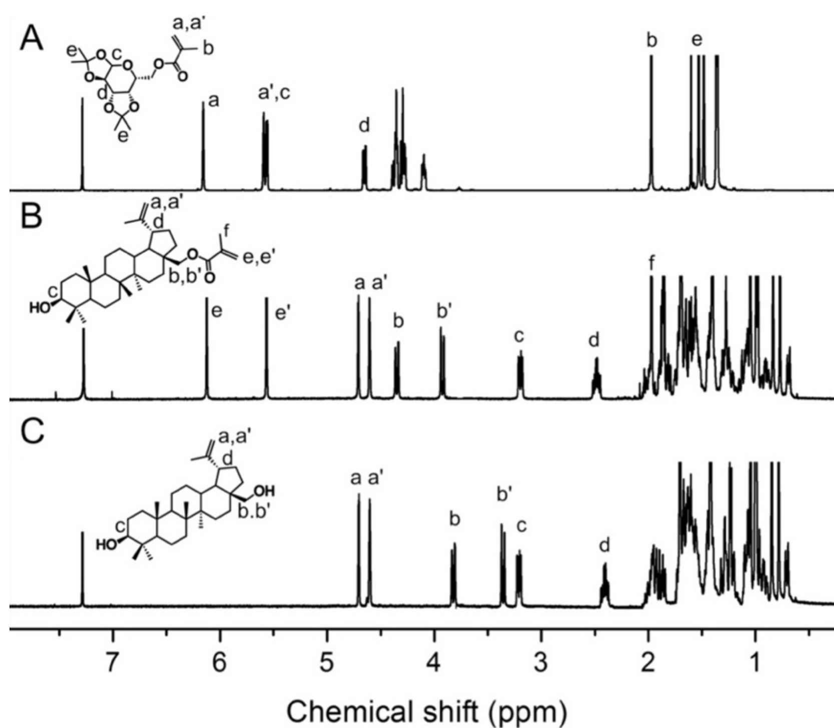


Figure 3.S1 ^{1}H NMR spectra of the synthesized monomers of (A) MIpGa, (B) MBet and (C) Betulin in $CDCl_3$.

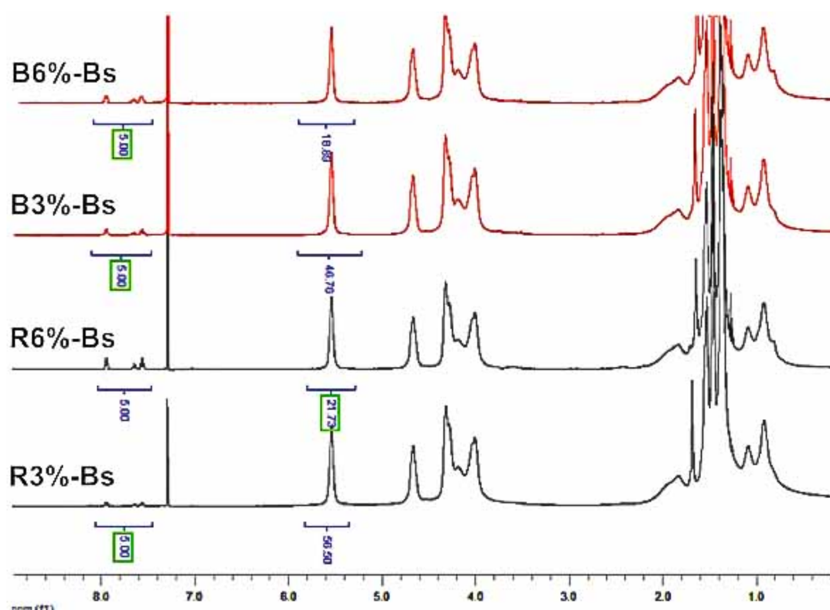


Figure 3.S2 ^{1}H NMR spectra of the glycopolymers functionalized with benzenesulfonyl group in $CDCl_3$. The peak at 5.53 ppm is attributed to the proton attached to the anomeric carbon in the sugar ring. The peaks at 7.40-8.05 ppm are from the benzenesulfonyl group.

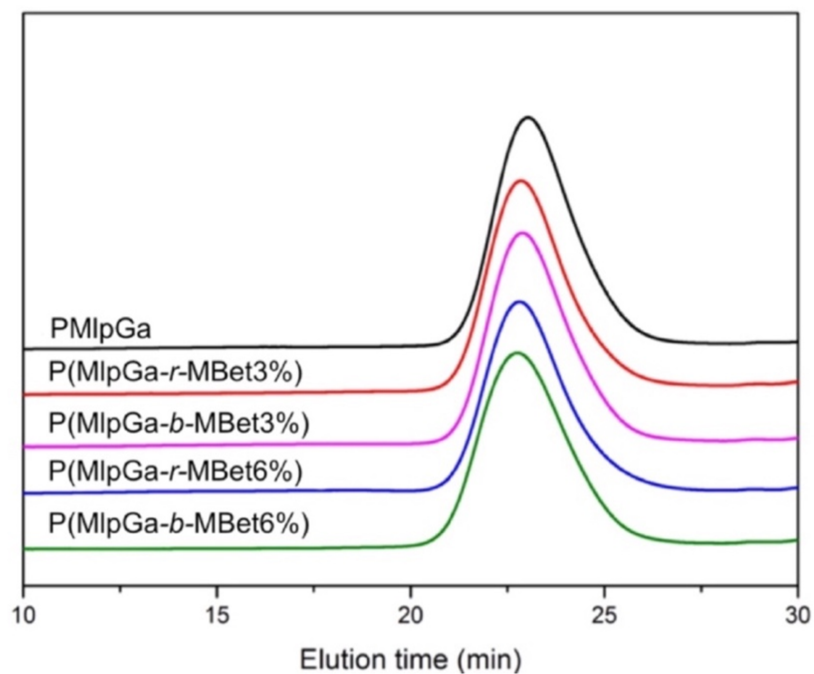


Figure 3.S3 SEC traces of the glycopolymers eluted with THF.

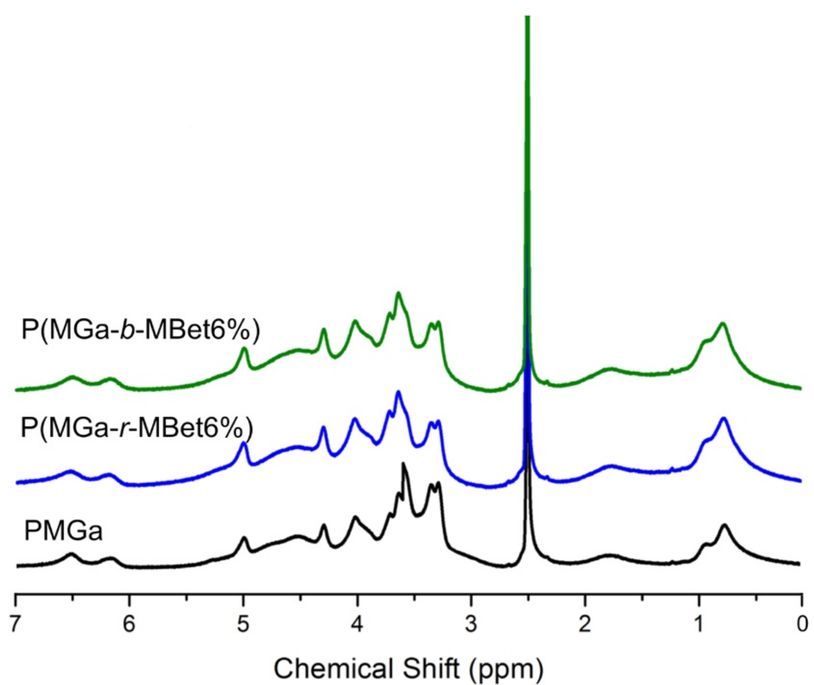


Figure 3.S4 ^{1}H NMR spectra of the deprotected glycopolymers in DMSO-d_6.

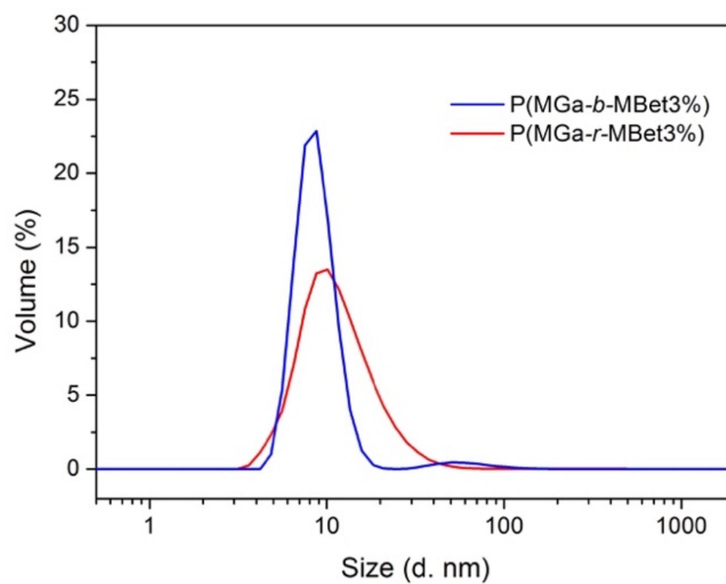


Figure 3.S5 Volume-average size distribution of the micelles formed by P(MGa-*r*-Mbet3%) and P(MGa-*b*-Mbet3%) at concentration of 1.0 g/L.

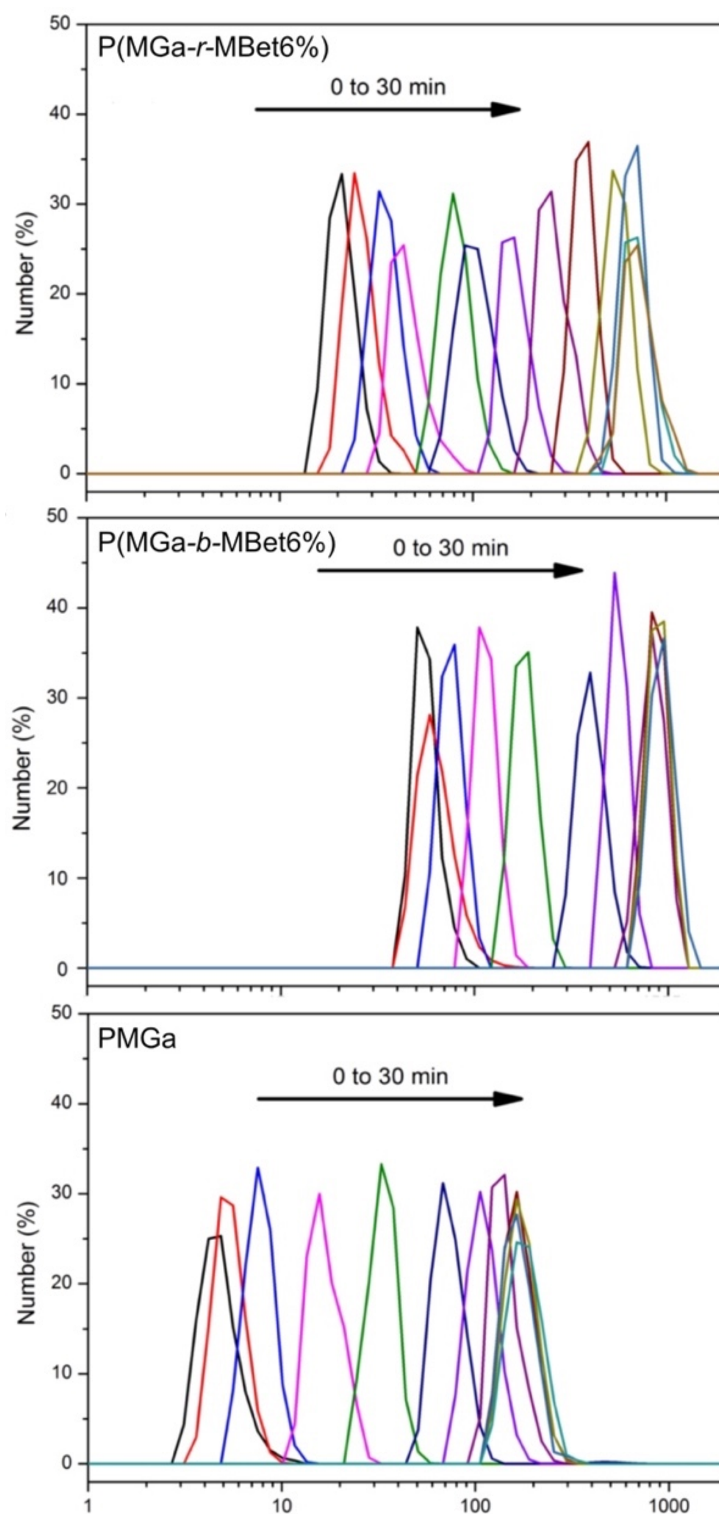


Figure 3.S6 Evolution (0 to 30 min) of the number-average size of the aggregates of selected glycopolymers (0.8 mL, 1 g/L) in water upon the addition of the lectin RCA_{120} (0.3 mL, 1 g/L) at room temperature, measured by light scattering.

3.6 Acknowledgments

We wish to thank Prof. Xiangzheng Hu of Tianjin University of Science and Technology, who provided betulin as a gift. The authors thank Mr. S. Essiembre for his technical support and Dr. M. Zhang for his help with TEM. Financial support from NSERC of Canada and FQRNT of Quebec is gratefully acknowledged. Z. Ma is grateful to the China Scholarship Council and FRQNT for a scholarship. The authors are members of CSACS funded by FQRNT and of GRSTB funded by FRSQ.

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Chapter 4
Core-crosslinked micelles made of glycopolymers bearing dopamine and cholic acid pendants*

Abstract

A series of block glycopolymers bearing galactose, dopamine and cholic acid (CA) pendants have been synthesized by RAFT polymerization. These copolymers can self-assemble into micelles in water. The dopamine moieties, located at the hydrophobic core, can self-polymerize in a weakly basic solution, stabilizing the micelles in both water and organic solvent (DMSO). The crosslinked micelles are smaller in size than the uncrosslinked precursors. Introducing more CA groups into the copolymers promotes the self-assembly to form larger aggregates, control the crosslinking of the stabilized micelles and facilitates the encapsulation of hydrophobic compounds such as Nile Red (NR). The amount of CA comonomers added also helps to control the crosslinking density which affects the loading and release of NR. The core crosslinked micelles displayed a slow but sustained NR release, and interact effectively with lectin (RCA_{120}), demonstrating their potential use as a biocompatible multifunctional platform for targeted release of drugs.

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*Z. Ma and X. X. Zhu, "Core-crosslinked micelles made of glycopolymers bearing dopamine and cholic acid pendants". *Mol. Pharmaceut.* **2018**, 15, 2348-2354.

4.1 Introduction

Amphiphilic block copolymers are usually composed of hydrophobic and hydrophilic segments, which spontaneously self-assemble in aqueous solutions to form core-shell micelles above their critical micelle concentration (CMC).[1-3] Polymeric micelles have potential applications in medicine, controlled drug delivery and diagnostic imaging, etc.[4-6] The micelles are rather unstable and may disintegrate at lower concentrations, causing the premature release of the entrapped substances at unexpected locations with serious side effects.[7-9] Crosslinked micelles are more stable and help to control the release of the encapsulated substances.[10-14] However, the crosslinking agents such as glutaraldehyde[15] and isocyanates[16] are often toxic and prone to leaching out in the body upon matrix biodegradation. Therefore, alternative stratagies of crosslinking are much needed. Dopamine (DA) is a natural compound, that can control its crosslink by self-polymerization time or pH-dependent ligand-metal coordination, making it suitable in the preparation of stable micelles.[17-21] It was found a small amount of dopamine (5% or less) was enough to stabilize the micelles by crosslinking.[22-24] Xin et al. found catechol-Fe^{3+} coordinated micelles could resist high volume dilution without losing their integrity during systemic circulation.[25] Wu *et al.* made core crosslinked micelles with improved stability via bubbling air to crosslink the DA containing biodegradable triblock copolymer.[22] Hwang *et al.* reported that polymeric micelles with DA-Fe^{3+} coordinated core crosslinks provide robustness, which are in response to pH.[26] However, the drug loading capacity and controlled release for the core crosslinked micelles are not as good as expected.

Cholic acid (CA) is an interesting and useful building block, due to its biocompatibility, amphiphilicity and self-assembling capacity, which was selected to make copolymers for investigating the effect of crosslinking density of the DA-grafted block copolymers to improve drug loading capacity and controlled release.[27-31] In order to generate robust micelles with high stability and good biocompatibility for targeted release, we designed and prepared a series of block glycopolymers with different contents of DA and CA via RAFT polymerization and studied their self-assembly behavior. Such micelles were loaded with a hydrophobic model drug for release studies. In this work, we demonstrate the principle that a low degree of crosslinking can help to stabilize the micelles formed by the amphiphilic copolymers by introducing a small amount of another biological compound, dopamine. It helps with the stabilization of the micelles during the transport and circulation of the drug delivery vehicle. The commonly used hydrophilic PEG is efficient in reducing nanoparticle uptake by the reticuloendothelial system and in prolonging micelle circulation,[32, 33] but inhibits cellular

uptake, leading to low drug delivery efficiency,[34] and sometimes, causes hypersensitivity.[35, 36] We also seek to replace PEG by a glycopolymer block, in which the sugar residues display good biocompatibility and biodegradability, to facilitate cell recognition due to specific sugar-lectin interactions.[37-39] The choice of sugar, molecular weight, and composition of the polymers can determine the lectin binding affinity.[40-43] We have already studied the effects of polymer structure and polymer chain length on the self-assembly of glycopolymers [41, 44] and the cluster effect for their binding with lectins.[44] In this work, two different lectins were used to study the binding specificity of the glycopolymers to the lectins and the result may be useful for their eventual application as drug carriers. Although the copolymers are not designed to be biodegradable, their molar masses are low enough for them to be eventually metabolized and eliminated from the body.

4.2 Experimental section

4.2.1 Materials

All reagents were purchased from Aldrich and used without further purification unless otherwise stated. *6-O*-Methacryloyl-*1,2:3,4*-di-*O*-isopropylidene-*D*-galactopyranose (MIpGa), [45] *p*-nitrophenyl acrylate (NPA)[46] and methacrylate of the PEGylated cholic acid (MCA)[47] monomer were synthesized according to the literature. *4*-Cyano-*4*-[(propyl sulfanylthiocarbonyl)sulfanyl] pentanoic acid was used as the chain transfer agent (CTA).[48] The *2,2′*-azoisobutyronitrile (AIBN) was recrystallized twice from methanol. Milli-Q water was used for the experiments, and *1,4*-dioxane was purified by treatment with sodium metal and redistillation.

4.2.2 Polymer synthesis

For RAFT polymerization, a macro-CTA (PMIpGa) was first synthesized. MIpGa (1.0 g, 3.0 mmol), CTA (5.82 mg, 0.02 mml) and AIBN (1.0 mg, 0.006 mmol) were dissolved in 4 mL of *1,4*-dioxane. The mixture was purged with argon for 30 min and then stirred at 75°C for 4 h. The polymerization was quenched by cooling in an iced bath. The macro-CTA was purified by precipitating with methanol three times and drying under vacuum. To synthesize the block copolymers, the macro-CTA (500 mg, 0.01 mmol), NPA (16.5 mg, 0.1 mmol), MCA (61 mg, 0.1 mmol) and AIBN (0.5 mg, 0.003 mmol) were dissolved in 4 mL of *1,4*-dioxane. After purging with argon for 30 min, the polymerization was carried out in a preheated oil bath at 75 °C and quenched after 12 h. By changing the ratio of NPA to MCA (1:0, 1:1 and 1:2), three

pre-copolymers were prepared. The copolymers were purified by precipitating with methanol three times to remove unreacted RAFT reagents, followed by drying under vacuum. After reacting with dopamine[49], deprotecting isopropylidene and dialysis, three block copolymers PMGa-*b*-P(ADA-MCA) were obtained and named GDC 97-3-0, GDC 94-3-3, and GDC 91-3-6; where the numbers indicate the molar percentages of the monomers bearing Ga, DA, and CA, respectively, in the copolymers.

4.2.3 Characterization

^{1}H NMR spectra of the monomers and polymers in DMSO-d_6 were recorded on a Bruker AV400 spectrometer. Size exclusion chromatography (SEC) was performed on a Breeze system from Waters equipped with a 717 plus autosampler, a 1525 Binary HPLC pump, and a 2410 refractive index detector. THF was filtered through 0.2 μm nylon Millipore filters and used as eluent (flow rate: 1 mL/min). Polystyrene reference samples (2500-608000 g/mol) were used as calibration standards for molar masses. Dynamic light scattering (DLS) was performed on a Malvern Zetasizer NanoZS instrument (Malvern CGS-2 apparatus) equipped with a He-Ne laser at a wavelength of 633 nm and a scattering angle of 173°. The polymer suspensions (1.0 g/L) were filtered through 0.45 μm Millipore filters. Transmission electron microscopy (TEM) was performed on a FEI Tecnai 12 TEM equipped with a Gatan 792 Bioscan 1k × 1k wide-angle multi-scan CCD camera at 80 kV. The samples were prepared by placing a drop of polymer solution (0.1 g/L in water) on carbon-coated copper grids (300 mesh, Carbon Type B, Ted Pella, Inc.). The solution was frozen, followed by the removal of water through freeze-drying. Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). The amounts of pyrene were chosen to reach a saturation concentration of 6×10^{-7} M. The emission spectra were recorded in the range of 350-500 nm with a fixed emission at 335 nm. The slit widths for excitation and emission were 5.0 and 2.5 nm, respectively. The intensity ratios of the third over the first emission bands (I_3/I_1) were recorded and plotted against the polymer concentration. The critical micelle concentration (CMC) was taken as the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low polymer concentrations.

4.2.4 Preparation of micelles

To prepare the micellar solution, 8 mg of the copolymer were directly dissolved in 4 mL of PBS buffer solution (0.01 M, pH 7.4), the mixture was equilibrated by shaking for 24 h at room

temperature, then the micellar solution was filtered through a Millipore 0.45-μm PTFE membrane. To prepare the crosslinked micelles, the same method was used to make the non-crosslinked micelles, but the pH of the PBS solution was adjusted by 0.05 M NaOH to pH 8.5 and the mixture was agitated in a water bath at room temperature for 24 h to allow the self-polymerization of dopamine. The micellar mixture was also filtered through the same PTFE membrane. For both micelles with and without crosslinking, the filtration was used to remove any large particles that may have formed to produce samples for the subsequent light scattering experiments.

4.2.5 Lection recognition and loading and release of Nile Red (NR) and doxorubicin (Dox)

The experimental conditions used for the NR loading and lectin recognition are based on a previous report.[44] The Dox-loaded micelles were prepared by the nanoprecipitation method.[50] Briefly, 0.5 mg of Dox·HCl was dissolved in 2 mL of DMSO and neutralized by adding 5.0 μL of triethylamine. The solution was stirred for 2 h at room temperature. Then, the Dox solution was added to 5 mL of the as-prepared micellar solution, either crosslinked or not. The micellar solution was incubated at room temperature for 2 h, then filtered through 0.45-μm filters and transferred into a dialysis tubing (MWCO 6,000-8,000 Da) placed in a PBS buffer (0.01 M, pH 7.4) for 24 h to remove any untrapped Dox and trimethylamine. The loading capacity was determined by taking 1 mL of the micellar solution, mixing with 1 mL DMSO, measuring the fluorescence intensity on a fluorescence spectrometer (excitation 485 nm, emission 590 nm), and comparing with a calibration curve for Dox in DMSO/PBS (v/v=1/1).

For *in vitro* drug release, the tubing was immersed in 50 mL of PBS (0.01 M, pH 7.4) and shaken at room temperature. At predetermined times, 1 mL of the surrounding solution of the tubing was collected and the released molecules were quantitively measured to calculate the amount released. This was repeated until no change in emission was observed, suggesting the complete release of the encapsulated Dox.

4.3 Results and discussion

4.3.1 Synthesis of glycopolymers

Dopamine is an efficient crosslinker. Xin et al. found that PEG with a dopamine end group could form stable micelles.[25] It is important to keep the dopamine content low, enough to

stabilize the micelles, but not too high to impair eventual metabolism and elimination of the polymers. For this purpose, the dopamine content was kept at 3 mol%. The same monomers were made before[31, 41, 44, 51] and again successfully made as confirmed by ^{1}H NMR spectra (Figure 4.S1). Block copolymers were synthesized via RAFT polymerization (Scheme 4.1). The molecular weight of the copolymers ranged from 41 to 58 kDa with relatively narrow dispersity (Table 4.1).

Scheme 4.1 Synthesis of the block copolymers.

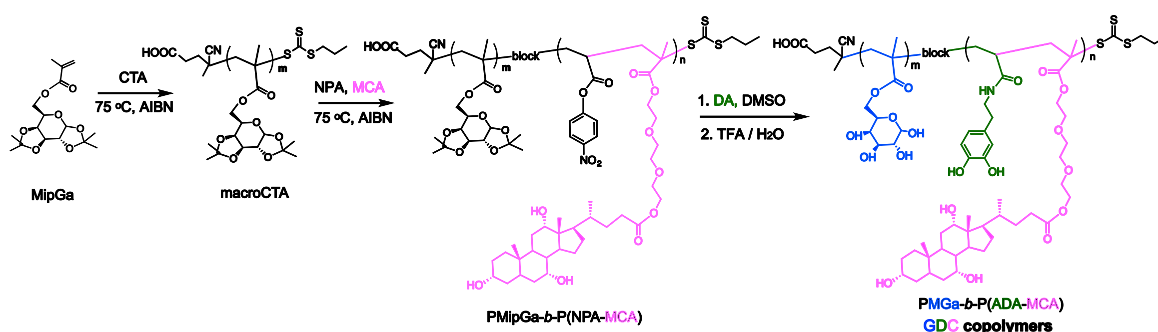

Table 4.1 Composition and characteristics of the block copolymers

Copolymers[a]	DA (mol%)[b]	CA (mol%)[b]	M_n (g/mol)[b]	M_n (g/mol)[c]	Đ[c]	CMC (mg/L)[d]
macroCTA	0	0	41,000	34,000	1.18	-
GDC 97-3-0	2.9	0	48,700	42,000	1.17	5.0
GDC 94-3-3	3.3	2.9	54,000	46,000	1.16	3.8
GDC 91-3-6	3.1	6.1	58,100	47,000	1.17	2.4

[a] The numbers in the abbreviated names of the copolymers represent the approximate molar percentages of Ga, DA and CA moieties in the copolymer. [b] Calculated from the ^{1}H NMR spectra. [c] Determined by SEC. [d] Determined with pyrene fluorescence data.

The conversion of the active ester groups to amide bonds with dopamine was almost quantitative as shown by NMR spectroscopy (Figure 4.S1). After dopamine substitution, the ^{1}H NMR spectra of copolymers show the appearance of the dopamine proton signals at 6.9-6.5 ppm and the disappearance the active ester signals at 8.30 and 7.38 ppm. The chemical compositions of the copolymers (Table 4.1) are calculated from the proton signal integrations of the ^{1}H NMR spectra (Figure 4.S1). The SEC traces (Figure 4.S2) show narrow and mono-disperse peaks indicating the successful synthesis of the block copolymers. After the dopamine

substitution and the deprotection in TFA/H_2O (8/2, v/v), the final glycopolymers were obtained. The ^{1}H NMR spectrum of glycopolymers show the disappearance of isopropylidene group and the appearance of new peaks of dopamine at δ = 6.64, 2.91 and 2.70 ppm (Figure 4.S3), indicating the block glycopolymers were synthesized successfully.

4.3.2 Formation of the core crosslinked micelles

Amphiphilic block copolymers are capable of forming micelles in water. The CMC values were measured by fluorescence spectroscopy with pyrene as a probe and are estimated to be about 5.0, 3.8 and 2.4 mg/L for GDC 97-3-0, GDC 94-3-3, and GDC 91-3-6, respectively. Introducing more CA into the copolymers increases the hydrophobicity and promotes self-assembly. Therefore, in this work, we designed and synthesized a series of block copolymers with 3 mol% of dopamine and studied the effect of introducing of cholic acid on the control of the crosslink of dopamine. The DA pendants in the hydrophobic core can undergo oxidative crosslinking in weakly basic solution (pH=8.5), stabilizing the micelles. We add an affix "X" to indicate the crosslinked samples. The self-polymerization process was followed by UV-vis spectroscopy (Figure 4.1). Initially, the solution has a characteristic absorbance of dopamine at λ_{max} = 280 nm. After changing the pH of the solution to 8.5 with NaOH (0.05 M), a progressive increase in the absorbance at 300 and 350 nm is observed over time. These two characteristic peaks represent respectively the formation of the dopaminochrome and the oxidation of dopamine.[52, 53] The changes in the UV-vis spectra of the copolymers in Figure 4.1B are in agreement with the dopamine results, where the absorbance decreases at 280 nm and slightly increases at 300 and 350 nm, suggesting the oxidative crosslinking of dopamine moieties. The crosslinked structure was confirmed by (transmission electron microscopy) TEM, DLS and ^{1}H NMR. Before crosslinking, the micelles displayed spherical structures (Figure 4.3) of 100, 113 and 164 nm in diameter (DLS, Figure 4.2, listed in Table 4.2), for GDC 97-3-0, GDC 94-3-3, and GDC 91-3-6, respectively. After crosslinking at pH = 8.5 overnight, the micellar size decreased to around 67, 88 and 113 nm (Table 4.2), due to the crosslinking. The same trend was found in TEM where the micelles shrank in size and became darker in color, indicating the polymerization of dopamine. Notably, the micellar sizes measured by TEM were smaller than those measured by DLS, due to the dehydration of the polymers during the sample preparation. In the ^{1}H NMR spectrum of the crosslinked micelles (Figure 4.S3), the typical peaks of DA and CA were broadened or disappeared, since these pendants are located at the micellar core, indicating the stabilization of the micelles in the organic solvent.

Table 4.2 Characteristics, loading capacities and cumulative release of NR from the micelles

Copolymers	Micellar Size (nm) [a] no NR	Micellar Size (nm) [a] loaded with NR	NR Loading Capacity (mg/g)[b]	NR Cumulative Release (%) [b, c]	NR Cumulative Release (%) [d]
Before crosslinking					
GDC 97-3-0	100	122	19.2	80	91
GDC 94-3-3	113	172	22.9	76	90
GDC 91-3-6	165	253	31.7	71	84
After crosslinking					
GDC 97-3-0:X	66	68	3.1	18	31
GDC 94-3-3:X	88	89	10.5	25	41
GDC 91-3-6:X	113	114	19.9	30	57

[a] Determined by DLS. [b] Determined by UV-Vis absorbance. [c] At the release point of 12 h. [d] At the release point of 60 h.

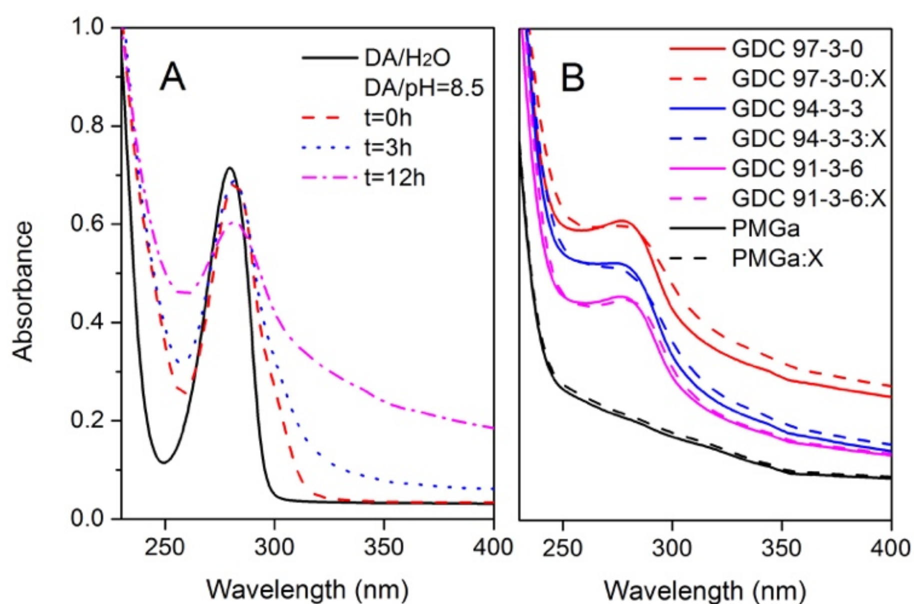

Figure 4.1 UV-vis spectra of (A) dopamine (DA) during self-polymerization at pH=8.5 and (B) glycopolymers before and after crosslinking.

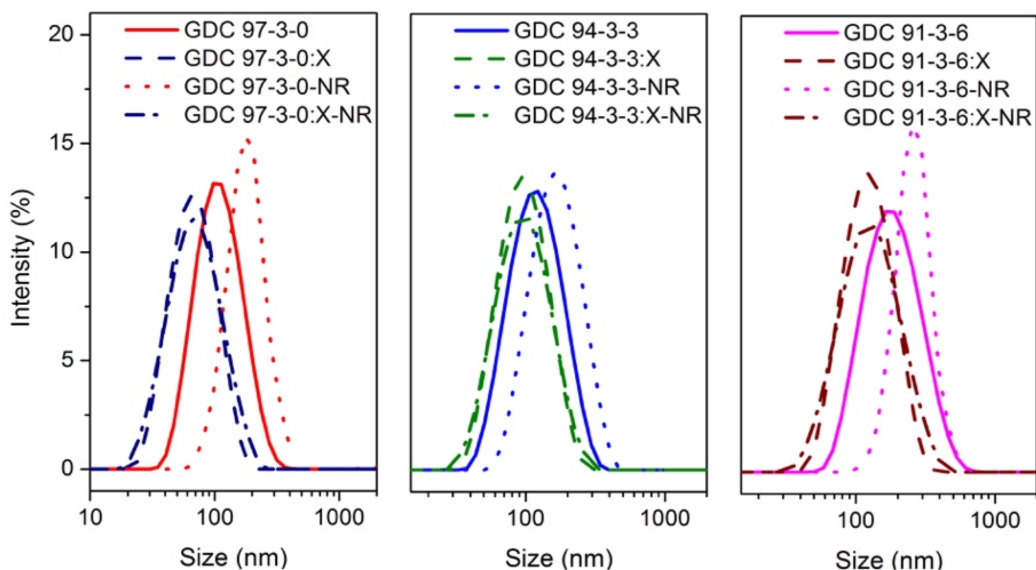

Figure 4.2 Intensity-average size distributions of the micelles in aqueous solution formed by copolymers before and after loading NR, as measured by DLS.

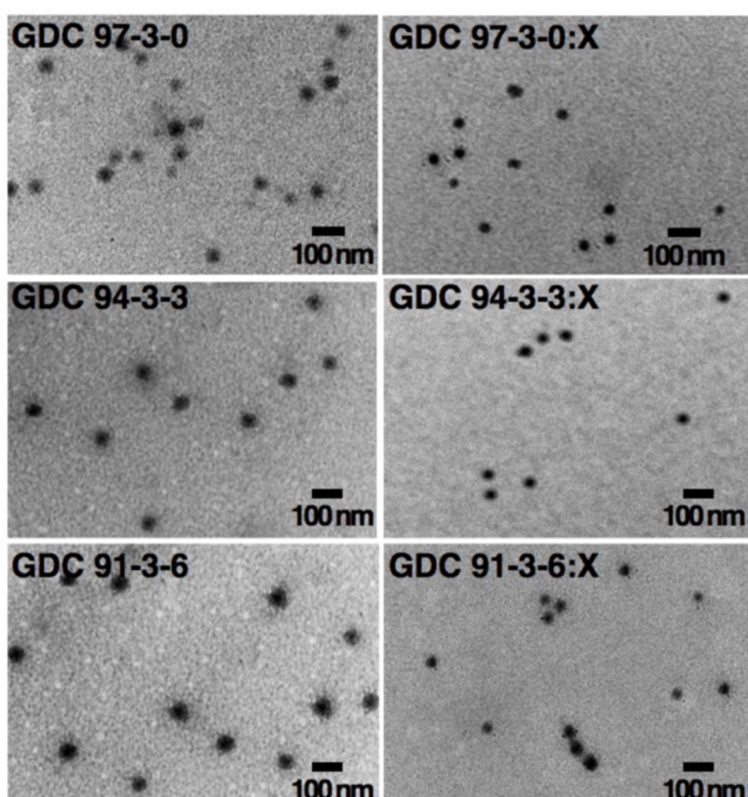

Figure 4.3 Representative TEM images of the micelles.

4.3.3 Loading and release of NR

Nile Red (NR) has poor solubility in water and was used as a model molecule to be loaded into the core of the micelles. The sizes of the NR-loaded micelles (Figure 4.2) were much larger

than the micelles before loading with NR. DLS measurements gave mean D_h values at 122, 172 and 253 nm (Table 4.2), respectively, for the NR-loaded micelles. For the crosslinked micelles, they were first crosslinked in weak basic media and then loaded with NR that was pre-dissolved in a minimal amount of THF, followed by the removal of THF with rotary evaporation and the rehydration of the system by the addition of water to reach the same volume. The size of the crosslinked micelles remained unchanged after loading with NR, with a slight increase in the size distribution. The NR loading capacity of the micelles formed by GDC 97-3-0, GDC 94-3-3, and GDC 91-3-6 were 19.2, 22.9 and 31.7 mg/g (Table 4.2), respectively. After crosslinking, all three polymers showed lower NR loading capacities: 3.1, 10.5 and 19.9 mg/g (Table 4.2). The introduction of CA moieties in the second block may have decreased the crosslinking density and facilitated the encapsulation of the NR. The hydrodynamic size, loading capacity and cumulative release of NR of the micelles are listed in Table 4.2.

The effect of crosslinking on the release of NR was studied in PBS solution (pH=7.4) at room temperature. Figure 4.4A shows that NR release from the crosslinked micelles is slower than from the uncrosslinked micelles. At 12 h, about 80, 76 and 71% of NR was released from GDC 97-3-0, GDC 94-3-3 and GDC 91-3-6, but after crosslinking these values reduced to 18, 25 and 38% (Table 4.2), respectively. The cumulative release of NR from crosslinked micelles gradually increase with increasing CA content in the copolymers. The NR release from the micellar core into the aqueous media was efficiently slowed down by the core crosslinked structure and controlled by the crosslinking density.

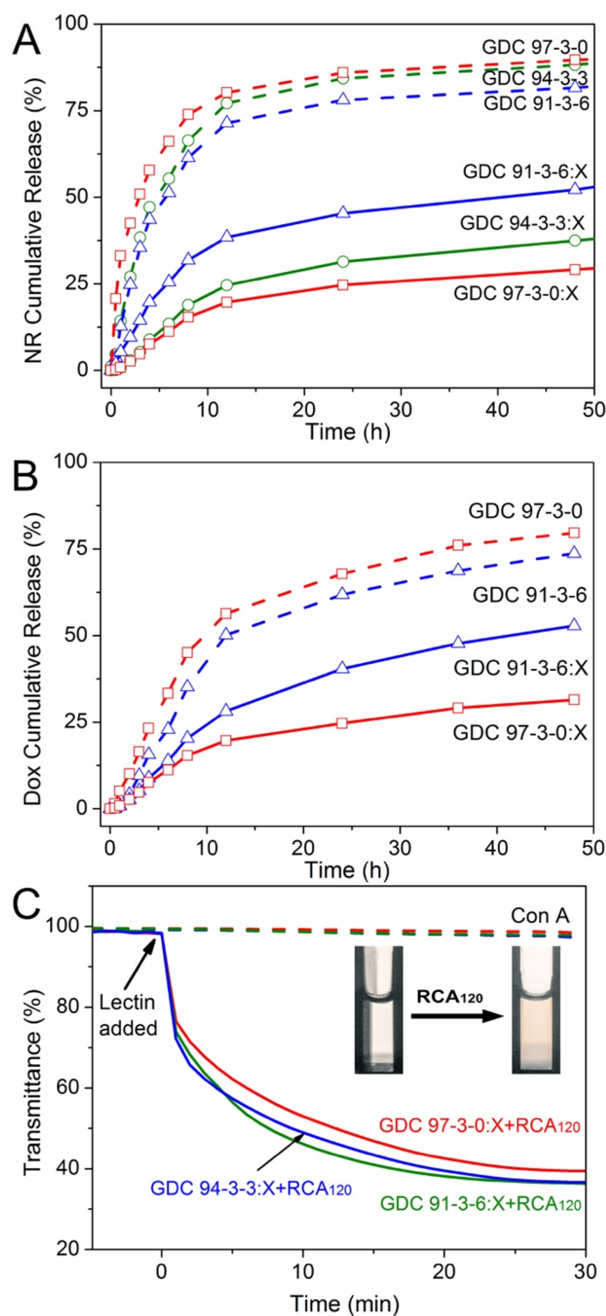

Figure 4.4 (A) NR release profiles from the uncrosslinked micelles (dashes) and crosslinked micelles (solid lines) in PBS solutions (pH=7.4). (B) Dox release profiles from the uncrosslinked micelles (dashes) and crosslinked micelles (solid lines) in PBS solutions (pH=7.4). (C) Transmittance change at λ = 420 nm of the solution of crosslinked micelles (1 mg ml^{-1}) upon the addition of RCA_{120} (solid lines) and Con A (dashes).

4.3.4 Loading and release of Dox

The micelles formed by GDC 97-3-0 and GDC 91-3-6 before and after crosslink were selected as representative samples to study the loading and release of Dox. The micelles formed

by GDC 97-3-0 and GDC 91-3-6 exhibited Dox loading capacity of 7.4 and 18.2 mg/g, respectively, these values decreased to 1.9 and 8.4 mg/g upon crosslinking. Figure 4.4B shows that 56.3% and 50.1% of Dox were released at the point of 12 h in the cases of GDC 97-3-0 and GDC 91-3-6, respectively. The crosslinked micelles GDC 97-3-0:X and GDC 91-3-6:X release 19.6% and 28.1%, respectively. The release of Dox from the crosslinked micelles was significantly slower under the same conditions. This is consistent with the results of NR release mentioned above. The total amount of the drug releases from the crosslinked micelles increases with higher CA content in the copolymers since the CA units effectively lower the degree of crosslinking.

4.3.5 Lectin recognition of glycopolymers

Glycopolymers have the advantage of specific interactions with lectins, important in cell adhesion and signal transduction. The multivalent binding of glycopolymers to lectins results in the formation of aggregates.[54-56] Ligand-lectin binding abilities of the core crosslinked micelles were determined by specific binding with RCA_{120}, a lectin that specifically binds to galactosyl residues. Another nonspecific lectin Con A served as a control. The lectin agglomeration capability was monitored by observing turbidity changes with UV-vis spectroscopy at 420 nm. After the addition of RCA_{120} into the clear micelle solutions, the binding between lectin and the sugar units of the copolymers caused aggregation, which leads to a gradual increase in the turbidity of the samples in water (Figure 4.4C inset). The light transmittance of the mixed solution of GDC 97-3-0:X, GDC 94-3-3:X and GDC 91-3-6:X gradually decreased with time until a plateau was reached (Figure 4.4C). Even though the content of glycomonomer varied by a few percentages with the incorporation of MCA (the monomer containing cholic acid), the difference in the light transmittance is not obvious and remained approximately the same within experimental error for the binding with RCA_{120}. As a control experiment, Con A solution was added to a blank PBS buffer with the same polymers under the same condition, but no obvious change in transmittance of the mixture solution was observed, indicating the specific binding interaction between the RCA_{120} and the core crosslinked micelles.

4.4 Conclusion

We designed and prepared three block copolymers containing galactose, DA and CA pendants via RAFT polymerization. The copolymers have a methacrylate backbone and are

not designed to be biodegradable, but the use of RAFT polymerization methods allows the control of their molar masses which were designed to be low enough for them to be cleared from the body. These copolymers can all self-assemble into micelles. The hydrophobic pendants are located at the core of the micelles, where the DA units self-polymerize in a weakly basic solution to crosslink the core of the micelles. The crosslinked micelles are able to remain intact in water. The NR release profiles of the core crosslinked micelles show a slow but sustained drug release. The micelles formed by these glycopolymers can selectively bind to RCA_{120}. The use of dopamine units in the glycopolymers is a useful and convenient alternative solution to crosslink micelles to enhance their stability during circulation for pharmaceutical applications as drug carriers for targeted release. The degree of crosslinking is designed to be very low and this strategy is meant to stabilize the micelles for a limited duration and the crosslinked dopamine may also be degraded as reported previously [57, 58]. The dopamine content was kept low at 3 mol% and was shown to be effective. It is possible to reduce even further the amount of dopamine used for crosslinking in the future. The effort made in this work should lead to the stabilization of the polymeric micelles during the transport and circulation of such drug delivery vehicles, which should eventually lead to a more efficient and more effective system.

4.5 Supporting information

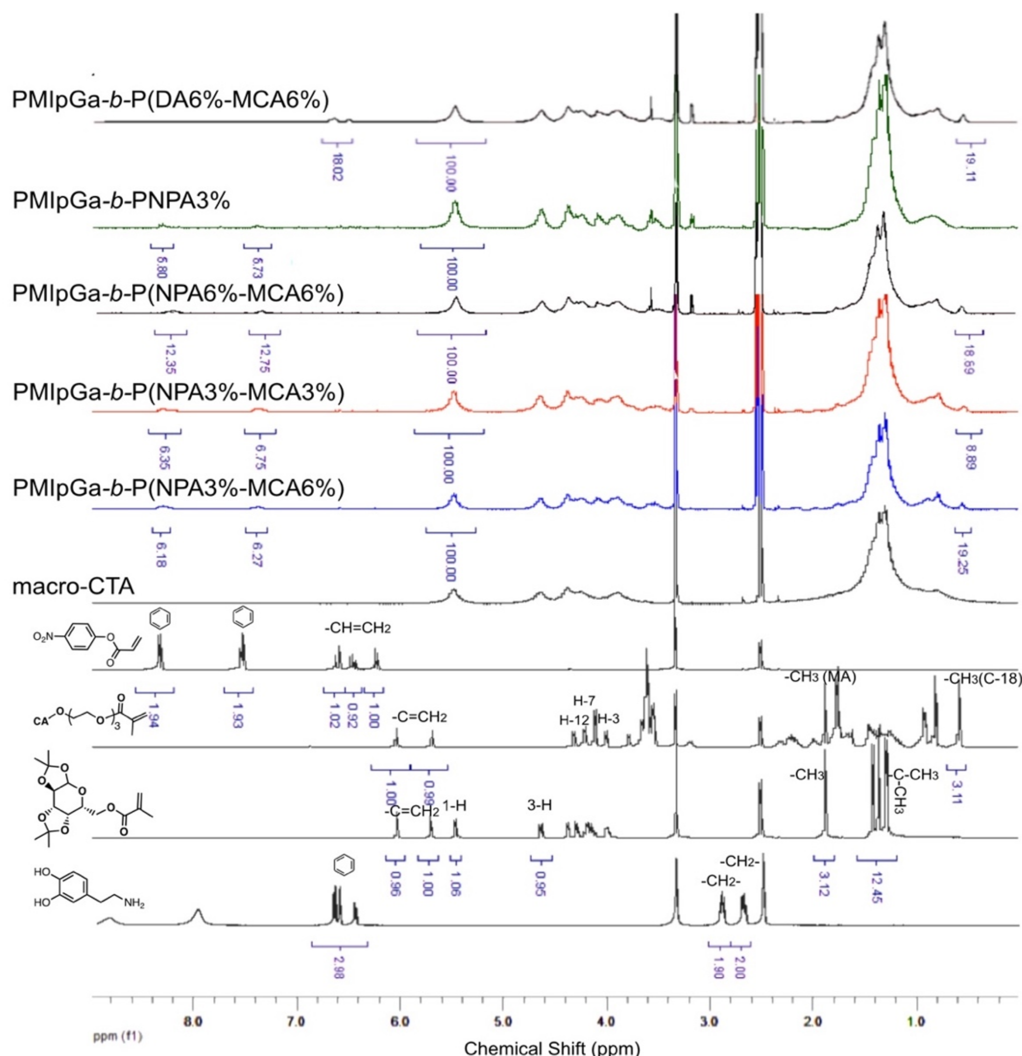

Figure 4.S1 ^{1}H NMR spectra of the monomers and the copolymers in DMSO-d_6. The peak at 5.53 ppm is attributed to the proton attached to the anomeric carbon in the sugar ring. The peaks at 8.30 and 7.38 ppm are from the benzene ring of the NPA. The signal at 0.63 ppm is assigned to the methylene group of cholic acid. After dopamine substitution, ^{1}H NMR spectra of copolymer PMIpGa-*b*-P(DA3%-MCA6%) show the dopamine peaks at 6.90-6.50 ppm and the disappearance of the active ester peaks at 8.30 and 7.38 ppm. The dopamine substitution is almost quantitative as estimated from the integration of the ^{1}H NMR signals. The molar percentage of DA and MCA can be calculated from the integration ratios of the peaks at δ = 6.90-6.50 ppm and 0.63 ppm. The spectrum confirms that the H-3, H-7 and H-12 protons of cholic acid remained unchanged in the MCA monomer. Previously, we also made copolymers containing 6 mol% dopamine. But they were not selected for the Nile Red loading and release studies.

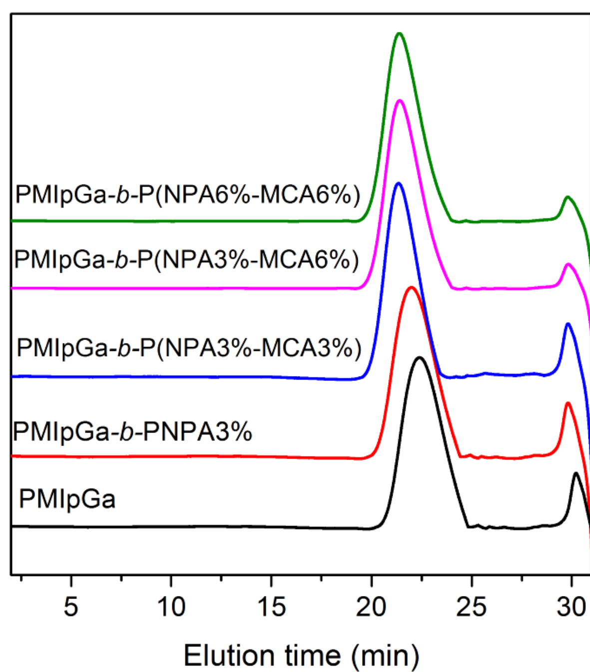

Figure 4.S2 SEC elution traces of the macro-CTA (PMIpGa) and the copolymers, all protected with the isopropylidene (Ip) group to keep them soluble in THF.

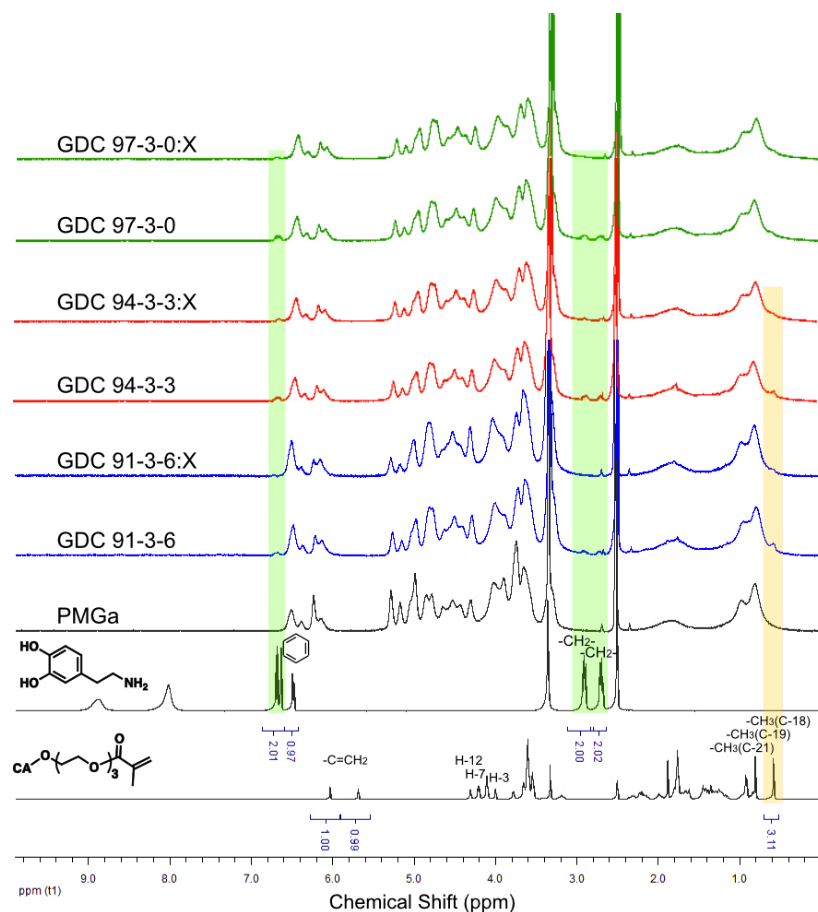

Figure 4.S3 ^{1}H NMR spectra of the copolymers and the crosslinked micelles (X) in DMSO-d_6. ^{1}H signals of the small molecules are indicated in the spectra. The disappearance of selected ^{1}H signals upon crosslinking is shown in the shaded areas.

4.6 Acknowledgments

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Chapter 5

Enzymatic conversion of galactose-polymers into copolymers containing galactonic acid by glucose oxidase*

Abstract

The conversion of a glycopolymer into a copolymer containing sugar acid groups though the use of enzymes such as glucose oxidase (GOx) represents a useful green chemistry synthetic method. GOx can oxidize a small carbohydrate substrate such as glucose into gluconic acid, but did not show activity on the corresponding methacrylic glycomonomers or glycopolymers. To solve this problem, we introduced a poly(ethylene glycol) (PEG) spacer between the carbohydrate (galactose) pendant and the methacrylic unit by anionic polymerization and found that GOx showed enzymatic activity on the galactose pendants when the PEG spacer is sufficiently long. The PEGylated galactose pendants can be successfully converted into galactonic acid yielding a copolymer. The PEGylated glycopolymers showed stronger binding to the sugar-specific lectin than the glycopolymers without the PEG spacer and reduced binding when some of the sugar pendants were converted to acid groups.

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*Z. Ma, A. Cunningham, and X. X. Zhu, "Enzymatic conversion of galactose-polymer into copolymers containing galactonic acid by glucose oxidase", to be resubmitted for publication.

5.1 Introduction

Polysaccharides play pivotal roles in many cellular activities such as mediating signal transfer, targeting bacterial infection, and serving as glue in cell adhesion and cell growth, where the sugar-binding proteins, known as lectins, are usually specific and multivalent.[1, 2] Because of their biodegradability and biocompatibility, polysaccharides are candidates for various bioapplications, including enzyme immobilization, environmental remediation, drug/gene delivery and bioimaging.[3-6] Glycopolymers are synthetic polymers containing sugar moieties with the ability to mimic the functions of natural carbohydrates.[7-10] Well-defined glycopolymers have been synthesized by the use of atom transfer radical polymerization (ATRP),[11] reversible addition-fragmentation chain-transfer polymerization (RAFT)[12-14] and post-modification reactions.[15-17] Glycomonomers can be copolymerized with other functional monomers to obtain copolymers with desired properties for specific applications.[18-20] Copolymers containing sugar and oxaborole moieties were synthesized by free-radical polymerization to obtain hydrogels with self-healing properties.[21] Copolymers bearing galactose and acid-labile ortho ester residues were made for an acid-triggered degradable drug delivery system to enhance tumor-targeting properties.[22] pH-responsive glycopolymers with different hydrophobicities (C8 and C6 alkyl chains) showed various types of multi-micellar assemblies such as spherical aggregates, fractals, and micellar clusters, depending on the pH of the medium.[23] A series of amphiphilic glycopolypeptide analogues with redox-responsive disulfide linkages were prepared through a combination of RAFT polymerization, ring-opening polymerization, and “click” coupling reactions, for the purpose of serving as redox-responsive, biocompatible materials.[24] Since the reactivity and solubility of monomers are different, the reagents used in the polymerizations are not always benign, making the preparation of biocompatible glycopolymers challenging.

Glucose oxidase (GOx, EC 1.1.3.4) is a well-characterized enzyme consisting of two identical subunits with two FAD co-enzymes.[25, 26] GOx shows high glucose specificity, but it can also oxidize other sugars and mono-, di-, tri-, and oligosaccharides with varying enzymatic activity. GOx converts sugars into their acid derivatives in water, which makes possible the preparation of enzymatically converted copolymers containing oxidized sugar acid derivatives under mild reaction conditions. To the best of our knowledge, there is no report on the oxidation of sugar pendants of glycopolymers via GOx. The GOx activity on galactose (Ga) is lower than on glucose, but still reaches an activity ca. 51%.[27] Therefore, we selected Ga as the starting point since four of the -OH groups can be easily protected, leaving one primary hydroxyl group

at position 6 for the synthesis of the glycomonomer. A PEG spacer was introduced to enhance the accessibility of the sugar pendant group. The effect of the PEG spacer length on the oxidation of the glycopolymer is studied in this work, followed by glycopolymer-lectin binding studies.

5.2 Experimental section

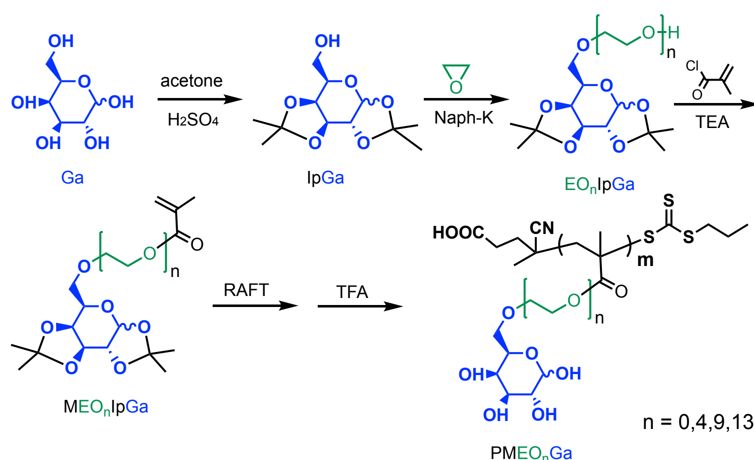
5.2.1 Materials

All reagents were purchased from Sigma Aldrich and used without further purification unless otherwise stated. Ethylene oxide (EO) was passed through a calcium hydride column and condensed using dry ice and acetone for quantification by volume before transfer to a reaction vessel. THF was purified by treatment with sodium metal and redistillation. Potassium naphthalene (Naph-K) was prepared directly in dry THF from naphthalene (> 99%) and potassium (98% in mineral oil) with a concentration of 0.45 M, as previously reported.[28] *4*-Cyano-*4*-[(propylsulfanylthiocarbonyl)sulfanyl] pentanoic acid was used as the chain transfer agent (CTA). *2,2'*-Azoisobutyronitrile (AIBN) was recrystallized twice from methanol. Milli-Q water was used for all experiments.

5.2.2 Synthesis of PEGylated glycomonomers

Scheme 5.1 shows the synthesis of the PEGylated glycomonomer. According to a previously published procedure, *1,2:3,4*-di-*O*-isopropylidene-*D*-galactopyranose (IpGa) was synthesized first.[29] For the PEGylation sugar, all glassware and needles were flame-dried under vacuum and purged with argon three times before use. To prepare EO_9IpGa, for example, IpGa (1.30 g, 5.0 mmol) was placed into a 100 mL flask charged with 20 mL of dry THF. Naph-K (1.0 mmol, 0.20 equiv.) was introduced dropwise into the flask via a canula and a pressure difference using argon. Dry ethylene oxide (2.3 mL, 10 equiv., chilled in dry ice/acetone) was introduced into the flask and polymerized at 45 °C for 48 h. The reaction mixture was neutralized and quenched with concentrated HCl (1 mL). The mixture was filtered to remove the insoluble salt, and THF was removed by rotary evaporator. The product was washed with hexane (20 mL × 5) to remove naphthalene and dried under vacuum. Other PEGylated IpGa with PEG spacers of different lengths were synthesized by the same procedure but with different amounts of ethylene oxide. These PEGylated galactose sample with protecting groups were named EO_nIpGa where the number n indicates the number of ethylene oxide (EO) units.

Scheme 5.1 Synthesis of the glycopolymers.



For the preparation of the monomer MEO_9IpGa, EO_9IpGa (1.45 g, 2.0 mmol) and triethylamine (0.7 mL, 5 mmol) were dissolved in CH_2Cl_2 (30 mL) and cooled to 0 ºC. A solution of methacryloyl chloride (5.0 mmol, in 10 mL of CH_2Cl_2) was added dropwise over a period of 1 h while maintaining continuous stirring at 0 ºC. The resulting mixture was gradually warmed to room temperature and stirred for 48 h. The insoluble salt was filtered off, and the crude product was concentrated and washed with 5% aqueous NaOH and brine. The glycomonomer was reprecipitated from its THF solution in hexane three times to afford a light-yellow oil (yield: 77%). ^1H NMR ($CDCl_3$, 400 MHz) δ 6.13 (m, 1H, =CH*H*), 5.58 (m, 1H, =C*H*H), 5.53 (d, 1H, Ga-*H* at 1 position), 4.62 (dd, 1H, Ga-*H* at 3 position), 4.30 (m, 2H, Ga-*H* at 2 and 4 position), 3.95-3.59 (m, Ga-*H* at 5 and 6 position and CH_2CH_2O), 1.95 (s, 3H, $CH_3CR=CH_2$), 1.53-1.33 (m, 12H, $(CH_3)_2COO$). ^{13}C NMR ($CDCl_3$, 101 MHz) δ 161.40, 138.20, 126.28, 109.58, 108.92, 96.73, 77.75, 77.75, 77.43, 77.12, 72.96, 71.53, 71.13, 71.03, 70.98, 70.93, 70.85, 70.83, 70.73, 70.68, 70.30, 67.20, 67.18, 62.08, 26.38, 25.33, 24.84, 18.43.

Other glycomonomers with different PEG spacers lengths were synthesized by the same procedure.

5.2.3 Synthesis of glycopolymers

The polymerization and the reaction conditions were as reported previously.[8] A series of glycopolymers with different molar masses and PEG spacers were synthesized by RAFT and are listed in Table 5.1.

Table 5.1 Characteristics of the glycopolymers with different PEG spacer

Glycopolymers[a]	[M]/[CTA][b]	Yield (%)[c]	M_n (Da)[d]	Đ[d]
PMIpGa-5K	20	89.0	4,800	1.15
PMIpGa-20K	100	86.4	19,900	1.19
PMEO$_4$IpGa-6K	20	87.0	6,100	1.23
PMEO$_4$IpGa-15K	50	85.3	15,200	1.18
PMEO$_9$IpGa-7K	20	71.2	7,100	1.26
PMEO$_9$IpGa-18K	50	69.5	18,000	1.22
PMEO$_{13}$IPGa-7K	20	66.4	7,200	1.24

[a] The subscripts indicate the number of EO units in the glycomonomer and the last numbers indicate the approximate molar mass of the glycopolymer. [b] The feed ratio of monomer [M] to CTA. [c] Calculated from the ^{1}H NMR spectra. [d] Determined by SEC.

5.2.4 Characterization

^{1}H NMR spectra of the monomers and polymers in $CDCl_3$ were recorded on a Bruker AV400 spectrometer. Size exclusion chromatography (SEC) was performed on a Breeze system from Waters equipped with a 717 plus autosampler, a 1525 Binary HPLC pump, and a 2410 refractive index detector. THF was filtered through 0.2 μm nylon Millipore filters and used as eluent (flow rate: 1 mL/min). Polystyrene standards (2500-608000 g/mol) were used for calibration.

5.2.4.1 Enzymatic assays

Glucose oxidase from *aspergillus niger* (GOx, type X-S, 155 unit/mg) and horseradish peroxidase (HRP, EC 1.11.1.7, 148 unit/mg) were purchased from Sigma Aldrich. GOx-HRP solutions containing GOx (100 unit/mL) and HRP (100 unit/mL) in PBS buffer were prepared and stored at 6 °C. Stock solutions were prepared of the Ga (10 mM) and $ABTS^{2+}$ (10 mM) in PBS buffer (10 mM, pH=7.4). Glycopolymer solutions containing 10 mM of galactose residues on polymers for enzymatic reaction were prepared by dissolving the appropriate quantity of glycopolymer in PBS buffer and stored at 6 °C. The GOx-HRP cascade reaction was carried out in a UV cell on a Cary 500 UV-vis spectrophotometer (Agilent Technologies) according to a previous report.[30] Enzymatic activities were determined by recording the ABTS absorbance changes at 414 nm. All the assays were replicated thrice with different batches of samples. The

concentration of the sugar residues and of the enzymes were varied, but the [ABTS] / [sugar] and [HPR] / [GOx] ratios were always kept at 2, and 1, respectively, throughout this study.

5.2.4.2 Lectin recognition

Glycopolymer-lectin binding experiments have been evaluated with a galactose-binding lectin, Ricinus communis agglutinin 120 (RCA_{120}, a 120 kDa tetramer from castor bean, Aldrich). The RCA_{120} solution (42 μM) in PBS buffer was freshly prepared before lectin recognition. The experimental conditions are based on previous reports.[31, 32]

5.3 Results and discussion

5.3.1 Synthesis of glycopolymers

The synthesis of PEGylated glycomonomers and glycopolymers is illustrated in Scheme 5.1. IpGa with a primary alcohol group was used as the initiator for the anionic polymerization of ethylene oxide to afford PEGylated IpGa. Three PEGylated IpGa samples with different PEG lengths were analyzed by ^{1}H NMR spectroscopy (Figure 5.S1). The proton signals at 5.53 and 4.62 ppm were respectively assigned to the protons at positions 1 and 3 on the sugar ring. They are used as reference to compare the signals at 3.59 to 3.95 ppm, which correspond to the protons on the PEG chain and the protons at positions 5 and 6 on the sugar ring. The PEG spacer of EO_nIpGa were determined to have 4, 9 and 13 ethylene oxide (EO) units (Table 5.1). After reacting with methacryloyl chloride, new proton peaks at δ 6.13, 5.58 and 1.95 ppm are attributed to the methylacrylate group. A series of glycopolymers with different molar masses and PEG spacer lengths were synthesized by RAFT polymerization. The glycopolymers were analyzed by ^{1}H NMR spectroscopy and SEC. Typical ^{1}H NMR spectra for the glycopolymer with a PEG spacer of 9 EO units with galactose pendants before and after deprotection are shown in Figure 5.S2. The protons of the isopropylidene (Ip) protecting groups are visible at 1.2-1.6 ppm. The featured proton resonance signals at 5.43 and 4.55 ppm were assigned to the protons attached to the anomeric carbon of the sugar ring. The signals at 0.7-1.2 and 1.5-2.1 ppm may be assigned to the protons of methylene and methyl groups in the polymer backbone. The SEC traces (Figure 5.S3) show narrow and monodisperse peaks, suggesting the successful synthesis of the glycopolymers with relatively narrow molar mass distributions. The characteristics of the copolymers are listed in Table 5.1. The Ip protecting groups were removed by treatment with in TFA/water (8/2, v/v) for 2 h. After dialysis against Milli-Q water for 3 days, the final glycopolymers were dried and collected. The disappearance of the proton

signals of the Ip protecting groups indicates their successful removal. New ^{1}H NMR peaks at 4.0-5.1 ppm are attributed to the free secondary alcohol protons of the sugar.

5.3.2 Galactose oxidation

The oxidation of galactose was evaluated by a cascade enzyme reaction with *2,2'*-azino-bis(*3*-ethylbenzothiazoline-*6*-sulfonic acid) diammonium salt (ABTS) as a chromogenic substrate (Scheme 5.2). GOx catalyzes the oxidation of galactose into galactonolactone and yields H_2O_2 which will further convert colorless $ABTS^{2+}$ into chromogenic $ABTS^{+\cdot}$ by reacting with HRP. $ABTS^{+\cdot}$ is detectable at 414 nm by UV-vis spectroscopy.[27] Therefore, the oxidation of Ga is directly related to the final absorbance of $ABTS^{+\cdot}$. The galactonolactone spontaneously breaks down into galactonic acid afterward. Zhang and coworkers optimized this cascade enzymatic reaction and found the [ABTS] / [sugar] and [HRP] / [GOx] ratios at 2 and 1, respectively, showed fast sugar oxidation and slow enzyme inhibition.[30] Therefore, the same ratios were kept in this work, and [GOx] and [Ga] are noted in the following discussion.

Scheme 5.2 Schematic representation of cascade reaction.

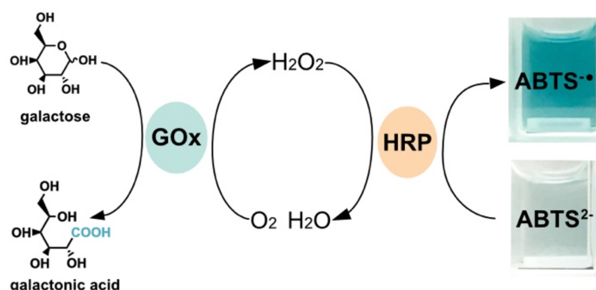


Figure 5.1A shows the UV-vis absorption at 414 nm of ABTS solutions containing variable amounts of galactose and 4 unit/mL of GOx as function of time. The galactose concentration varied from 0.05 to 0.5 mM, and the solvent color changed from colorless to dark green (Figure 5.1A inset). At [Ga] = 0.3 mM, the absorbance value is ca. 0.8 (15 h). Then [Ga] was kept constant at 0.3 mM, while the amount of GOx was varied from 3 to 9 unit/mL (Figure 5.1B). Increasing the amount of enzyme gradually shortens the time to reach a plateau, leading to a slightly higher concentration of $ABTS^{-\cdot}$ at the same measurement time. The Ga oxidation with 6 unit/mL of GOx reached a plateau within 12 h. However, adding more enzymes caused the absorbance to increase first and then to decrease, due to the inhibition of the enzyme by the rapid galactonolactone accumulation.[30] In this case, we selected [Ga] and [GOx] as 0.3 mM

and 6 unit/mL, respectively, for the oxidation of glycomonomers and corresponding glycopolymers.

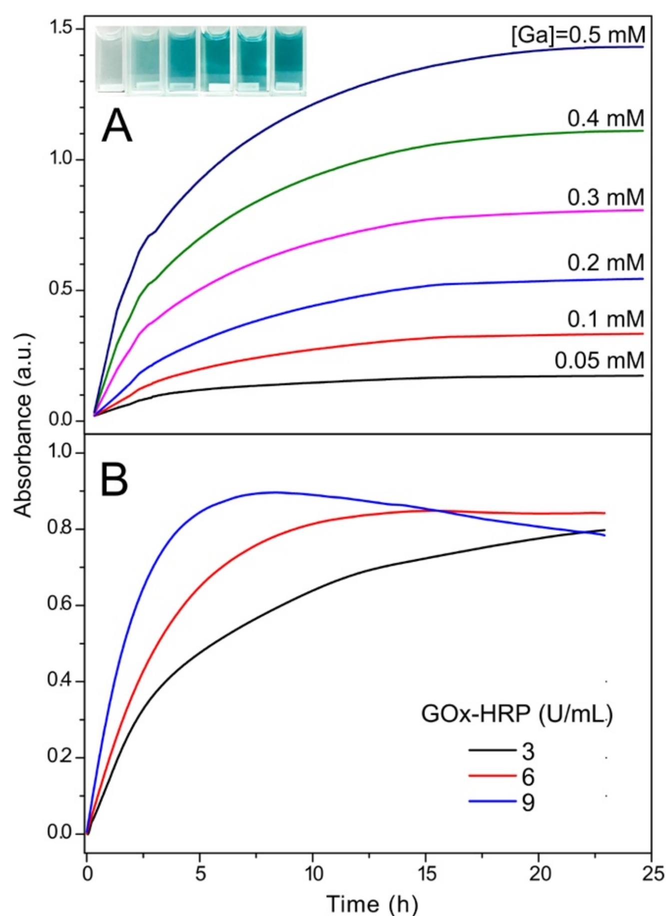


Figure 5.1 UV-vis absorption at 414 nm of PBS buffer at 35 °C solutions containing (A) variable Ga and constant GOx (4 unit/mL), (B) constant Ga (0.3 mM) and variable GOx. Adding more enzyme shortens the time to reach a plateau but may cause inhibition due to the rapid galactonolactone accumulation. Note: The [HRP] / [GOx] and [ABTS] / [sugar] ratios were constant at 1 and 2, respectively.

5.3.3 Glycomonomer and glycopolymer oxidation

The isopropylidene protection groups of PEGylated glycomonomers and polymers were removed by treatment with trifluoroacetate acid for 2 hrs. After neutralization and purification, the deprotected glycomonomers and glycopolymers were obtained and their enzymatic conversions were texted. Figure 5.2A shows the absorbance changes of the buffer solutions of representative PEGylated galactose (0.3 mM) with different PEG spacers. With PEG spacer, the absorbance showed a decrease from 0.8 (Ga, Figure 5.1B) to ca. 0.4 for PEGylated sugar.

These results suggest that the steric hindrance of the PEG spacers may reduce the enzymatic activity. The difference among the PEGylated sugars with variable PEG length is small since the effect of hindrance may be similar regardless of the spacer. After the addition of the methacrylate group through an ester linkage, the absorbance of the glycomonomer solutions is further reduced to ca. 0.15 (Figure 5.2B), probably due to the hydrophobicity of the methacrylate group. For all glycopolymers with or without a PEG spacer, we used appropriate polymer concentrations to keep a constant number of Ga residues (0.3 mM) on the polymers in the PBS buffer. The actual oxidation of the glycopolymer without the PEG spacer by GOx is negligible (Figure 5.S4, less than 0.02). After the addition of 5-fold GOx-HRP (30 unit/mL of GOx and 30 unit/mL of HRP), the absorbance did not change. This is attributed to the sugar pendants being too close to the polymer backbone for the Ga substrate to gain access to the active sites of the enzyme. Introducing a flexible spacer should make the sugar substrate more accessible. For $PMEO_4Ga$, a glycopolymer with a tetra-EO spacer, there is no obvious change in absorbance (Figure 5.2C). Two glycopolymers of different molar masses made of a glycomonomer with a PEG spacer of 9 units of EO were prepared and tested. Interestingly, under the same enzymatic oxidation conditions, an absorbance increase (ca. 0.15, 50 hrs) for $PMEO_9Ga$-7K was clearly obtained, which is close to its corresponding glycomonomer. The glycopolymer with a higher molar mass ($PMEO_9Ga$-18K) shows a slight decrease in their absorbance, suggesting a negative effect of the molar mass of the polymer unfavorable for the enzymatic reaction, likely due to steric hindrance. Increasing the PEG length ($PMEO_{13}Ga$-7K) further does not increase enzymatic conversion of the glycopolymer, which remains approximately at the same level as the corresponding PEGylated glycomonomers.

Polymers with oligo(ethylene glycol) side-chains are known to exhibit thermoresponsiveness.[33-35] The aqueous solutions of both $PMEO_9IpGa$-7K and $PMEO_9IpGa$-18K show sharp phase transitions (Figure 5.3) with cloud points (T_{cp}) at 22.1 and 24.5 °C, respectively. Their T_{cp}s seem to depend on the molar mass. The phase transitions of both glycopolymers are reversible with a certain hysteresis (dashes lines are cooling curves in Figure 5.3). The phase transition of $PMEO_9IpGa$-7K is broader with a larger hysteresis, which may be ascribed to the formation of interchain hydrogen bonds. After deprotection, the pendant sugar groups become more hydrophilic, leading to the loss of their thermo-responsive properties.

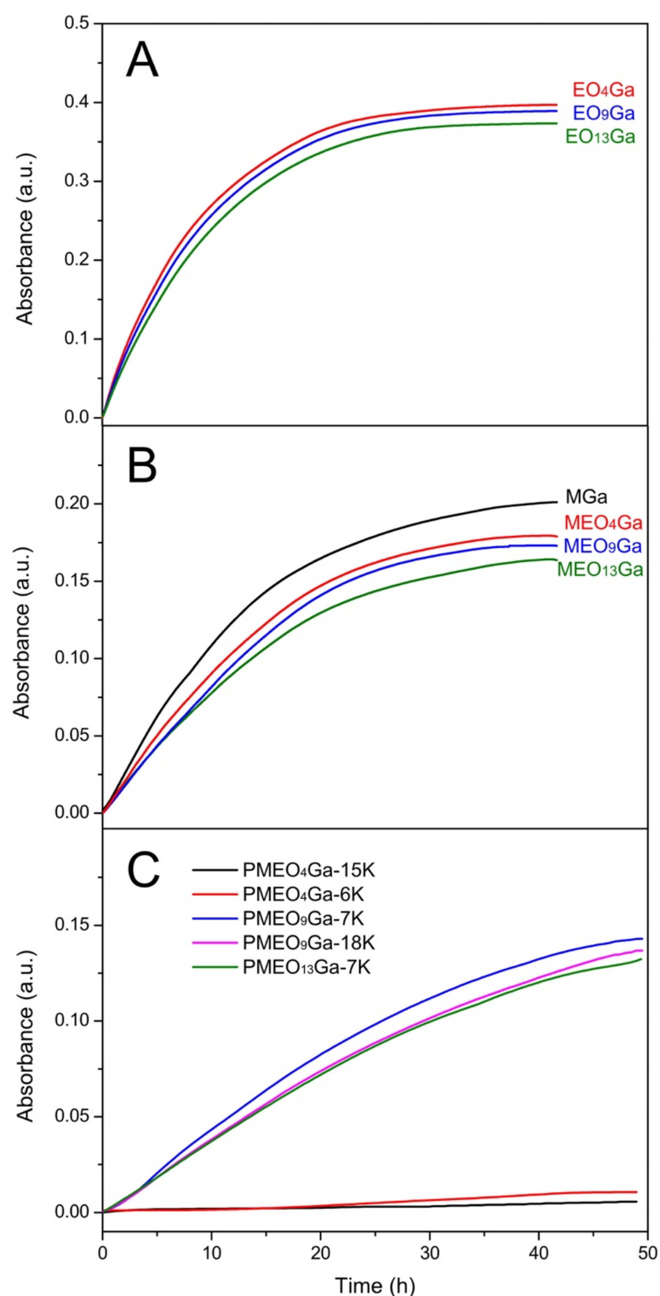


Figure 5.2 UV-vis absorption curves as a function of enzymatic reaction time at 414 nm of PBS solutions at 35 ºC containing GOx (6 unit/mL) and (A) PEGylated galactose (0.3 mM), (B) the glycomonomers (0.3 mM), and (C) glycopolymers (containing 0.3 mM of Ga residues on the polymers) with various PEG spacers (4, 9 and 13 units of EO) and of different molar masses. The [ABTS] / [sugar] and [HRP] / [GOx] ratios were kept at as 2 and 1, respectively. A solution containing ABTS (0.6 mM), GOx (6 unit/mL) and HRP (6 unit/mL) was used as a control.

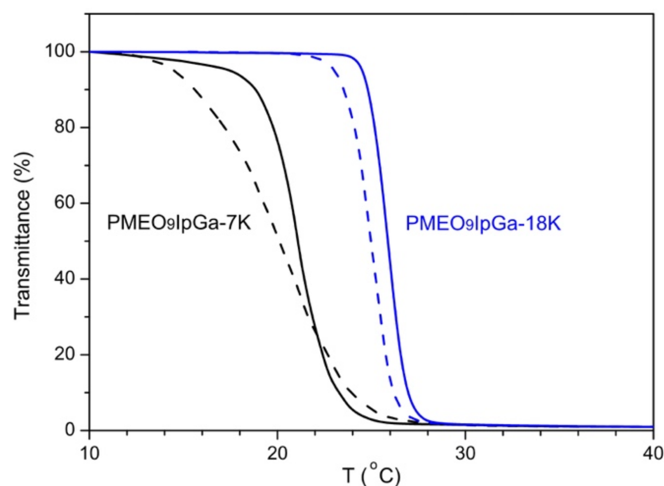


Figure 5.3 Transmittance variation of aqueous solutions of selected glycopolymers (1.0 g/L) during heating (solid lines) and cooling (dashes), measured at λ = 400 nm and heating or cooling rate of 0.5 ^{o}C/min.

5.3.4 Lectin recognition of glycopolymers

The multivalent binding of glycopolymers to lectins results in the formation of aggregates, making the binding between glycopolymer and lectin a very effective method for ascertaining the bioactivity of these materials. Glycopolymer-lectin binding studies were performed using turbidimetric assays, in order to assess the effect of the PEG spacer on such biochemical interactions. Typically, these tests can be carried out by mixing the galactose conjugated glycopolymer with a galactose-binding lectin (RCA_{120}).[36] The binding between the glycopolymer and lectin may lead to aggregation and eventually precipitation (Figure 5.4A inset). A solution of RCA_{120} (ca. 42 µM) in PBS buffer was freshly prepared and transferred into a dry quartz microcuvette. After equilibration for 5 min at 25 ^{o}C, a glycopolymer solution was added and the absorbance at 420 nm recorded for 30 min. The lectin showed affinity for all glycopolymers, while the response over time was dependent upon the PEG spacer length in the glycopolymer (Figure 5.4A). The glycopolymer PMGa showed the weakest interaction, which may be strengthened by increasing the molar mass of the polymer. A short PEG spacer ($PMEO_4Ga$-6K) leads to the formation of stronger and more stable glycopolymer-lectin clusters, and a longer PEG spacer further improves the sugar-lectin binding, shown by an increase in turbidity. Therefore, the PEG spacer has an important effect on the interaction of the glycopolymers with lectins.

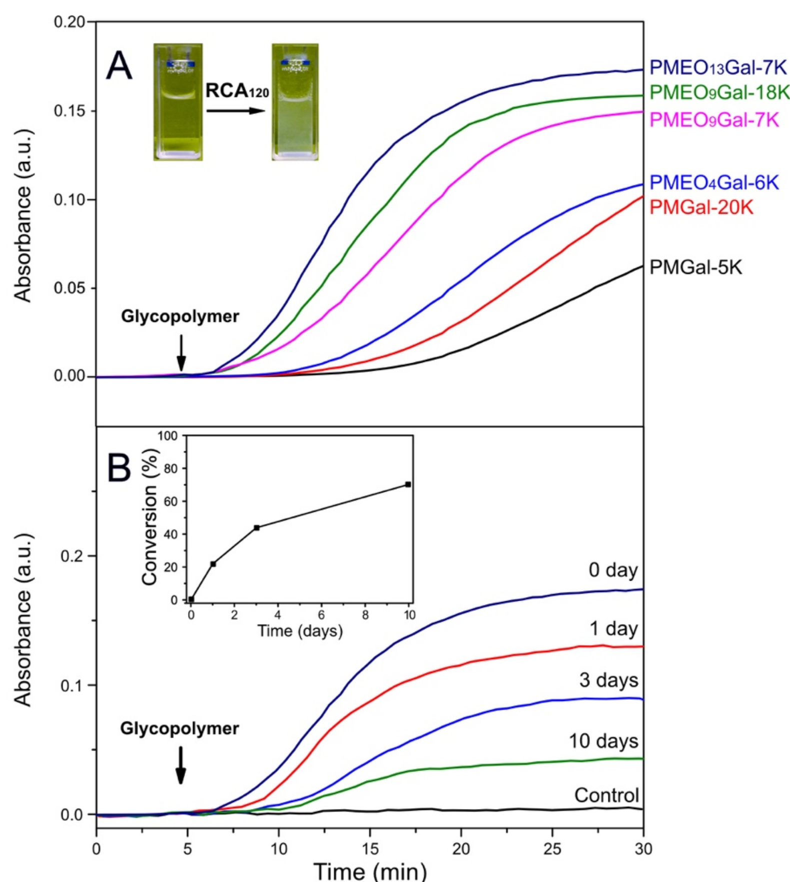


Figure 5.4 Turbidity measurements of a solution of 350 μL of RCA_{120} (42 μM) after adding 200 μL of (A) glycopolymers (containing 250 mM of galactose residues) with different PEG spacer lengths, and (B) oxidized $PMEO_{13}Ga$-7K samples obtained after different reaction times with GOx (varying from 0 to 10 days). Inset shows the degree of conversion as a function of enzymatic reaction time. A solution containing RCA_{120} (27 μM) and GOx (6 unit/mL) was used as a control.

The glycopolymer with the longest PEG spacer, $PMEO_{13}Ga$-7K showed the strongest binding to lectin and fastest enzymatic oxidation. Therefore, it is selected to study its interaction in its oxidized form at different stages with RCA_{120}. The concentration of the glycopolymer was kept low in the enzymatic reaction. The glycopolymers were concentrated by withdrawing 250 ml of the glycopolymer solution periodically at different time intervals during the enzymatic oxidation to remove water by freeze-drying. Then 0.3 mL of PBS buffer solution was added into the lyophilized product, the mixture was equilibrated by shaking for 1 h at room temperature, and then filtered through a Millipore 0.45 μm PTFE membrane. Since it is hard to remove GOx from the polymer solutions, the interaction between GOx and RCA_{120} was tested as a control experiment. The turbidity remained at ca. 0 (Figure 5.4 B). After the

enzymatic oxidation, the glycopolymer-lectin binding was significantly weaker, and decreased with increasing reaction time from 1 to 10 days. These results indicate that the homo-glycopolymer was converted into a copolymer with a gradual decrease in the galactose content so that the interaction with lectin became weaker with time, which also make possible the calculation of the sugar conversion, according to the formula $(A_0 - A)/A_0 \times 100\%$, where A_0 and A denote the absorbance at time 0 and a specific conversion time point, respectively. The conversion within 1, 3 and 10 days is 21, 46 and 73% (Figure 5.4B inset), respectively. We were unable to obtain enough pure oxidized glycopolymer for additional characterization and the removal of GOx proved also to be difficult. In the future work, we may opt for the use of an immobilized enzyme[37, 38] with can be more easily remove or the use of an alumina oxide column to remove the enzyme after the reaction.

5.4 Conclusion

Despite the known specificity for glucose, GOx can be used to oxidize oligosaccharides and other sugar substrates such as galactose. In this work, a series of glycopolymers with PEGylated galactose pendants were first synthesized and their enzymatic oxidation by GOx was studied by monitoring a GOx-HRP cascade reaction using ABTS as a chromogenic substrate. Glycopolymers without a PEG spacer or with short PEG spacers could not be oxidized by GOx, probably due to the inaccessibility of the sugar pendants due to steric hindrance. Even though the enzymatic activity on the sugar moieties on the glycopolymers is lower than on free sugar, the oxidation by GOx took place when the sugar substrate becomes accessible by the use of a long and flexible spacer. The PEG spacer also enhances the glycopolymer-lectin recognition, facilitating the formation of aggregates. These are the first examples of glycopolymers with sugar pendant groups that are oxidized to yield copolymers containing oxidized sugar acids. Such copolymers may prove to be useful for tissue engineering or as targeting agents for drug delivery systems. Issues regarding cytotoxicity, bioavailability, targeting effects, and especially in other applications of these glycopolymers are to be subjects of further studies.

5.5 Supporting information

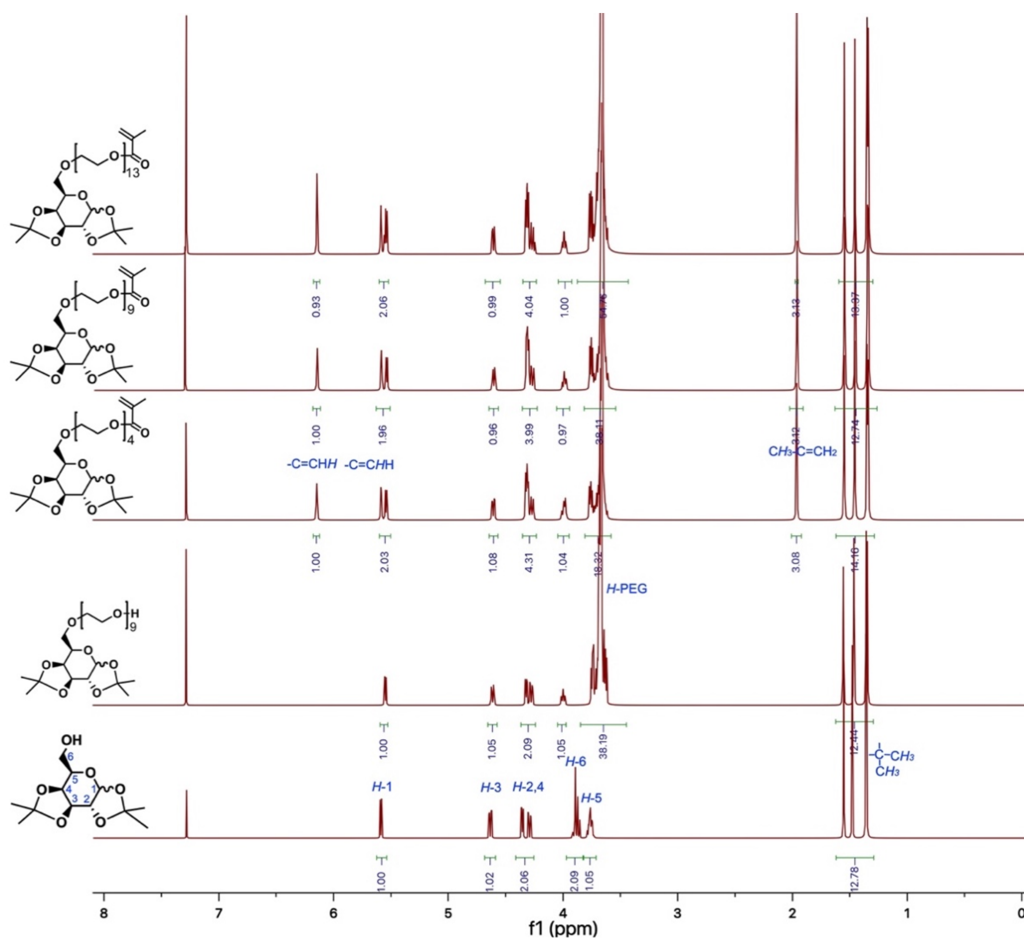


Figure 5.S1 ^{1}H NMR spectra of the representative products and glycomonomers in $CDCl_3$.

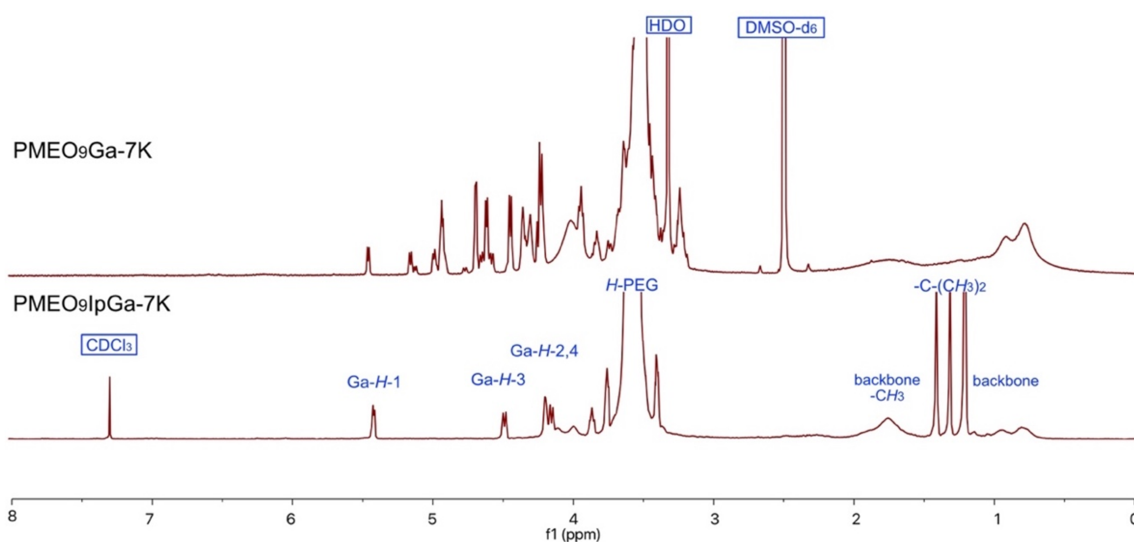


Figure 5.S2 ^{1}H NMR spectra of the representative glycomonomers with isopropylidene (Ip) groups in $CDCl_3$ and without Ip in DMSO-d_6.

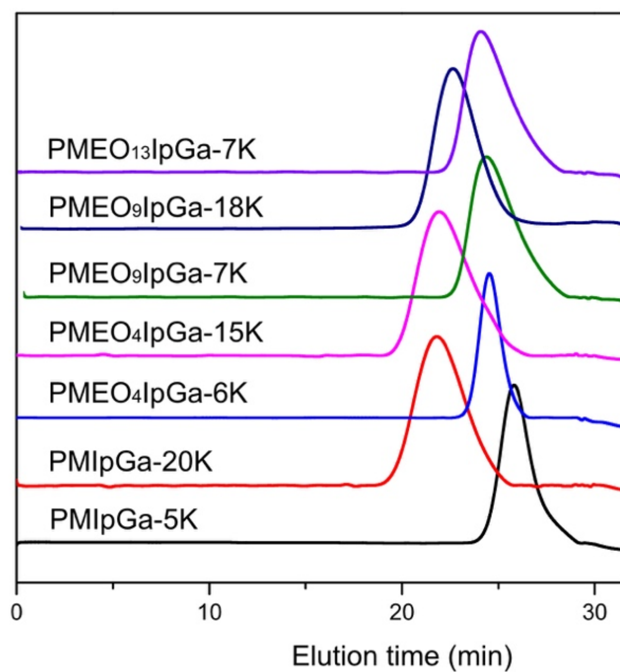


Figure 5.S3 SEC elution traces of the glycopolymers, all protected with the isopropylidene (Ip) group to keep them soluble in THF.

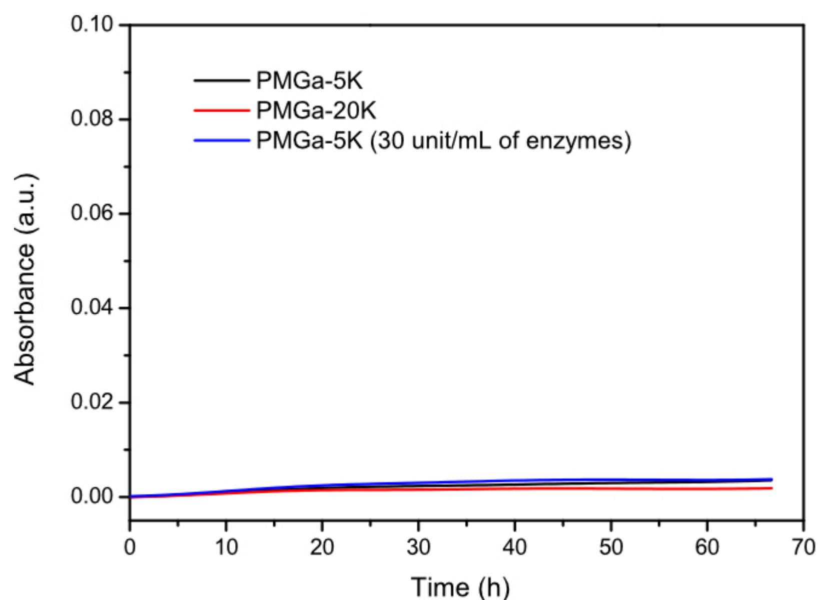


Figure 5.S4 UV-vis absorption curve of PMGa solutions (including 0.3 mM of Ga residues on the polymers) as a function of enzymatic reaction time at 414 nm at 35 ^{o}C containing ABTS (0.6 mM) and GOx (6 unit/mL).

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Chapter 6
Conclusions and future work

6.1 Conclusions

Glycopolymers possess promising properties making them useful in biomedical and pharmaceutical applications.[1,2] To improve biocompatibility and/or to endow bio-functionalities, natural compounds and biocompatible blocks (such as PEG, PLA, and PCL) can be introduced into these polymers.[3-6] Natural hydrophobic biocompounds, such as betulin, cholic acid and dopamine (Figure 6.1) are interesting building blocks for making amphiphilic copolymers. The copolymers can maintain some properties of biocompounds, such as hydrophobicity, bioavailability, biocompatibility and self-assembling capacity. In this work, three galactose-bearing copolymers have been designed and successfully synthesized by reversible addition-fragmentation chain-transfer (RAFT) polymerization. Betulin and cholic acid are rather hydrophobic in nature and can be conjugated with methacrylate or other polymerizable groups and then copolymerized with glycomonomers. These amphiphilic copolymers can self-assemble into micelles with a hydrophobic core and a hydrophilic corona. The micellar shell containing galactose pendants can bind to a galactose-binding lectin, RCA_{120}. The specific bio-recognition may be potentially useful as a biocompatible carrier for targeted release of drugs.

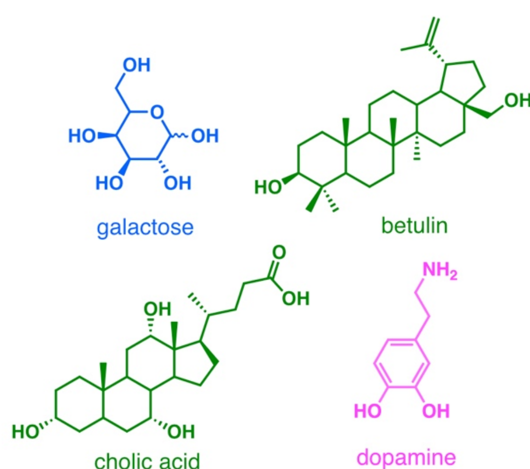

Figure 6.1 Structure of galactose, betulin, cholic acid and dopamine

6.1.1 Amphiphilic glycopolymers

Glycopolymers are mostly hydrophilic in nature and can serve as interesting and useful alternatives for the commonly used PEG. Many glycopolymers have been reported and investigated as biomaterials. Such polymers may be made amphiphilic by copolymerizing with monomers bearing other biocompounds that are more hydrophobic such as betulin and cholic acid or other bile acids. The random and block copolymers manifest different aggregation properties and varying encapsulation and release behaviors.

Betulin was conjugated with methacrylate and then copolymerized with glycomonomers to prepare random and block copolymers by RAFT polymerization. For easy comparison, these glycopolymers are designed to have similar molar masses and monomer compositions. The micelles made of random copolymers are smaller in size than those made of block copolymers, which facilitated the loading of a hydrophobic model compound such as Nile Red and released it faster. The hydrophilic sugar blocks of the block copolymer micelles are more exposed to bind to lectins, which may bind to lectin more easily than random copolymer micelles.

Block copolymers bearing cholic acids and galactose pendants were successful synthesized by RAFT polymerization. These copolymers were shown to self-assemble into micelles. These micelles are unstable and may disintegrate at lower concentrations. The micelles can be stabilized by the introduction of various amounts of repeat units bearing dopamine which is another biocompound that can serve as a crosslinker of the micellar core. The dopamine moieties, located at the hydrophobic core, can self-polymerize in a weakly basic solution, forming crosslink structure to stabilize the micelles in aqueous solution. The crosslinking of dopamine changed the micellar sizes and the release kinetics of the encapsulated agents. The CA pendants promote the self-assembly to form larger micelles, control the cross-linking of the stabilized micelles and facilitate the encapsulation of Nile Red. The crosslinked micelles showed a slow but sustained Nile Red release and interacted with lectin (RCA_{120}) effectively.

6.1.2 Enzymatic oxidation of the glycopolymers

The transformation of glyco-homopolymers into copolymers bearing other functional groups is of interest for the application of such polymers. Enzymatic conversion is a green and convenient way for such transformations with good specificity and high yield. To the best of our knowledge, there have been no reports on enzymatic conversion of glycopolymers. Glycopolymers containing PEGylated galactose as pendant groups are synthesized by a

combination of anionic and RAFT polymerizations. We explored the conversion of glycopolymers by glucose oxidase (GOx). By testing the use of GOx in the oxidation of galactose-bearing polymers, we have found that (1) no enzymatic activity was observed by using the directly methacrylated polymer; (2) attaching a PEG spacer that is sufficiently long can render the galactose substrate accessible to the active site of GOx; (3) GOx shows good activity on the PEGylated galactose substrates; (4) copolymers bearing carboxylic acid functional groups can be obtained by enzymatic oxidation. Even though the enzymatic activity on the sugar moieties on the glycopolymers is lower than on the free sugar, the oxidation by GOx can take place when the sugar substrate becomes accessible by the use of a long and flexible spacer.

6.1.3 Lectin recognition

The choice of the sugar[7], the molecular weight[8], and composition[9] of the glycopolymers can determine the lectin recognition and binding affinity. Random and block copolymers formed micelles with different hydrophilic shells,[10] which may affect their lectin binding. The block copolymers facilitated the formation of larger aggregates and clustered faster than the random copolymers upon the addition of lectins, since the hydrophilic galactose block are more exposed to bind to lectins. The crosslinked micelles made by block glycopolymers with galactose, dopamine, and cholic acid pendants also displayed RCA_{120} affinity. For the homo-glycopolymers, the polymers with higher molar masses promote the interaction between glycopolymer and lectins. The glycopolymers with PEG spacers enhance their lectin binding, facilitating the formation of larger aggregates. The polymers bearing galactose pendants showed reduced binding affinity to the lectin after the enzymatic conversion of some of the galactose into galacturonic acid. The binding of the glycopolymers should make them useful in lectin separation and targeted drug delivery.

6.2 Perspectives

Various glycopolymers have been made and tested showing promise for use as biomaterials. Issues regarding the cytotoxicity, drug delivery, and especially targeting effect are subjects of further studies.

6.2.1 Enzymatic oxidation of glucose-bearing polymers

We used galactose to study the enzymatic oxidation in our previous work, since it is easier to use in the steps of protection and functionalization. However, GOx has a specificity for glucose which is the most abundant monosaccharide in nature. Glucose-bearing monomers and copolymers are difficult to synthesize due to the arrangement of the five hydroxyl groups. It is become even harder to make mono-PEGylated glycose by anionic polymerization. To address this issue, it is possible to use a different synthetic strategy. The linear glucose derivative may be used as a potential starting material, which will generate *1,2:5,6*-Di-*O*-isopropylidene-*α*-*D*-glucofuranose after reacting with acetone under acidic conditions. After deprotection, it can regenerate glucose by ring extension.[11] The PEGylated glucofuranose monomers can be synthesized in a procedure similar to that described in Section 5 synthesis of PEGylated glycomonomers. PEGylated glucose-bearing copolymers may be obtained either by polymerization of the protected monomers and subsequent deprotection of the polymers or by polymerization of the glycomonomers deprotected beforehand (Figure 6.2). The effect of the PEG length and molar mass of the glycopolymers on the enzymatic oxidation may be studied. Glucose-bearing polymers may be oxidized more effectively by GOx due to its specificity toward glucose, which could generate copolymers with glucuronic acid groups. Its properties may be characterized and tested for applications in biomineralization and as hydrogels.

The removal of the enzyme GOx after the enzymatic conversion proved to be difficult. We were unable to obtain sufficient quantities of pure oxidized glycopolymer for further studies. GOx may be immobilized and used for easier removal after the reaction to obtain the oxidized copolymers.

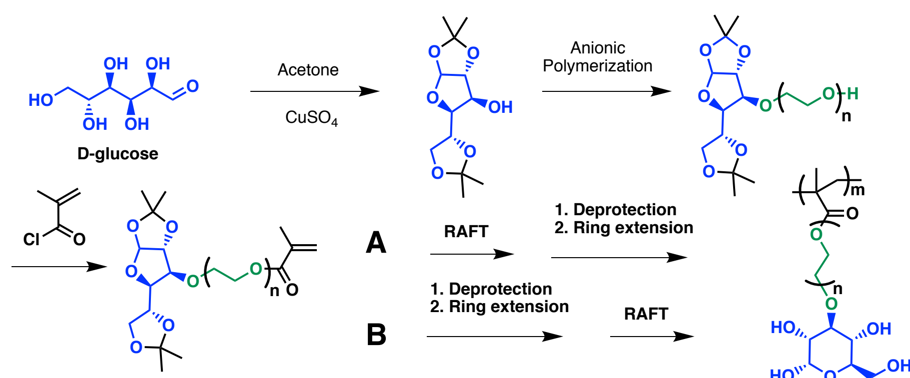

Figure 6.2 Synthetic scheme of PEGylated glucose-containing copolymers for GOx oxidation.

6.2.2 Enzymatic oxidation of polymers bearing amino sugar pendants

A hydroxyl group on the carbohydrate may be replaced by an amino group, forming an amino sugar, which also exists in nature. More than 60 amino sugars are known, the most abundant being glucosamine.[12] Glucosamine-bearing monomers and polymers are easy to prepare by amidation reaction which do not need protection and deprotection steps. In a previous work, we found the glucosamine-based monomers and polymers were not oxidized by GOx. The use of a spacer may improve the enzymatic activity. We would like to explore the use of a PEG spacer between the polymer chain and the amino sugar moiety. The PEGylated glycomonomers and glycopolymers with glucosamine pendants may be synthesized by amidation chemistry using active ester precursors.[13] Copolymers with active ester groups possessing PEG spacers of varying lengths may be synthesized (Figure 6.3). Then the amino sugars can be attached to the side chain by amidation chemistry. Enzymatic oxidation on such glycopolymers by GOx may be studied and compared with the glycopolymers bearing galactose or glucose.

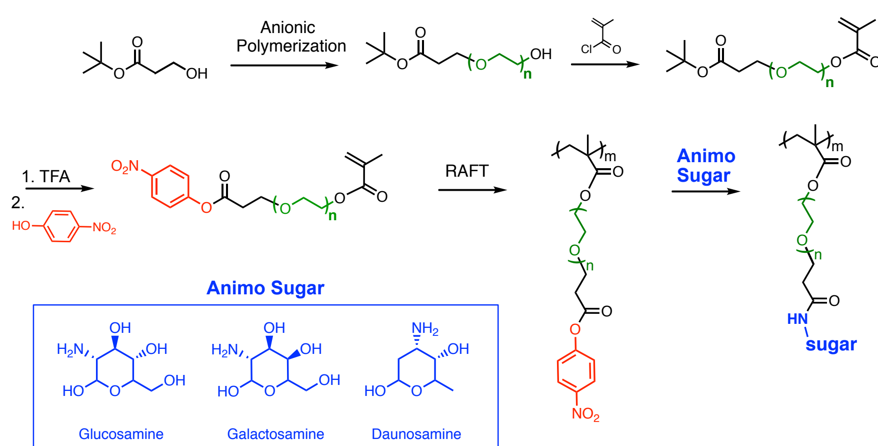

Figure 6.3 Synthetic scheme of PEGylated copolymers bearing different amino sugars for GOx oxidation.

6.2.3 Star-shaped glycopolymers with a porphyrin core

Porphyrins are macrocyclic molecules that play a key role in essential biological processes such as oxygen transport and photosynthesis.[14, 15] Porphyrins and their derivatives can generate singlet oxygen under irradiation, which have been tested for photodynamic therapy to kill cancer cells. Some porphyrin-based drugs have been developed for photodynamic therapy. However, most them are poorly soluble in water and cannot be directly administered

intravenously. The delivery efficacy may be improved by conjugation to hydrophilic polymers such as glycopolymers.

A star-shaped copolymer consisting a porphyrin core and four galactose-containing arms can be designed and synthesized. *5,10,15,20*-Tetrakis(*4*-hydroxyphenyl) porphyrin has four hydroxyl groups which can react with bromoisobutyryl bromide to yield an ATRP initiator. Then ATRP of glycomonomers will afford star-shaped glycopolymers (Figure 6.4). Preliminary UV-visible and fluorescence spectroscopic studies indicate that the luminescent property of the porphyrin core can be retained within the star polymer, promising for use as a biological probe and in photodynamic therapy. Their cytotoxicity and solution properties may be studied for their applications as biomaterials. More detailed studies can be conducted to quantify the singlet oxygen production[16] and the lectin affinity.[9] Drug loading and release may be investigated for potential pharmaceutical applications.

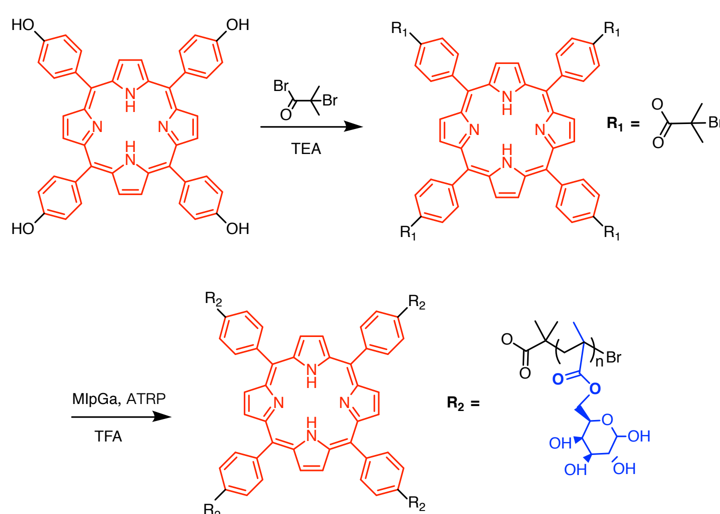

Figure 6.4 Synthetic scheme of star-link galactose-containing copolymers with a synthetic tetraphenyl porphyrin core.

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