Stimuli responsive polypeptides

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of a degree of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____  _____ (**Antonios Kapetanakis**)

ID No. **10121501**

Date: **24/09/2015**

to my mother

Acknowledgements

More than 4 years have passed by, but it still feels like the first day. It was a long journey full of new unexpected experiences, useful knowledge, happy moments, sad moments, people coming while others were leaving all of these happening at the same time. There are quite a few characters in this journey, who played a very important role not only in my academic life but they also helped me to adjust in this new place and I am grateful that I shared with most of you many nice moments. I will try to refer to each one separately and not to forget anyone.

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Mark Byrne, Robert Murphy, Antonios Kapetanakis, Joanne Ramsey, Sally-Ann Cryan, Andreas Heise. Star-Shaped Polypeptides: Synthesis and Opportunities for Delivery of Therapeutics, **Macomolecular Rapid Communications**, IN PRESS

Conference Presentations

Antonios Kapetanakis, Tushar Borase, Mark Byrne, Andreas Heise. Synthesis of Glycopeptide star polymers using ppi dendrimer as initiator. **243rd ACS National Meeting**, San Diego/USA, March 2012. (poster presentation)

Antonios Kapetanakis, Tushar Borase, Mark Byrne, Andreas Heise. Synthesis of 8-arm star glycopolypeptide copolymers from PPI dendritic initiators. **European Polymer Congress**, Pisa/Italy, June 2013. (oral presestation)

Abstract

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Great progress has been made over the last years on the synthesis of biodegradable and biocompatible polymers. Specifically synthetic polypeptides posses these characteristics and have been highlighted as potential candidates for a variety of applications such as drug and gene delivery or tissue engineering. Inspired by this, the aim of this PhD project was to synthesize a library of various polypeptides of linear and star-shaped architecture with combined selective binding and stimuli responsive properties. The synthesis of the polypeptides was achieved by N-carboxyanhydride ring opening polymerization (NCA-ROP) of the corresponding amino-acids.

Selective binding properties were introduced to the polypeptides by conjugation with sugar molecules, i.e. galactose. Copper catalyzed [3+2] Huisgen cycloaddition reaction was the technique applied in order to obtain glycosylated polypeptides. Their binding to biological model systems was investigated and confirmed by highly specific lectin-binding experiments. This synthetic strategy was applied to all the synthesized polypeptides that are described in chapters 2 to 5.

More specifically, in chapter 2 and 3 the synthesis of homo and block star glycopolypeptides was attempted. In chapter 2, γ-propargyl L-glutamate NCA was polymerized using a second generation poly(propyleneimine) (PPI) dendrimer as initiator and conjugated with azido-galactose through Cu catalyzed "click" chemistry. To increase the functionality of the system a second block was added to the polymeric chain. Moreover, a simple loading experiment was conducted to study the ability of the star-glycopeptides to host guest molecules. Solubility problems and the inability to selectively deprotect each polypeptide block separately led to the synthesis of a different type of star glycopolypeptides, which is described in chapter 3. In this case, DL-propargylglycine NCA and ε-carbobenzyloxy-L-lysine NCA were polymerized using PPI dendrimer as initiator to obtain random and block star copolymers. After conjugation with galactose, enzyme linked lectin assays (ELLA) were performed to prove their bio-recognition. Moreover, the successful complexation with plasmid DNA was demonstrated for one of the glycopolypeptides due to the interaction of negatively charged DNA molecule and the positively charged poly(lysine) on the polypeptide chain.

In chapters 4 and 5 the addition of temperature as a stimulus was attempted and its effect on the lectin binding was investigated. For that purpose, in Chapter 4, linear polypeptides were synthesized by the polymerization of γ-propargyl L-glutamate NCA, followed by the

subsequent co-click of 1-azido-2-(2-methoxyethoxy) ethane (mEO_2) and azido-galactose. Varying the ratios of the two units on the polymer backbone, the hydrophilicity as well as the Cloud Point temperature (T_{cp}) could be modified. Moreover, it was demonstrated that lectin binding can be thermally controlled. In chapter 5, a similar study was performed for star polypeptides using again γ-propargyl L-glutamate NCA, which was polymerized by PPI dendritic initiator. After the co-click of (mEO_2) and azido-galactose the same effect of temperature on the lectin-binding was observed. Moreover, a comparison between linear and star glycopolypeptides revealed the effect of the structure and molecular weight on cloud point and helicity.

Chapter 1

Introduction

1.1 N-Carboxy-α-amino anhydrides.

N-Carboxy-α-amino-acid anhydrides (NCA, also known as 4-alkyloxazolidine-2,5-diones) can be obtained by the decomposition of alkoxycarbonyl-α-amino acid chlorides (Leuchs' method)[1] or the action of phosgene on α-amino acids (Fuchs-Farthing method)[2]. They are well-defined, colorless and generally crystalline substances. Furthermore, they are highly reactive compounds because of the activation of the carbonyl functionality at C-5 position of the anhydride, which makes them suitable for the synthesis of polypeptides by ring opening polymerization. NCAs are quite unstable and show high sensitivity to moisture. Extra care has to be taken when amino acids contain side chains with amino or hydroxyl functionality. In this case, the side chains have to be protected, in order to avoid undesirable by-products or polymerization of the NCA.

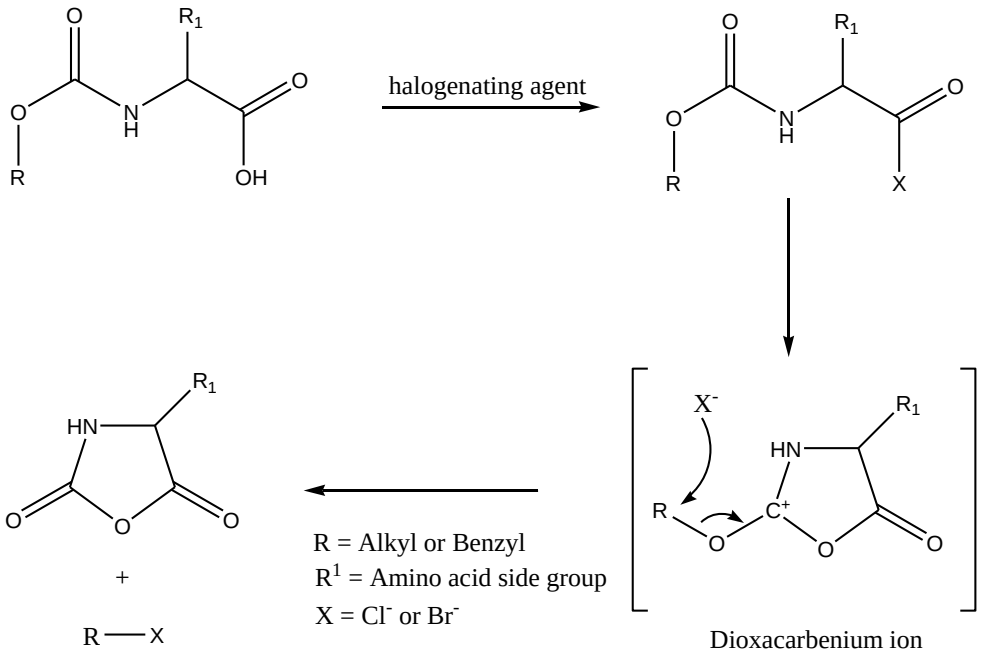
1.1.1 Leuchs' method for NCA synthesis.

NCAs were first discovered by Hermann Leuchs in 1906, when he attempted to purify N-ethoxycarbonyl or N-methoxycarbonyl amino acid chlorides by distillation. They are therefore also referred to as Leuchs' anhydrides. A few papers were published by Leuchs, between 1906 and 1908 demonstrating the synthesis of different NCAs as well as their ring opening polymerization[3,4].

Leuchs' method (Scheme 1.1) involves the reaction of an N-alkoxycarbonyl amino acid with a halogenating agent[1,3-6] to form an N-alkoxycarbonyl amino acid chloride or bromide. Upon heating (50 – 70 °C) a cyclic dioxacarbenium ion intermediate is formed, followed by the nucleophilic attack of the halogenide on the alkyl group, resulting in the formation of the NCA and the alkylhalide byproduct.

It is also necessary to mention that the type of the halogen plays an important role in the formation of the NCA. Bromide ions are better nucleophiles than chlorides, making phosphorus tribromide a strong halogenating agent[7]. Furthermore, an increase in the electrophilic nature of the N-alkoxycarbonyl group can increase the rate of cyclization, as illustrated below:

Ethyl- < Methyl- < Allyl- < Benzyloxycarbonyl

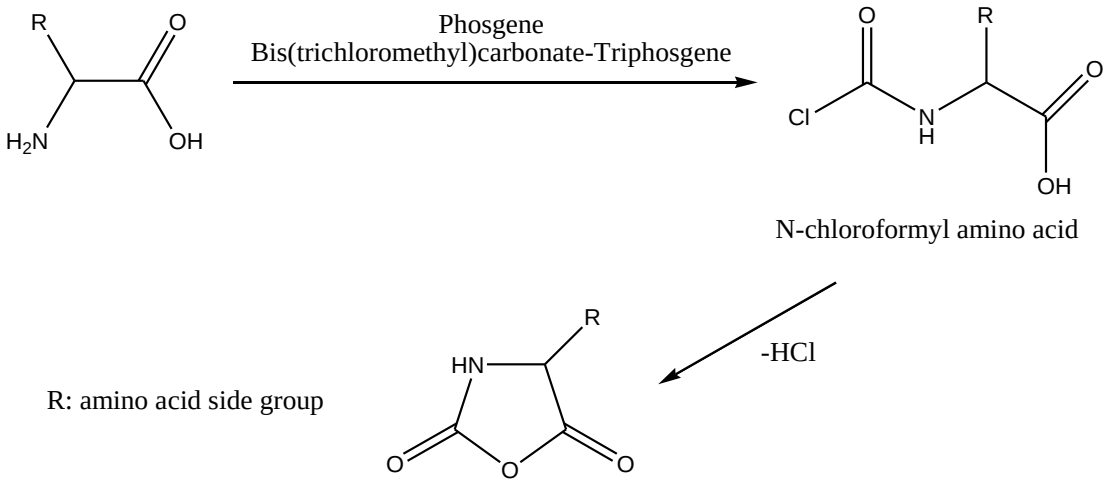


Scheme 1.1: Leuchs' method for the synthesis of NCAs

1.1.2 Fuchs-Farthing method for NCA synthesis.

This method involves the direct phosgenation of the α-*N*-unprotected amino acids. Cyclization proceeds through the formation of an *N*-chloroformyl amino acid intermediate and the loss of a second HCl molecule completes the NCA formation. Fuchs was the first who reported the synthesis of NCAs by bubbling phosgene gas into an alkaline solution of an amino acid[8]. The method was refined by Farthing by using heat and an inert solvent such a dioxane[9].

The drawback of using phosgene is the lack of stoichiometric control as well as the formation of contaminants in the reaction solution, such as amino acid chloride hydrochlorides. For that reason, the use of trichloromethyl chloroformate (diphosgene – phosgene dimer) or bis(trichloromethyl) carbonate (triphosgene – phosgene trimer) is favored[10,11,12]. They can both be used as replacements for phosgene gas in most reactions and they have many advantages concerning the safety in the laboratory setting and they both show better stoichiometric control than phosgene[13].



Scheme 1.2: Fuchs-Farthing method for NCA synthesis[2]

1.2 NCA-ROP polymerization

NCA ring-opening polymerization (NCA ROP) is the most frequently used synthetic methodology to generate homopolypeptides[14]. These synthetic polypeptides can be monodisperse and obtained in a highly controlled manner to form random coil[15], α-helix[16] or β-sheet[17] structures. These secondary conformations play an important role in the self-assembly of the polypeptides leading to different structures, chemical properties as well as making them suitable for biomedical or pharmaceutical applications[18,19].

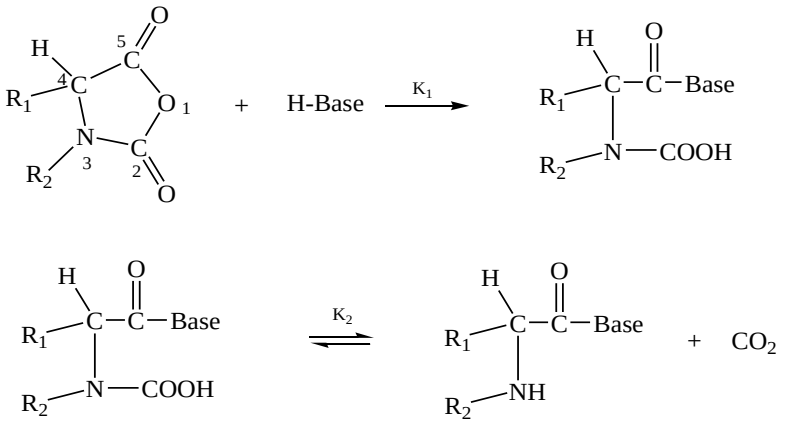
Until 1997 two main mechanisms were mostly investigated regarding the ring-opening polymerization of NCAs. These are the Normal Amine Mechanism (NAM) and the Activated Monomer Mechanism (AMM).

1.2.1 Normal Amine Mechanism

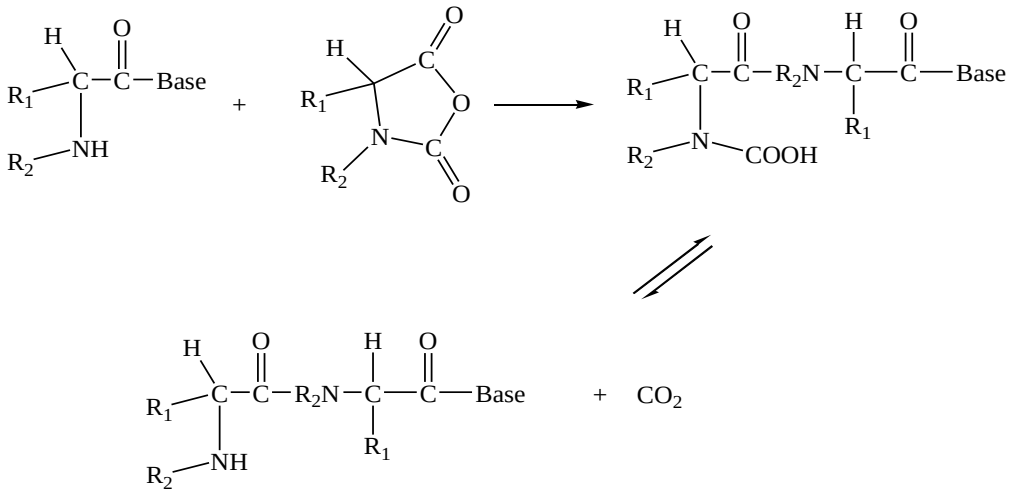
This mechanism involves the polymerization of NCAs using nonionic initiators, bearing one mobile hydrogen atom, such as primary amines, secondary amines, alcohols and water. The NCA ring opening is caused by a nucleophilic attack on the C-5 position of the NCA monomer, followed by the formation of unstable carbamic acids, which in turn, are converted to primary amines with subsequent release of CO_2. The newly formed amino group is free to attack the next molecule of NCA. The reason primary amines are the most favorable initiators is that they are more nucleophilic than the ω-amino groups of the propagating chains. As a result, the initiation rate is faster than propagation, leading to polypeptides with low polydispersity indices[20,21]. In many cases, secondary reactions can occur during NCA ring opening polymerizations. Common impurities are HCl, which can react with the amino active sites and form HCl salts[22], as well as water, which can lead to complete hydrolysis or

formation of oligopeptides[23]. Another unwanted side reaction can occur from the attack of the amino group at the 2C position instead of the favorable 5C, resulting in the formation of the ureido acid chain end[24]. To limit the attack at the 2-CO group, highly nucleophilic initiators are necessary to be used, i.e. hexylamine.

I. Initiation



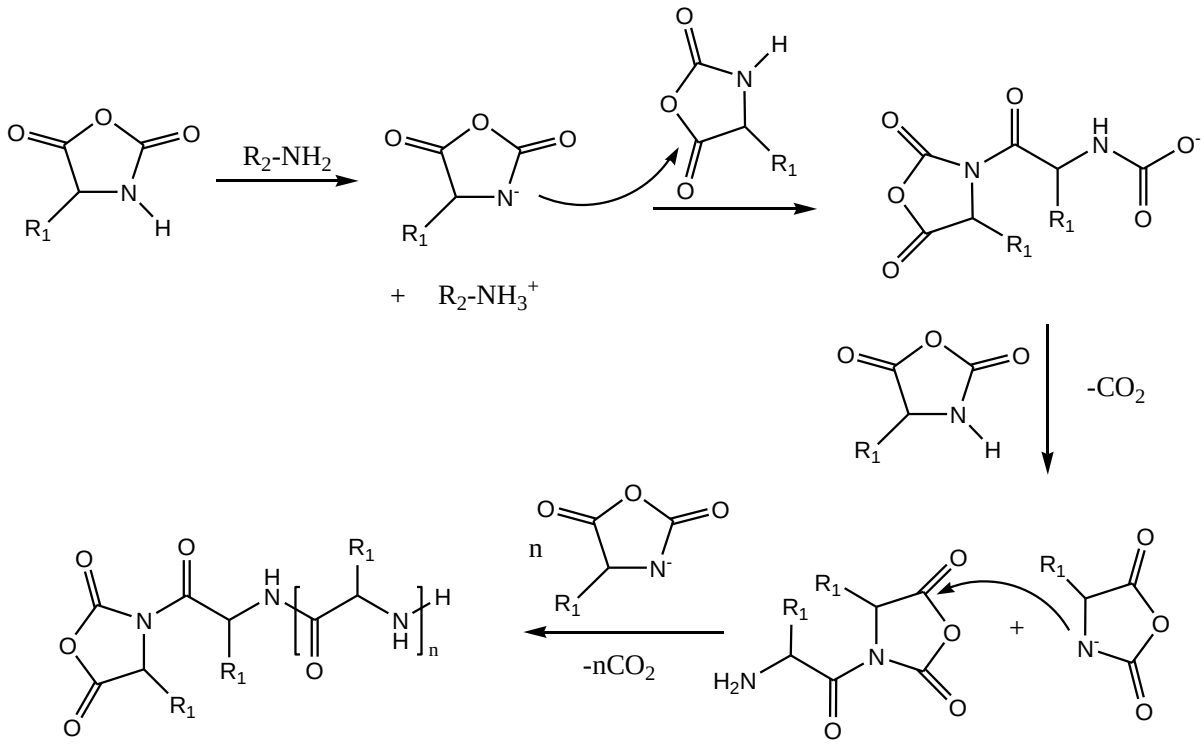
II. Propagation



Scheme 1.3: Normal amine mechanism[25].

1.2.2 Activated Monomer Mechanism

The activated monomer mechanism applies to N-unsubstituted NCAs, bearing a proton at the 3-N position. In contrary to NAM, the initiator acts as a base[26] resulting in the removal of the proton of the 3-N of NCA, forming the corresponding anion. The resulting anion then attacks the 5-CO of another NCA molecule to create a new anion, releasing carbon dioxide. Chain propagation takes place through further nucleophilic attack by the next NCA anion on the NCA endgroup of the growing polymer. The initiators mostly used in this procedure are tertiary amines[27], which are more basic than primary or secondary amines. In this mechanism, propagation rate is faster than initiation rate[28], leading to higher PDIs, and generally higher molecular weight polypeptides than the ones obtained from NAM.

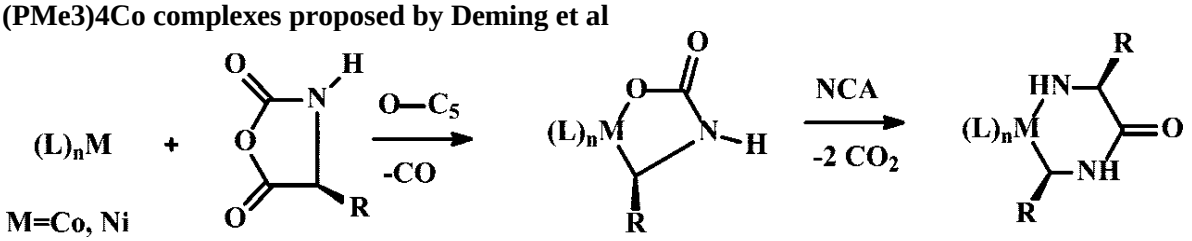


Scheme 1.4: Activated monomer mechanism.

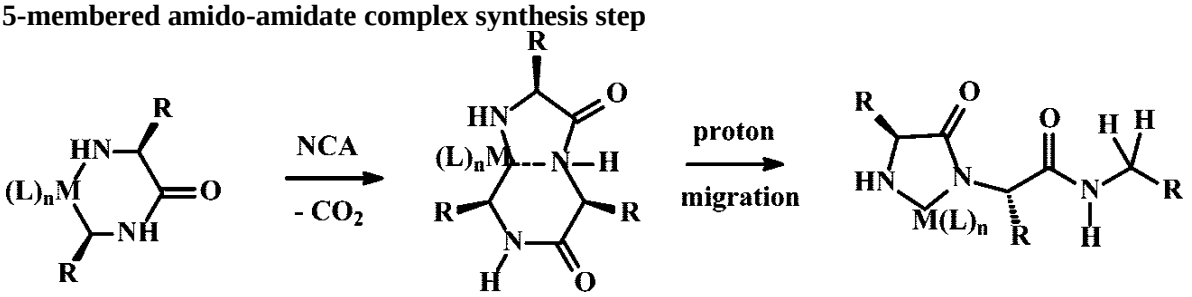
1.2.3 Living polymerization of NCAs

Although many different mechanisms and techniques were proposed for the NCA polymerization, it was in 1997 when the first living NCA polymerization was discovered by Deming[29]. The word 'living' refers to the NCA polymerization that is highly controlled leading to polymers with an unchanged end group and narrow molecular weight distribution. What Deming achieved, was the synthesis of homo- and block polypeptides with defined molecular weights and low PDIs, by using the zero valent nickel complex bipyNi(COD)[30] (bipy=2,2′-bipyridyl), (COD=1,5-cyclooctadiene) or $(PMe_3)_4CO$[31] complex as initiators. These newly discovered organonickel initiators were able to overcome termination reactions, keeping the chain end 'alive', by reacting with the NCAs to form an amido-amidate propagating intermediate, which is the main requirement for living NCA polymerization by transition metal initiators. Drawbacks in this mechanism is the presence of metals in the polypeptides and the lack of reactivity with N-substituted NCAs such as proline[32].

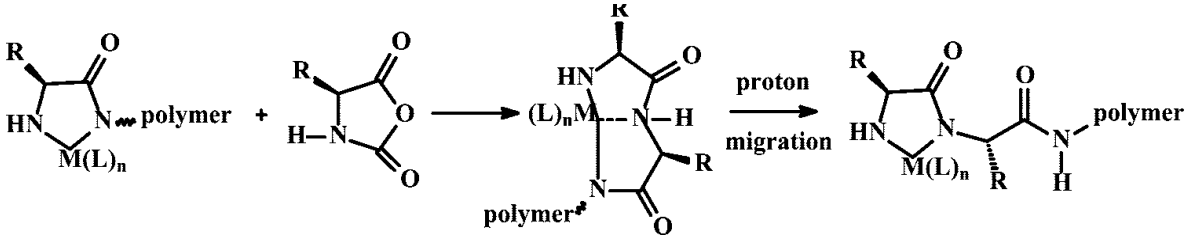
Initiation reaction of the mechanism for the polymerization of NCAs with bipyNi(COD) or (PMe3)4Co complexes proposed by Deming et al



5-membered amido-amidate complex synthesis step



Propagation reaction for the polymerization of NCAs with bipyNi(COD)



Scheme 1.5: Transition metal mediated NCA polymerization proposed by Deming et al.[30]

After Deming's work, many others have investigated and reported different mechanisms to control the NCA polymerization. The most important are mentioned briefIy below. In 2003 Schlaad[33] et al. reported the NCA polymerization using primary amine hydrochloride salts as initiators. In 2004, Aliferis, Iatrou and Hadjichristidis[34] reported the living polymerization of NCAs combining the use of primary amines as initiators with high vacuum techniques (HVT). Also in 2004, Vayaboury[35] et al. investigated the polymerization of ε-trifluoroacetyl-L-Lysine NCA in DMF with n-hexylamine, using Schlenk techniques under nitrogen and low temperatures to increase the amount of living chains. In 2007, Lu et al.[36] reported the polymerization of BLG-NCA using hexamethyldisilazane, leading to polypeptides with low PDI, predictable molecular weights and very high yields. In 2008 Peng et al.[37] studied the ROP of Z-Lys-NCA using the novel platinum complex {[bis(diphenylphosphino)ethane][*N*-

((1*S*,2*R*)-2-amido-1,2-diphenylethyl)-4 methylbenzenesulfonamidato]-platinum [(dppe)Pt(MBS-NH)]} as initiator, claiming that their system follows the mechanism proposed for the amido-amidate metal complexes of Ni proposed by Deming. Finally, in 2011, Ling et al.[38] investigated the ability of the rare earth metal complexes to initiate NCA polymerization, synthesizing polypeptides with low polydispersities.

1.3 Star Polypeptides

Star polymers are a typical type of branched polymers, which consist of several linear chains (arms) emanating from a central core[39]. The core is characterized by its functionality, which is attributed to the chemical bonds that can form with the external parts of the molecule. The core, which can be polymeric, is attached to each arm through linear units, which can be either monomers or polymers and at the end of each arm the branched unit is attached. Another important characteristic of star polymers is their three dimensional globular structure and their segmental density, as compared with their linear counterparts of the same molecular weight. These characteristics are responsible for their acquirement of unique properties such as low solution viscosities[40,41], encapsulation capabilities[42,43] and a variety of different internal or external functionalities[44,45,46].

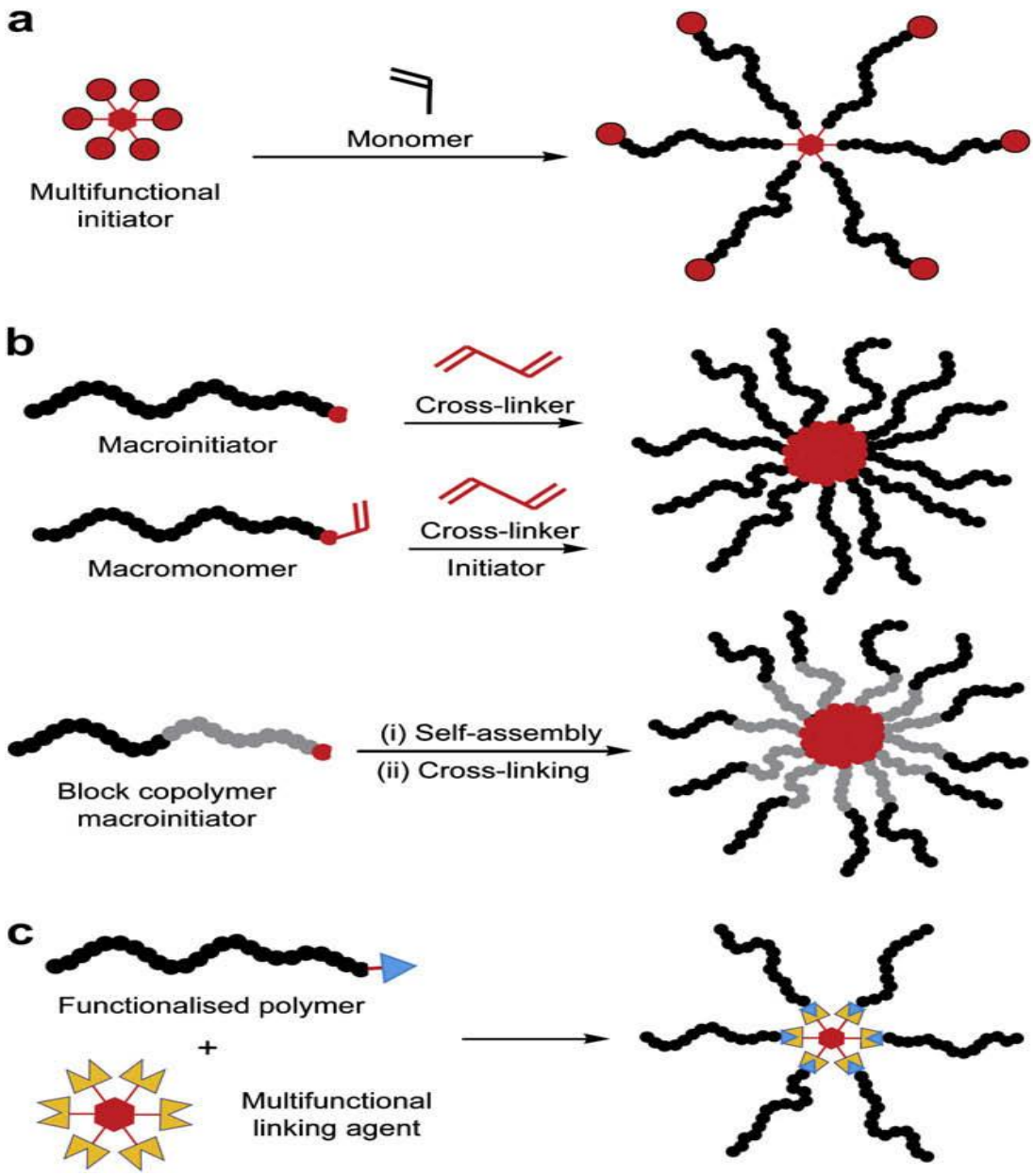
For all of the above, star polymers have received great scientific interest, since they were first synthesized sixty years ago. Especially over the last years, they were under investigation for various industries and companies, which produce engine oils, contact lenses, biomedical devices, etc.

There are three principal strategies for the construction of star-shaped branched polymers namely,

a) the core-first approach (divergent),

b) the grafting-to approach

c) the arm-first approach (convergent).



Scheme 1.6 Synthetic approaches for the preparation of peptide-based star polymers via (a) core-first, (b) arm first, and (c) grafting-to approaches[39].

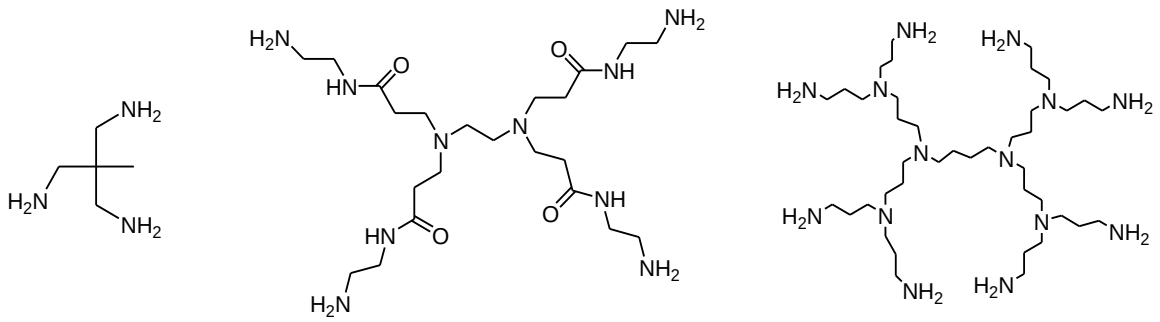
1.3.1 Core-first approach

The core-first approach is the most widely used for the synthesis of star polymers and it is based on the use of a well-defined multifunctional initiator, bearing a known number of initiating groups or a less defined multi-functional macromolecule. It involves the simultaneous initiation and growth of all the chains from the central core leading to the formation of star-branched polymers. It is desirable for all initiating sites of the central core to be equally reactive and the initiation rate must be higher than the propagation rate, in order to obtain star polymers with controlled molecular weight and arm length as well as low molecular weight distribution.

The advantage of the divergent method is the capability to obtain high molecular weight and well defined architectures. This method has been used for different polymerization techniques, promoting the introduction of a wide variety of monomers into the final polymeric structure[47].

One of the main disadvantages of the core-first approach lies on the fact that synthesis of well-defined initiators can be tedious and require many purification steps. Furthermore, the molecular weight, the number and the homogeneity of arms are difficult to characterized. In some cases, the number of arms can be calculated indirectly using methods such as end-group analysis or determination of the branching parameters comparing with a corresponding linear polypeptide with the same molecular weight. To analyze the arms, they have to be cleaved from the initiator core. This can be quite demanding and time consuming and in many cases the results are not more than an estimation. Star-branched polypeptides synthesized by this approach involve the polymerization of γ-benzyl-L-glutamate or carboxybenzyloxy-L-lysine NCA derivatives using 3, 4, or 6 amino-functionalized initiators[48,49].

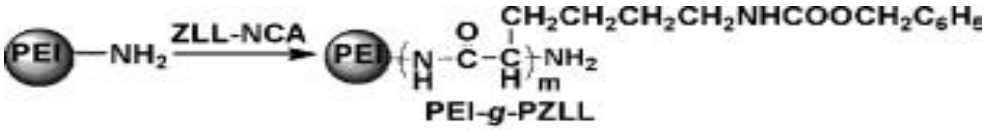
In many cases the synthesis of star-branched polypeptides was reported, using dendritic macroinitiators. Aoi et al[50] and Applehans et al[51] described the synthesis of star polypeptides, consisting of a highly increased number of arms, using a poly(trimethyleneimine) dendrimer as macroinitiator to obtain a 64-arm star of polysarcosine. Another example was reported by Okada et al. who combined a glycosylated NCA with a generation 3 PAMAM dendrimer to form a glycosylated star polypeptide ("sugar ball"), with low molecular weight distribution[52]. It was also proven from ^{13}C NMR studies that the initiation occurred from all the peripheral amino groups. One more example is provided by Aliferis et al. who studied the ROP of ε-butyloxycarbonyl-L-lysine and γ-benzyl-L-glutamate NCAs using G0-PAMAM dendrimer, G1-PPI dendrimer and 2(aminomethyl)-2-methyl-1,3-propanediamine (AMPDA)[53] as initiators. Both initiators containing tertiary amines in the core (PAMAM, PPI dendrimers) led to star shaped polypeptides with bimodality, uncontrolled molecular weights and higher polydispersity indices. In contrary to AMPDA, with no tertiary amine resulted in star shaped polypeptides with unimodality and narrow molecular distributions. The bimodality in the case of PAMAM and PPI dendrimers occurs due to the fact that both the primary amine peripheral groups and the tertiary amine groups in the interior can initiate the polymerization leading in star shaped polypeptides with high and low molecular weights respectively.



AMPDA **G0-PAMAM dendrimer** **G2-PPI dendrimer**

Figure 1.1. structure of 2(aminomethyl)-2-methyl-1,3-propanediamine (AMPDA), poly(amido amine) (PAMAM) and poly(propyleneimine) (PPI) dendrimers.

Apart from dendrimers, other branched macroinitiators have been used for the synthesis of star-branched polypeptides. Yan et al. demonstrated the synthesis of star poly(lysine) (PLL)[54] using a hyperbranched poly(ethylene imine) (PEI) core. Size exclusion chromatography (SEC) led to monomodal peaks proving the absence of tertiary amine initiated polymerization. However, polydispersity indices were higher than those obtained by dendrimer initiated ring opening polymerization (ROP). Excessive studies of PEI initiated ROP have been also made by HuaYu Tian[55,56] using benzyl protected histidine and BLG NCAs and lately the research extended by Liu et al. who synthesized a large number of star polypeptides using various PEI cores as well as polypeptide peripheries[57].



Scheme 1.7. Synthesis of star-branched polymer PEI-ZLL[54]

In addition, Thornton et al. demonstrated the synthesis of polyalanine star polymers, initiated by 4-armed poly(ethylene glycol) (PEG)[58]. ^{1}H NMR spectroscopy revealed good correspondence of the degree of polymerization with the amount of alanine NCA in the monomer feed. Furthermore, Karatzas et al. described the synthesis of $(PEG\text{-}b\text{-}PBLG)_4$ leading to star polymers with narrow molecular weight distributions. On the other hand, polymers synthesized using poly(propylene oxide) (PPO) as initiator resulted in the more polydispersed $(PPO\text{-}b\text{-}PBLG)_3$ star polypeptides[59].

Another type of star branched polymer, which was investigated in earlier studies, is the mikto arm stars, which consist of arms bearing different polymers. Hadjichristidis et al. reported the synthesis of ABC-type miktoarm star polymers consisting of two random coil-like arms of polystyrene (PS) and polyisoprene (PIP) combined with an α-helical polypeptide, poly(*tert*-butoxycarbonyl-L-lysine) (PBLL)[60] (Figure 1.2). These miktoarm polymers showed properties similar to roid-coil block copolymers and their secondary structure consists of rectangular cylinders.

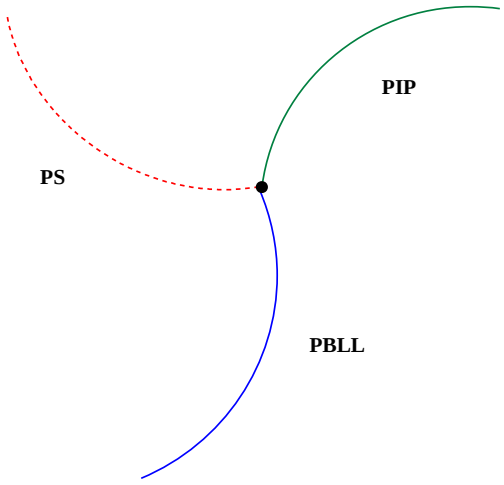
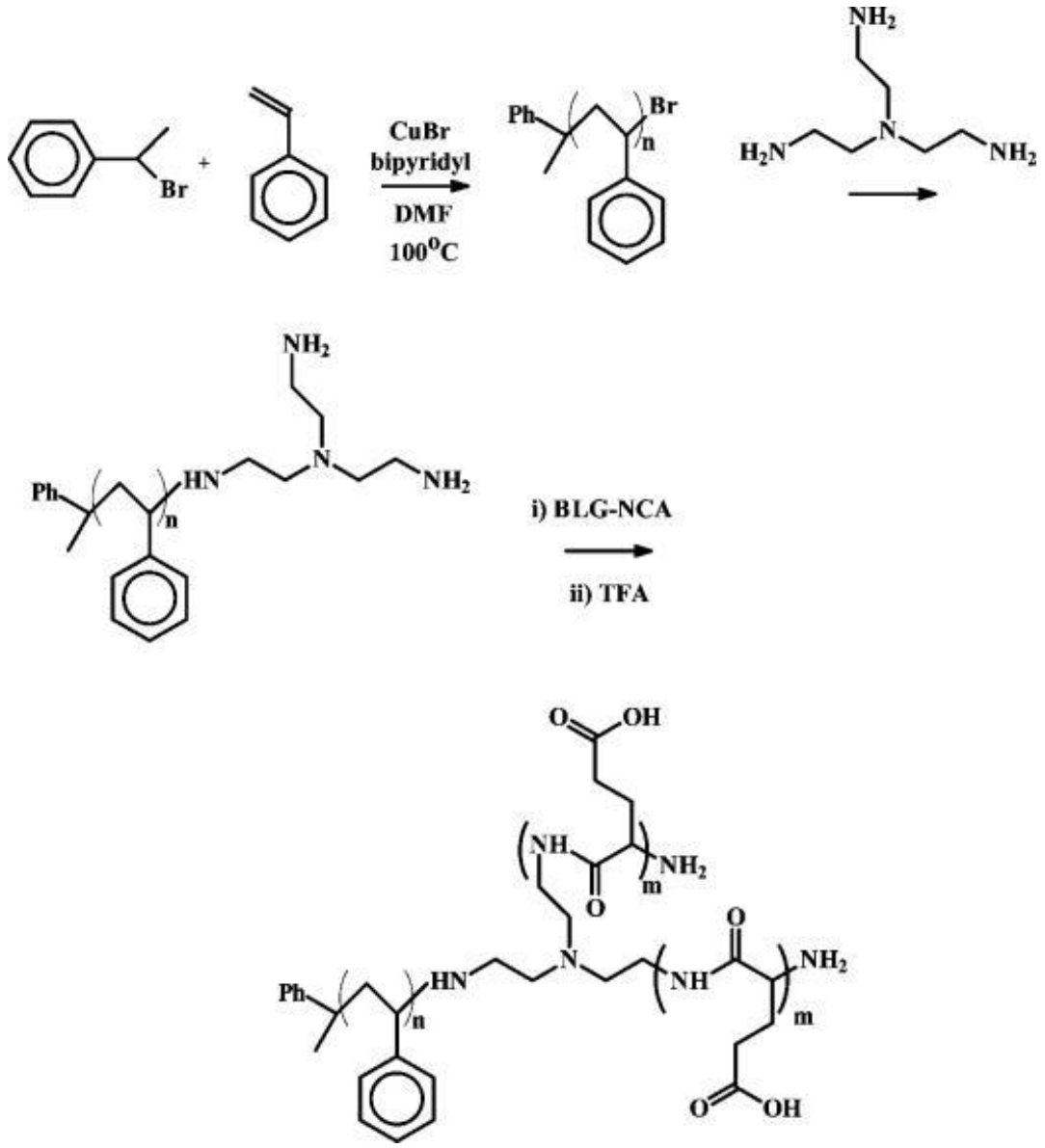


Figure 1.2. Mikto arm star copolymer synthesized from polystyrene (PS), polyisoprene (PIP) and poly(*tert*-butoxycarbonyl-L-lysine) (PBLL).

Another study about mikto arm polymers was presented by Babin et al. who synthesized $PS(PBLG)_2$ star copolymers[61,62]. He used ATRP to polymerize styrene and a large excess of 1-aminotriethylenetriamine to modify the bromide end group and obtain diamino PS chains. The amine end groups were used to polymerize BLG-NCA and form the desired copolymer. PBLG protective groups were cleaved using an excess of HBr in TFA leading to amphiphilic mikto arm star carrying polyglutamic acid arms. Both SEC and NMR studies confirmed the corresponding structure (Scheme 1.8).



Scheme 1.8 Synthesis of $PS(PBLG)_2$ miktoarm star copolymers[61].

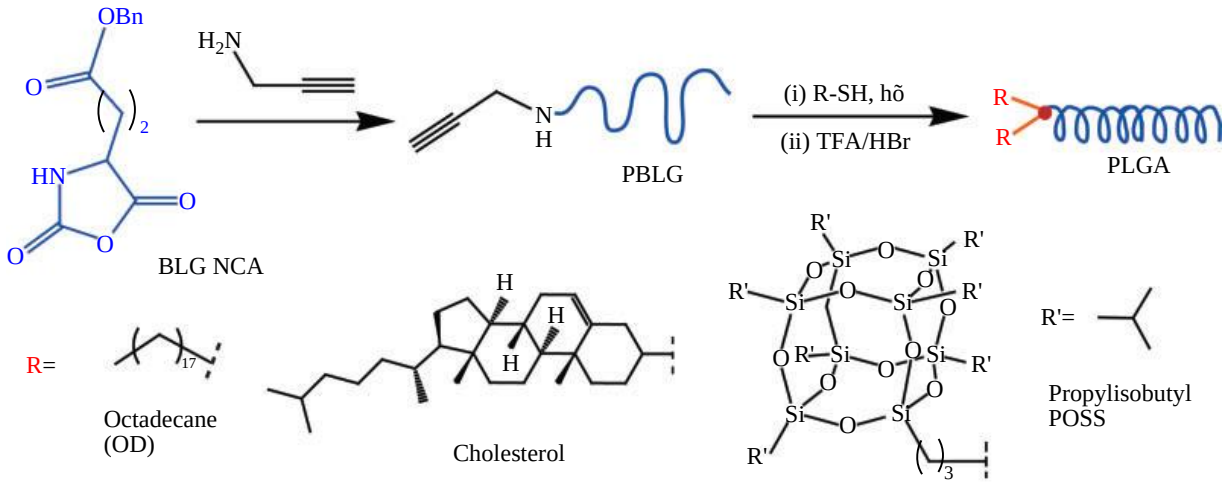
In a subsequent paper, Junnila et al. described the synthesis of 3 and 4-armes PLL hydrochloride miktoarm polymers using anionic polymerization and ROP techniques[63]. The star polymers obtained could form micelles in a non-ordered lattice bearing a PLL-HCl core surrounded by PS chains.

Lastly, click chemistry techniques have shown great potential as a synthetic route for the formation of mikto arm star polypeptides[64]. Rao et al. described the preparation of an AB_2 type miktoarm star polypeptides, using a tri-functional initiator containing a primary amine capable of NCA ROP and an alkyne group accessible to click with azide terminated linear chains[65]. With this method various combinations to the synthesis of mikto arm star polypeptides can be made, in order to obtain well defined star-shaped molecules with low PDIs.

1.3.2 Grafting-to approach

In the grafting-to approach, the core and the arms are being prepared independently. The functional groups of the core and the (living) end-groups of the arms can be coupled to form a star-shaped polypeptide. This method offers the advantage, that by using the appropriate experimental conditions, both the synthesis of the arms and the core can be accomplished in a precise manner and fully characterized. On the other hand, the main disadvantage of this method is that, in order to have a quantitative coupling, long reaction times and the use of an excess of ‘arms’ is necessary, leading to long purification steps. Moreover, it is quite difficult to produce star-polypeptides bearing a large number of arms, because of the steric hindrance around the core, leading to incomplete grafting.

Before 2009, the main example that was published about grafting-to approach was by Abraham et al., who described the synthesis of well defined, tri-armed polystyrene-b-PBLG (PS-b-PBLG) star polymers using a combination of ATRP and ROP techniques, resulting in star shaped block copolymers with controlled molecular weights and low molecular weight distributions[66]. More recent studies for the synthesis of star-shaped polypeptides use thiol-ene and copper click chemistry. For example, Ray et al. synthesized A_2B-type star-like polypeptides by coupling an alkyne terminated PBLG with octadecane, cholesterol, or polyhedral oligomeric silsesquioxane (POSS), using thiol-alkyne chemistry[67] (Scheme 1.9).



Scheme 1.9. Synthesis of star-like polymers consisting of octadecane, cholesterol, or POSS groups coupled to an alkyne terminated PBLG via thiol-alkyne chemistry, followed by deprotection to afford their PLGA derivatives[67]

Lastly, Kuo et al. described the synthesis of star-shaped polypeptides with polyhedral oligomeric silsesquioxane (POSS) derivatives as the core, by coupling alkyne terminated PBLG on it to form 8-armed stars. The PBLG groups adopted an α-helical conformation as it was shown by wide-angle X-ray diffraction, ^{13}C NMR spectroscopy, and FT-IR analysis[68].

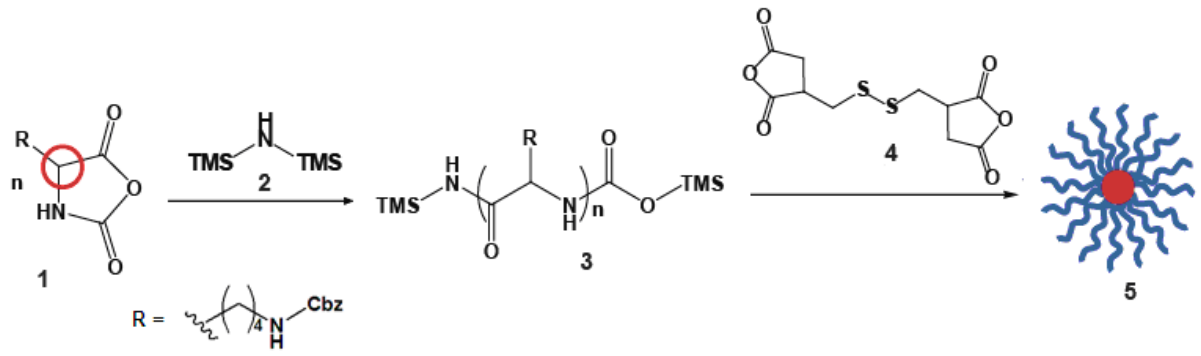
1.3.3 Arm-first (grafting through) approach

The grafting through approach, also referred to as convergent approach, describes the reaction of a living macroinitiator (MI) with a multifunctional cross-linker. In this way star-shaped polymers can be formed bearing macromolecular chains radiating from a densely cross-linked core. This type of star-shaped polymers is also called cross-linked star polymers (CCS) or large-core star polymers[39,69,70].

The main characteristic of this approach is that is suitable for the synthesis of star-shaped polymers with very large number of arms (>100). It is also possible to synthesize well-defined star polymers in a completely controlled manner, during all the synthetic steps. Furthermore, the living arms can be isolated and characterized before and after the linking reaction leading to star polymers with precise and entirely measurable functionalities. Moreover, functionality can be incorporated to the cross-linked core, which is capable of adopting a significant size depending on the size of the macromolecule. The drawback of this method is that, in many cases, a long reaction time is acquired and the reaction conditions have to be tightly controlled for the linking reaction or else incomplete conversion of MI to stars can occur and lead to broad arm number distributions. In this case, fractional precipitation or dialysis is required to obtain the pure star polymer.

Qiao et al. were the first who developed peptide-based CCS polymers composed entirely of amino acid building blocks using the arm-first approach[71]. They synthesized water soluble, biocompatible and biodegradable stars consisting of poly(L-Lysine) PLL arms radiating from a poly(L-cystine) (PLC) core (Scheme 1.10). The core could be functionalized via reaction with primary amines bearing different functional groups. These stars were capable of impounding hydrophobic drugs, such as the anti-cancer drug pirarubicin. Moreover, the

presence of the disulfide bond in the L-cystine blocks in the core makes the stars suitable for being cleaved by reducing agents such as dithiothreitol.



Scheme 1.10 Synthesis of star polymer **5** via a one-pot, arm-first approach. Z-*L*-lysine NCA **1** reacts with HMDS **2** to form linear living peptide **3**. The addition of *L*-cystine NCA **4** as cross-linker initiates the formation of star polymer **5**[71].

The same group, using the same approach, prepared CCS polymers with PBLG arms and PLC cores. Then the arms were functionalized after reacting with hydrazine, which displaced the benzyl protecting groups to afford pendant hydrazine groups[72], which are useful for attaching molecules via hydrazine linkers. Also, varying the reaction conditions, a library of stars with different properties and loading capacities was synthesized.

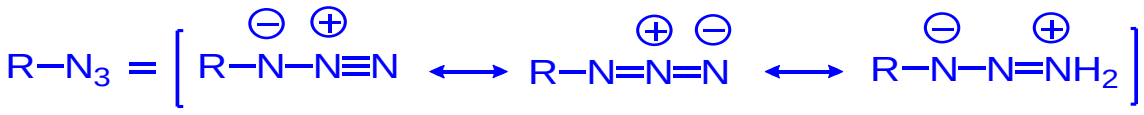
Another important study was made by Xing et al., who synthesized CCS polymers using α-methoxy-ω-amino poly(ethylene glycol) (MeOPEG1900-NH_2) as a macroinitiator for the ring opening polymerization of cystine and benzyl glutamate NCA derivatives[73]. The resulting star polymers were capable of encapsulating the hydrophobic drug indomethacin via hydrophobic interactions. After 200 hours, the drug was found to be 100% released after cleavage of the disulfide bonds in the core.

Furthermore, CCS polymers were prepared via RAFT polymerization, which involves the utilization of a MI terminated with a chain transfer end. Audouin et al.[74] reported the synthesis of CCS polymers, using divinyl benzene as the cross-linker and a styrenic terminated PBLG macromonomer, which prepared via ROP of benzyl glutamate NCA with an amino styrene initiator. Both RAFT and free-radical polymerization (FRP) were used to obtain CCS polymers with different molecular weight distributions and yields. It was shown that the stars synthesized with FRP had higher yields (43-73%), whereas RAFT led to stars with lower yields and in some cases resulted in gelation.

1.4 Click Chemistry

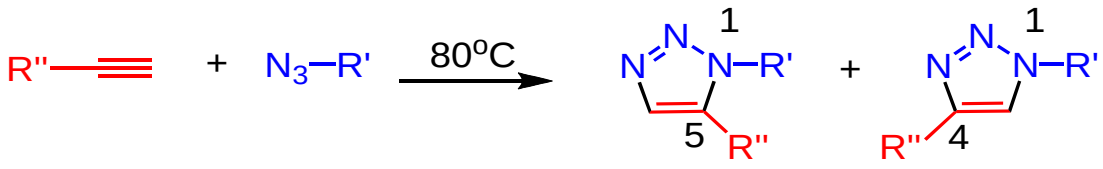
Click chemistry is an efficient, chemo-selective and highly reliable method to generate useful substances by joining small molecular units together in a stable and irreversible way, under mild reaction conditions. Click reactions have to fulfill some characteristics in order to be compatible with the above concept[75]. Initially, it has to be modular, wide in scope and give very high yields. It should also be stereo-specific and lead to the formation of harmless by-products that can be easily removed. The whole process should include simple reaction conditions, the use of a solvent that can be easily removed and finally, provide products that can be simply isolated under non-chromatographic methods.

Many reactions have been referred as good candidates for "click chemistry", but the most common and widely used is the Cu(I) catalyzed 1,3-dipolar cycloaddition of alkynes to azides to form 1,4-disubstituted-1,2,3-triazoles. To achieve this, many studies have been made dating back to 1893, when Authur Michael first discovered and reported the formation of triazole[76]. After that, was Linus Pauling who recognized the dipolar nature of azide in 1933[77] (**Scheme 1.11**).



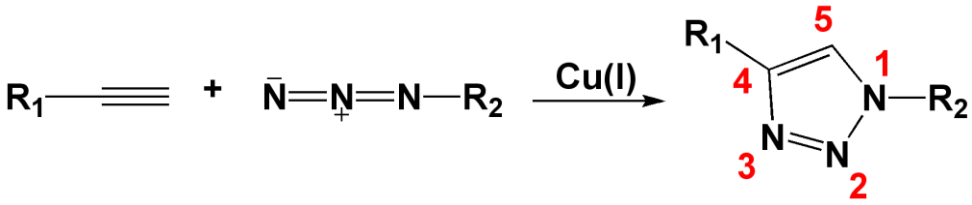
Sscheme 1.11 Dipolar nature of azide described by Pauling.

Almost 30 years later, in 1961, Rolf Hüisgen described the nature and the mechanism of this reaction, which was named after him as Hüisgen azide-alkyne 1,3-dipolar cycloaddition[78,79] (**Scheme 1.12**). Nevertheless, this reaction was non-regioselective and required high temperature and pressure conditions. For this reason this reaction was ignored for decades.



Scheme 1.12. Hüisgen azide-alkyne 1,3-dipolarcycloaddition.

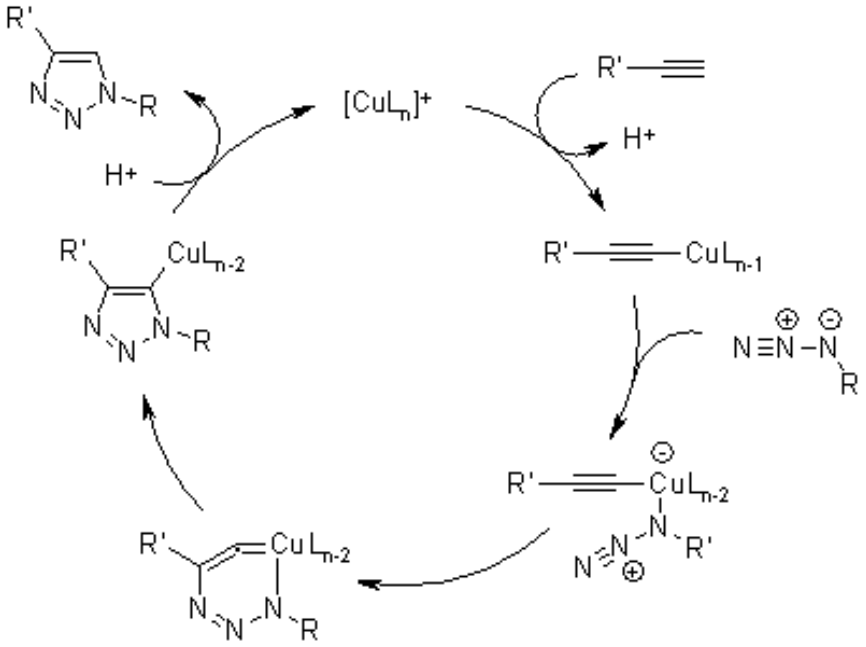
In 1984, L'Abbé first reported the use of copper as a catalyst of Hüisgen azide-alkyne 1,3-dipolarcycloaddition (CuAAC)[80], taking place as a side reaction for the synthesis of azidoallenes complex. No further process was observed until 2001, when two independent laboratories, one led by Sharpless[81] in the US and the other led by Meldal[82] in Denmark, introduced the CuAAC click reaction (**Scheme 1.13**).



Scheme 1.13. Click reaction as described by Sharpless and Meldal.

The innovation in this case was that the reaction was regioselective and occurred under mild conditions (room temperature, low pressure). Another main characteristic was that this reaction is very robust and insensitive to different reaction conditions like pH and solvent polarity. A possible mechanism, which was proposed by Jones et al[83]. and Ahlquist et al[84]. is

shown in Scheme 1.14. To explain briefly, Cu(I) forms a pi-complex with the triple bond of a terminal alkyne. Then, in basic environment, the terminal hydrogen is deprotonated to give a Cu acetylide intermediate. There are two copper atoms involved in the transition state. One is bonded to the acetylide and the other activates the azide. Then the azide reacts with the intermediate to generate a copper-azide-acetylide complex and subsequently cyclisation occurs, which is followed by protonation. The product is formed by dissociation and the Cu(I) complex is regenerated for further reaction cycles.



Scheme 1.14. CuAAC catalytic cycle.

A quite similar mechanism was proposed by Boren et al. , who described the same reaction using Ruthenium as a catalyst[85]. In this case 1,5-disubstituted 1,2,3-triazoles were formed, in contrary to the 1,4 disubstituted triazoles that were formed in the previous case. The main advantage of the Ruthenium catalyzed click reaction against Cu catalyzed click reaction is that it can also work on internal alkynes.

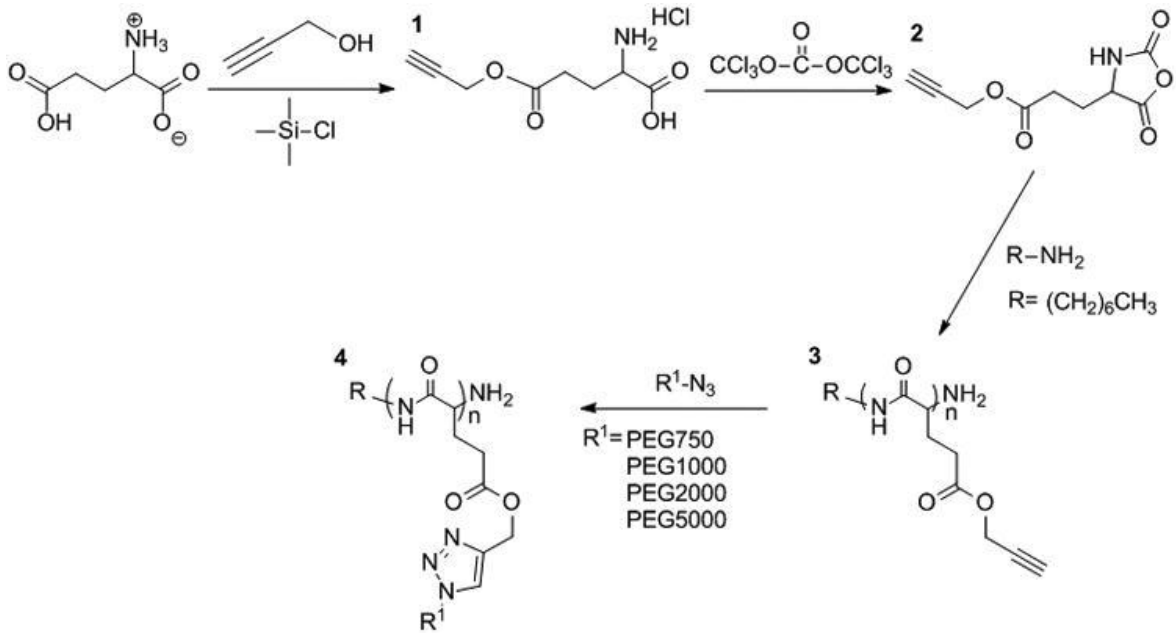
At this point, it is important to mention the potential of the triazoles for developing more stable polypeptides. Natural peptide bonds are unstable to proteolysis, whereas disulfide bonds are unstable to redox or thiol/disulfide exchange conditions. Many efforts have been made to replace the peptide bonds with non-natural stable structures. Some examples are the introduction of non-natural amino acids, terminal protection, cyclization and backbone modification to increase peptides' stability[86]. The 1,2,3-triazole constitutes a bioisostere of the amide bond. There are significant similarities in their physicochemical properties. They have similar sizes (distances between substituents are 3.8–3.9 Å in amides and 5.0–5.1 Å in 1,2,3-triazoles), similar dipole moment (amide ~ 4 Debye, 1,2,3-triazole ~ 5 Debye) as well as similar H-bond acceptor capacities [87,88]. The 1,2,3-triazole rings, possessing 2 sp^2-hybridized nitrogen atoms, N(2) and N(3), can function as weak hydrogen-bond acceptors. Also, the strong dipole moment of the 1,2,3-triazole ring can polarize the C(5) proton to such a degree that it can function as a hydrogen-bond donor similar to the amide NH. Another important feature of the triazole ring is that it has a large dipole that could align with that of the other amides in a given peptide secondary structure[89]. Furthermore, in contrary with amides, 1,2,3-triazoles are extremely stable to hydrolysis. Overall, since 1,2,3-triazoles can function as units that mimic the properties of peptide bonds, they can be potential surrogates of amide bonds for peptide modification.

1.4.1 Combination of click chemistry and NCA polymerization

Combining click chemistry with NCA polymerization can provide an effective and advantageous approach to quantitative post-polymerization functionalization. The clickable

backbone of the polypeptides is formed through direct polymerization of monomers and can be effectively attached to different side groups through click reactions.

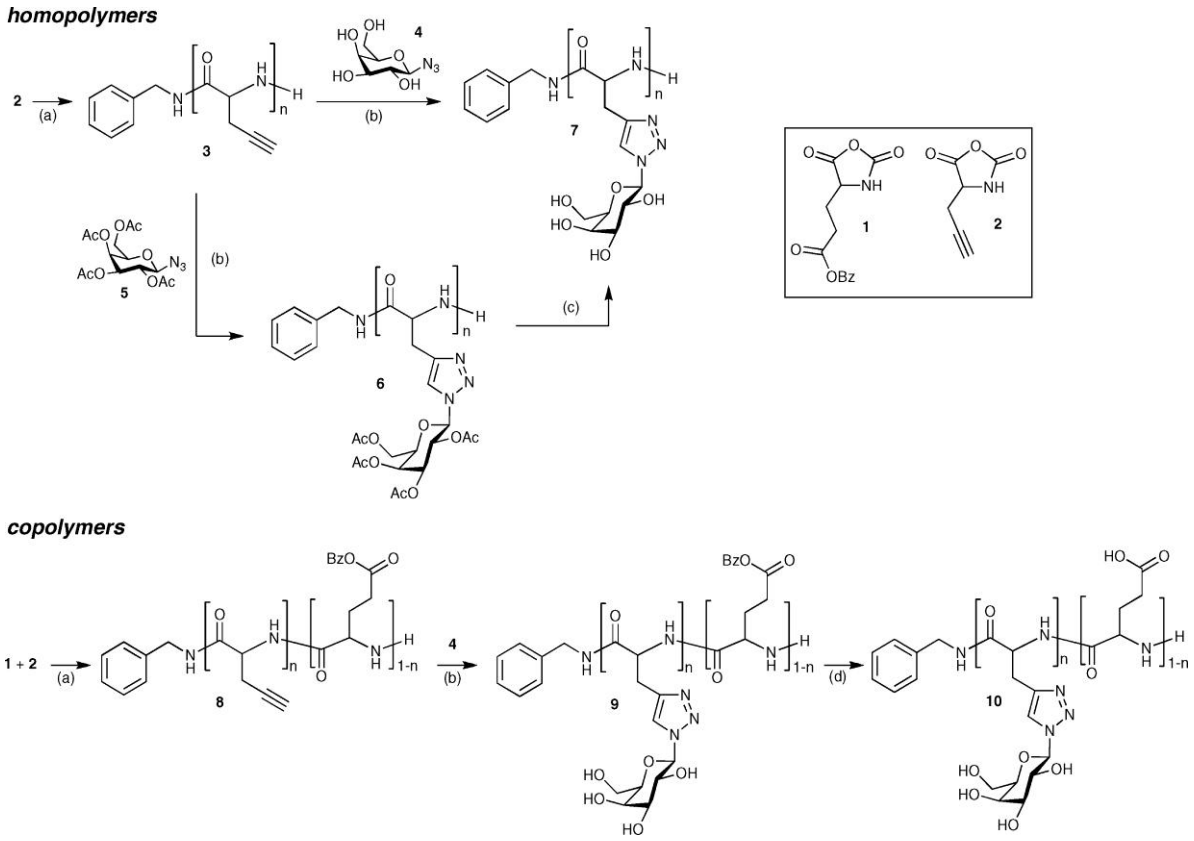
One of the first examples of click chemistry with an NCA polymerized polymer was reported by Engler et al., who first described the synthesis of an alkyne-modified NCA monomer, γ-propargyl L-glutamate (PLG), which was polymerized, via ring-opening polymerization, to form the poly(γ-propargyl L-glutamate) (PPLG), bearing an alkyne side group[90]. Afterwards, the alkyne side group was coupled with PEG-N_3 to obtain alpha helical post-modified polypeptides (**Scheme 1.15**).



Scheme 1.15. Synthesis of PPLG and side chain coupling via click chemistry[90].

Another study was presented by Huang et al., who synthesized glycopeptides using the non-natural amino acid DL-propargylglycine[17]. DL-propargylglycine NCA was homo and co-polymerized with γ-benzyl-L-glutamate (BLG) NCA, using benzyl amine as initiator, to obtain two clickable polymer backbones. Poly-propalgylglycine homo-polymer is stable to hydrolysis, in contrary with the co-polymer, which due to the propargyl ester, can be

hydrolized as a function of pH and temperature. Both polymers were glycosylated using azide functionalized galactose to obtain glycopeptides in high yields (**Scheme 1.16**). Acid deprotection chemistry was used to selectively hydrolyze the benzyl ester groups of the copolymers without any loss of galactose groups.



Scheme 1.16. Synthesis of glycopeptides with DL-propargylglycine using azide-functionalized galactose as described by Huang et al[17].

In addition to copper catalyzed click reaction, photoactivated thiol-ene or thiol-yne reactions gained great interest over the last years. The main advantage of thiol coupling reactions is that they can proceed smoothly without the presence of metal catalysts. They can also be easily controlled by a light source using facile and versatile chemical techniques, which make them suitable to combine with a big variety of functional groups. The mechanism is

straightforward and includes the thiol addition across the carbon-carbon double bond and it can be applied to both activated and non activated alkenes as well as almost any thiol group[91].

A characteristic example of the above was described by Sun and Schlaad[92]. They studied the reaction between thiol-ene type clickable polypeptides with poly(DL-allylglycine) and thiol functionalized sugars. Initially, poly(DL-allylglycine) homopolymer and poly(ethylene oxide)-b-poly(DL-allylglycine) copolymer were synthesized via controlled NCA polymerization, followed by radical thiol-ene additions with either methyl 3-mercaptopropionate or 1-thio-β-D-glucopyranose under thermal or photochemical conditions. Using trifluoroacetic acid (TFA) as solvent for the glycosylation with 1-thio-β-D-glucopyranose led to formation of glycopolypeptides from poly(DL-allylglycine) with only 50% degree of modification, under photoaddition techniques, in contrary with the block copolymer which reached almost quantitative yield for glycosylation. This aforementioned synthesis is very promising since no protecting group chemistry or metal catalysis were used.

1.5 Lectins

The most common way to investigate the biological activity and recognition of glycopolymers is by performing lectin-binding experiments. Lectins compose a diverse class of proteins that have the ability to selectively bind to mono-saccharides, oligosaccharides and glyco-conjugates in a highly specific and non-covalent way. This specific binding is due to the presence of a non-catalytic domain in the lectin molecule, which enables the recognition of carbohydrates and their interaction with the lectin. Lectins have been also named as agglutinins and form monomers, homo- and hetero- dimers and tetramers with an estimated size from 60 to 400 kDa[93]. Main source of lectins are animals and plants, but lectins have been also isolated from viruses, fungi and bacteria[94,95].

1.5.1 History of lectins

The first scientist who described lectins was Stillmark in 1888. He noticed that the crude extracts of castor beans contained a toxic substance, which he called '*Ricin'*, that could agglutinate human and animal red blood cells[96]. '*Ricin*' gained great attention at that time among bacteriologists and Ehrlich was the one that decided to focus his work on it and employ ricin as model antigen for immunological studies [97,98]. Some years later in 1926, J.B. Sumner was the first who crystallized the enzyme urease, which was isolated from jack bean, a protein that he named Concanavalin A, something that led to him being awarded with the Nobel Prize 21 years later. In 1936 Sumner and Howell reported that ConA was able to agglutinate cells and precipitate glycogen[99]. They also showed that agglutination of ConA was inhibited by sucrose, introducing this way the sugar selectivity of lectins. The same year, Landsteiner reported that the hemagglutinating activity of various seed extracts were different

when tested with erythrocytes from different animals[100]. This work was further investigated by Boyd and Shapleigh, who also came upon with the term 'Lectin', derived from the latin word 'legere', which means select[101]. Untill 1970s only a few lectins were isolated and even fewer were purified. Plant lectins that were early purified are: soya beans, green peas, wheat germ and mushroom (*Agaricus campestris)*[102]. The pace of purification of lectins was increased by the introduction of Affinity chromatography, which depends on the interaction between the lectin and a carbohydrate structure[103,104]. With this discovery, many lectins were characterized in a short period of time. Nevertheless, plants still remained the main source for lectins. Animal lectins that were isolated and characterized were mostly originated form eel, snail and horseshoe crab.

Lectins	Abbreviation	Sugar Specificity
Peanut Agglutinin	PNA	Galactose
Jacalin	AIA	Galactose/GalNAc
Erythrina cristagalli	ECA	Galactose/GlcNAc
Sophora japonica	SJA	GalNAc/Galactose
Ricinus communis Agglutinin I	RCA-I	GalNAc/Galactose
Griffonia simplicifolia Lectin I	GSL I	GalNAc/Galactose
Soybean Agglutinin	SBA	GalNAc
Dolichos biflorus	DBA	GalNAc
Vicia villosa Lectin	VVA	GalNAc
Griffonia simplicifolia Lectin II	GSL II	GlcNAc
Wheat Germ Agglutinin	WGA	GlcNAc/NANA
Succinylated Wheat Germ Agglutinin	SWGA	GlcNAc
Lycopersicon esculentum	LEA	GlcNAc
Solanum tuberosum	STA	GlcNAc/Sialic acid
Lens culinaris Agglutinin	LCA	Glucose/Mannose
Concanavalin A	ConA	Glucose/Mannose
Pisum sativum Agglutinin	PSA	Glucose/Mannose
Phaseolus vulgaris Leucoagglutinin	PHA-L	Complex Sugar
Phaseolus vulgaris Erythreoglubulin	PHA-E	Complex Sugar
Datura stramonium	DSA	LacNAc
Ulex europaeus Agglutinin I	UEA-I	Fucose

Table 1.1 List of plant lectins and sugars that they bind with[105].

1.5.2 Biological role of lectins

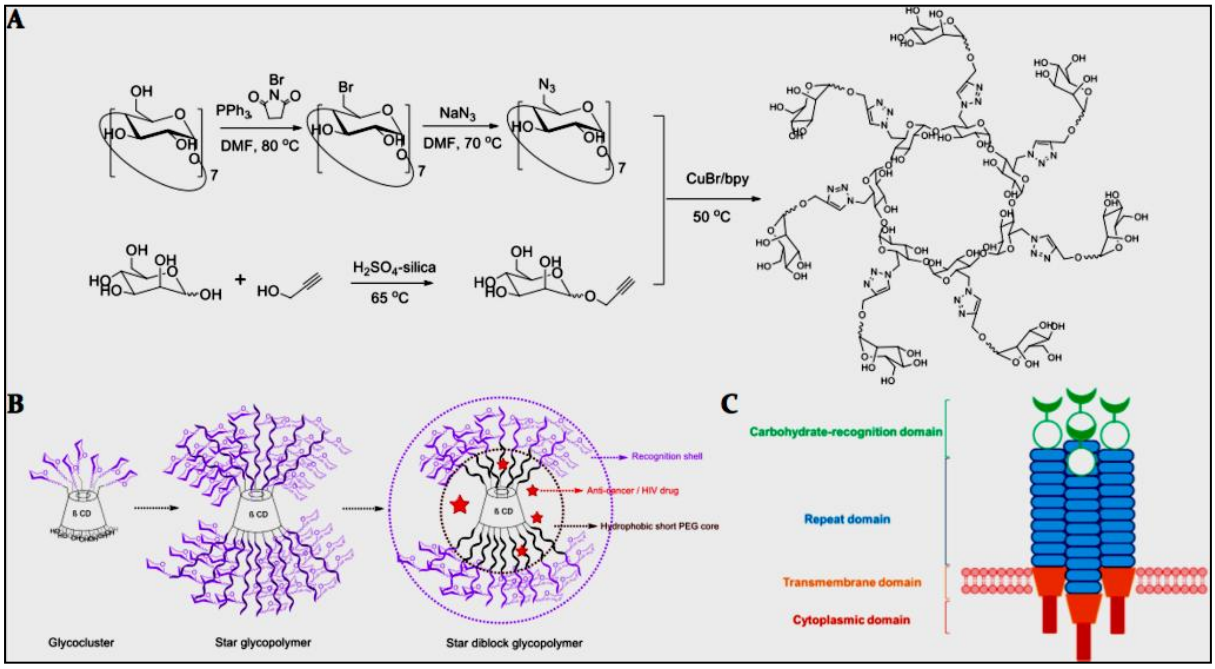
The biological role of lectins is based on hypotheses rather than pure knowledge. Due to their adhesion and agglutination ability, lectins are considered to play an important role in symbiotic and pathogenic interactions between some microorganisms and hosts. Initially the main focus was given to the investigation of the plant lectins, which at the beginning were the only ones that were recognised[106]. Based on that concept, it was reported that feeding bruchid beetles with 'the black neam' lectin resulted in the death of bruchid larvae. This led to the conclusion that lectins could protect legumes from attack by insects[107]. Moreover, lectins may play an important role in protection of plants against pathogenic microorganisms by inhibiting the sporulation and growth of different fungi[108]. Lectins are involved in sugar transport and carbohydrate storage and they as well take part in microbial adhesion to various surfaces; for instance they can bind to mucosal membrane and resist denaturation by acids and proteolytic enzymes[109]. Another theory about lectins is based its speculation on the fact that a lectin from a specific legume can bind to the surface of the polysaccharide of the corresponding rhizobial species, providing the plant with nitrogen, but cannot bind to bacteria from other legumes[110]. This was the lectin recognition theory, which led to many arguments due to inefficient evidence. Lastly, lectins can accomplish the attachment of a variety of cells or viruses to other cells, through the interaction with the surface carbohydrates of the cells that are going to be attached[111]. All the above characteristics of lectins resulted in their excessive use in biomedical research in applications such as agglutination, toxicity to cells, inhibition of fungal, bacterial and viral growth, anti-HIV property and anti-cancer activity[112,113,114].

1.5.3 Lectins and glycopolymers

Lectin binding experiments gained great interest over the last years, due to the facile way that they can be performed. Many research groups have focused on the synthesis of a broad variety of glycosylated polymers and their subsequent bio-recognition, which can be easily proved through lectin-binding assays. In this thesis, only a few of them will be reported. Huang et al. described the synthesis of homo- and block co-polypeptides poly(DL-propargylglycine) and poly(γ-benzyl-L-glutamate-co-DL-propargylglycine) and their subsequent glycosylation with azido-galactose[17]. The ability of the glycopeptides to interact with biological systems was investigated. A typical test includes the mixing of the glycopeptide with a lectin that is selective for the sugar attached to ti and a positive result is achieved when precipitation occurs. The lectin that was chosen for the above experiment was Ricinus communis Agglutinin (RCA_{120}) which is specific for binding with galactose moieties. The change in absorbance of different solutions of glycopeptides with RCA_{120} was measured and resulted in higher values for the higher concentrations of glycopeptides. In this case, lectin binding was immediate and completely dependent on the concentration.

Another study from the same group was performed by Byrne et al. who described the synthesis of star-glycopolypeptides using PPI dendrimer as initiator to obtain poly-glutamic acid derivatives[115]. The glycosylation was achieved using DMT-MM-mediated amide coupling chemistry to attach glucosamine in different ratios. Similarly as above, lectin binding analysis was performed using turbidimetric assays. The lectin that was used in this case was ConA, suitable for binding with glucosamine. Degree of glycosylation was found to affect the lectin-binding, i.e. a higher DS resulted in a quicker binding and precipitation. Moreover, comparing linear and star glycopolypeptides of similar DS and molecular weight, the linear ones demonstrated slower precipitation and decreased lectin-binding.

Chen et al. described the synthesis of glycopolymers-functionalized Ag nanoclusters (Gly-Ag-Ncs) using microwave irradiation, which were self assembled into micelles in water[116]. The bio-activity of the synthesized nanoclusters was tested with ConA lectin and showed an anticancer effect for cancer cells K562, inhibiting their viability in a dose dependant manner. Cytotoxic tests showed that these self-assembled polymers killed the K562 cancer cells under light irradiation. The above observations make these glycopolymers potential candidates for cancer imaging and therapy. Zhang and coworkers, synthesized cyclodextrin-based glycoconjugates via combination of CuAAC Huisgen coupling and Cu catalyzed radical polymerization.



Scheme 1.17. Synthesis of CD-Based Glycoclusters via CuAAC (A) and the Evolution Route from Glycocluster to Star Diblock Glycopolymer (B) and Schematic Structure of Human DC-SIGN Lectin (C)[117].

SPR (surface plasmon resonance) analysis showed that all these glycoconjugates can bind with the human lectin DC-SIGN and could prevent the binding of HIV gp120 protein to DC-SIGN at nanomolar concentrations. The aforementioned glycopolymers were also suitable for

loading anticancer and anti-HIV drugs, showing great potential for the synthesis of HIV-therapeutic and drug delivery systems. SPR analysis along with ITC (Isothermal titration calorimetry) techniques were used by Cameron et al. to investigate the interaction between mono- and polyvalent galactosides and the RCA_{120} lectin[118]. This was one of the few studies about thermodynamics and mechanisms of the lectin-sugar interaction. Avidities were found to greatly increased with multivalency due to a more than 4000-fold increae in K_a. Isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) data revealed that the cluster glycoside effect between glycopolymers and lectins occurred due to chelation and 'bind and slide'[119] mechanisms.

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Chapter 2

Synthesis and characterization of Star-shaped glycopolypeptides suitable for selective sugar-lectin binding

2.1 Introduction

Carbohydrates play a very important role in many biological processes including protein folding, inflammation, fertilization and hormone activities[1,2,3]. Consequently, a lot of research has been conducted on the preparation of synthetic glycopolymers that can mimic the natural carbohydrates. These artificial glycoconjugates have gained great interest not only due to their potential biomedical applications, such as drug delivery[4] and pathogen detection[5], but also due to their recognition properties[6]. Recent review papers from Cameron[7], Stenzel[8] and Kiessling**6** have described and summarized the latter as well as other applications.

Initially, the majority of the synthesized glycopolymers were acrylic in nature and their synthesis was based on controlled radical polymerization, displaying controlled molecular weights and glycosylation density. However, these glycoconjugates favor a random coil conformation and lack well defined secondary structures which might prevent some of the side-chain carbohydrate moieties accessing the biologically active sites. On the contrary, glycopolypeptides have the ability to adopt well defined secondary structures such as α-helix or β-sheet, which enable them to mimic naturally occurring glycoproteins. In the literature, the potential of glycopolypeptides in the field of therapeutics and medical diagnostics[9,10] as well as probing carbohydrate-protein interactions[11] has been discussed.

There are two different routes for the synthesis of glycopolypeptides: 1) ring opening polymerization of glycosylated NCA monomers and 2) post-polymerization glycosylation of polypeptide side chains. A recent study regarding the first synthetic route was reported by Deming et al. who succeeded in the synthesis of C-linked glycosylated-L-lusine NCA monomers, which were polymerized using transition metal initiators[12]. Different protected sugars were coupled to L-lysine in a four step procedure and then converted to the corresponding NCA by treatment with dichloro(methoxy)methane. Polymerization resulted in

high molecular weight polypeptides, which were soluble in water after deacetylation. The main issues with this strategy involve the use of protected sugars and the purification of the glyco-NCAs which requires high synthetic expertise. For the second synthetic strategy, various click-type coupling chemistries have been applied[13,14] and the one that gained great interest lately is the Cu catalyzed azide-alkyne [3+2] cycloaddition. This click chemistry is a highly versatile reaction, which can take place under various conditions like different solvents, pH and temperature[15,16]. Huang et al. demonstrated the synthesis of well defined - homo and block copolypeptides bearing alkyne functionality and their successful glycosylation[17]. Both protected and deprotected azido-galactose were used to investigate the effect of the free hydroxy group on the click reaction. NMR. FT-IR and SEC confirmed the successful glycosylation of the polypeptides. Moreover, lectin binding experiments confirmed the recognition of the glycopeptides from biological systems.

Byrne e al. described the synthesis of star polypeptides, performing NCA ring opening polymerization (ROP) techniques, to obtain well defined star-shaped polymers with different sizes and molecular weights. For that purpose, poly(propylene imine) (PPI) dendrimers were used bearing terminal amine groups, suitable to initiate NCA polymerization[18].

Based on the above studies and observations, our main interest in this first chapter was the synthesis of star-shaped polypeptides initiated by second generation PPI dendrimers and their subsequent glycosylation using Huisgen [3+2] cycloaddition reaction. γ-Propargyl-L-glutamate NCA was used for that purpose, due to its alkyne groups, which are suitable for 'click' chemistry reaction. To increase the functionality of our system a second block was added to the polymeric chain. Moreover, lectin-binding assays were performed to investigate the bio-recognition of the glycosylated polymers. This is a synthesis based chapter and the platform, which the next chapters were based on.

2.2. Experimental section

2.2.1 Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Diethyl ether was purchased from VWR. DMSO, ethyl acetate and ethanol were used directly from the bottle under an inert and dry atmosphere. PPI (polypropylene imine) dendrimer generation 2 was purchased from SyMO-Chem BV (The Netherlands). Ricinus communis (castor bean) Agglutinin RCA_{120} (10 mg/mL in buffered aqueous solution) and Concanavalin A (Con A, Type IV, lyophilized powder) from Canavalia ensiformis (Jack bean) were purchased from Sigma-Aldrich and used as received.

2.2.2 Instrumentation: ^1H spectra was recorded at room temperature with a Bruker Avance 400 (400 MHz), DMSO-d6, $CDCl_3$ and D_2O were used as solvents and signals were referred to the signal of residual protonated solvent signals. TMS was used as an internal standard for DMSO-d6 and $CDCl_3$. ATR-FTIR spectra were collected on a Perkin-Elmer Spectrum 100 in the spectral region of 650-4000 cm^{-1} and were obtained from 4 scans with a resolution of 2 cm^{-1}. A background measurement was taken before the sample was loaded onto the ATR unit for measurements.

2.2.3 Synthesis of 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose. To a solution of 1-α-Bromo-2,3,4,5-tetraacetyl-D-galactose (5.69 g, 13.8 mmol) in 60 mL CH_2Cl_2 at room temperature was added NaN_3 (4.5 g, 69.1 mmol), tetrabutylammonium hydrogen sulfate (4.7 g, 13.8 mmol) and 60 mL of a saturated solution of $NaHCO_3$. The reaction mixture was stirred vigorously at room temperature for 4 hours and then diluted with 500 mL ethyl acetate. The organic layer was washed with 200 mL of a saturated solution of $NaHCO_3$ and evaporated under reduced pressure. Azidogalactoside was obtained as a pale yellow solid.

Recrystallization from methanol yielded as white crystals (4.10 g, 80%). ^{1}H-NMR (400 MHz, $CDCl_3$, δ, ppm): 2.00, 2.08, 2.12, 2.19 (4s, 12H, 4 x CH_3CO), 4.00-4.04 (m, 1H, H-6a), 4.14-4.22 (m, 2H, H-5 and H-6b), 4.63 (d, 1H, H-1), 5.08 (dd, 1H, H-3), 5.17 (dd, 1H, H-2), 5.45 (dd, 1H, H-4)

2.2.4 Synthesis of 1-Azido-1-deoxy-β-D-galactopyranoside. 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose (650 mg, 1.74mmol) was dissolved in 10 mL anhydrous methanol in a Schlenk tube. To this solution a catalytic amount of anhydrous potassium carbonate (10 mg, 0.07 mmol) was added and the reaction mixture was vigorously stirred at room temperature under a nitrogen atmosphere for 3 hours. Amberlite IR-120 resin was washed with methanol and then added to and stirred with the reaction mixture for 1 hour. The resin was then filtered off under gravity and the resulting solution concentrated to yield white powder (305 mg, 86%). ^{1}H-NMR (400 Mhz, D_2O, δ, ppm): 3.50 (dd, 1H, H-2), 3.68 (dd, 1H, H-3,) 3.72-3.80 (m, 3H, H-5, H-6a, H-6b), 3.96 (d, 1H, H-4), 4.65 (d, 1H, H-1).

2.2.5 Synthesis of N-carboxyanhydride of γ-propargyl-L-glutamate (PLG-NCA). The preparation of PLG-NCA was carried out following a modified procedure reported by Hammond and coworkers[19]. To a solution of L-glutamic acid (10 g, 0.068 mol) suspended in propargyl alcohol (300 mL, 5.2 mol), 17 mL of chlorotrimethylsilane was added dropwise under nitrogen. The solution was stirred at 40 °C overnight and then the crude product was precipitated into diethyl ether. After filtering and recrystallizing from ethanol and drying under vacuum, a white solid (γ-propargyl L-glutamate hydrochloride) (**1**) was obtained to yield 13.3 g (0.06 mol, 88% yield). Product **1** (3.6 g, 0.016 mol) was suspended in dry ethyl acetate (120 mL) and the solution was heated to reflux under nitrogen. Triphosgene (2.2 g, 0.007 mol) was added dropwise and the reaction was left to reflux for 5 more hours. The

reaction solution was left to cool at room temperature and was filtered to remove any unreacted **1** Then it was cooled to 4 °C and washed with 120 mL of water, 120 mL of saturated sodium bicarbonate and 120 mL of brine all at 4 °C. The organic phase was then dried over anhydrous magnesium sulfate, filtered and evaporated to give viscous oil (2.1 g, 0.009 mol, 56% yield). ^{1}H NMR ($CDCl_3$, δ ppm): 2.21 (*J* = 4.52 Hz, 2H, $CHCH_2$), 2.38 (*J* = 2.19 Hz, 1H, C≡CH), 2.68 (*J* = 4.3 Hz, 2H, $COCH_2$), 4.48 (*J* = 2.11 Hz, 1H, $CHCH_2CH_2$), 4.76 (*J* = 4.4 Hz, 2H, OCH_2C≡CH), 6.58 (*J* = 2.07 Hz, 1H, NH).

2.2.6 Synthesis of star poly(γ-propargyl-L-glutamate) (3) initiated by propylene imine Dendrimer (PPI, 8 arm). PLG-NCA (**2**)(300 mg, 1.42 mmol) was dissolved in 10 mL of DMSO added to a Schlenk tube and left under nitrogen for 30 min. Then 26 mg (0.033 mmol ratio 40:1) of PPI dendrimer dissolved in DMSO (2 mL) was added to the reaction solution. The reaction mixture was left overnight to stir at room temperature. The polymer was precipitated into diethylether and collected by centrifugation and dried overnight under vacuum to yield 48% (155 mg). Following the same procedure for polymerization, polypeptides of 5, 15 and 30 monomer units were synthesized using PPI dendrimer as an initiator.

2.2.7 Glycosylation of star poly(γ-propargyl-L-glutamate). Star poly(γ-propargyl-L-glutamate) **(3)** 100 mg, (0.46 mmol of alkyne units), 1-Azido-1-deoxy-β-D-galactopyranoside (4) (141.46 mg, 0.69 mmol, 1.5 equivalent to alkyne) and triethylamine (33 μl, 0.12 mmol, 0.5 equivalent to alkyne) were dissolved in 6 ml of anhydrous DMSO in a Schlenk tube. The mixture left to stir under nitrogen atmosphere for 30 min. $(PPh_3)_3CuBr$ (43 mg, 0.046 mmol, 0.1 equivalent to alkyne) was then added in the solution and bubbled under nitrogen for another 30 min. Then, the Schlenk tube was placed in an oil bath at 30 °C for 72 h. Amberlite

Resin 120 was added to the mixture and left to stir for 24 h to remove the biggest part of the Cu catalyst. After filtration to remove the resin, the solution was added dropwise to an excess of THF solution, re-dissolved in DMSO, precipitated twice in THF and dialyzed against water for 72 h. The polymer then was lyophilized to obtain white solid (101 mg, 42% yield).

2.2.8 Synthesis of star shaped poly(γ-benzyl-L-glutamate-*b*-γ-propargyl-L-glutamate). γ-benzyl-L-glutamate NCA (250 mg, 0.95 mmol) was dissolved in 5 ml of DMSO in a Schlenk tube. A solution of PPI dendrimer 8arm (9.17 mg, 0.011 mmol) in 1ml of dry DMSO was added after the NCA was dissolved. The reaction left to stir at room temperature under a dry nitrogen atmosphere, until the NCA had been completely consumed as monitored by SEC and FTIR. After 16h, 601.35 mg (2.85 mmol) of γ-PLG-NCA dissolved in 1 ml of DMSO as the second monomer was added to the solution. The reaction was stirred at room temperature for 24h until the second monomer was completely consumed. The reaction mixture was precipitated into diethyl ether, centrifuged and dried under vacuum to obtain a pale yellow viscous solid (187 mg, 72% yield).

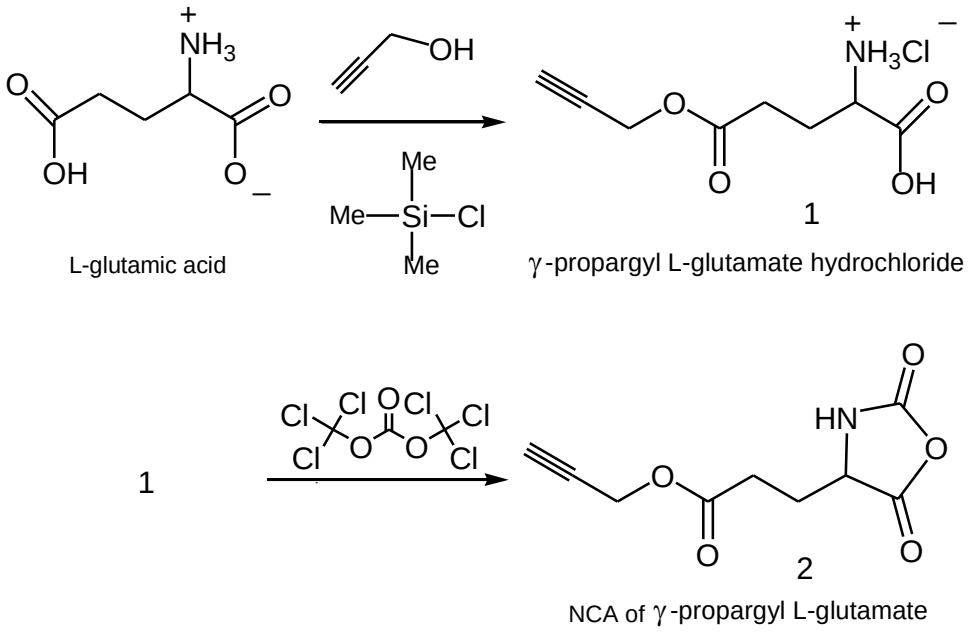
2.2.9 Synthesis of star shaped Poly(ε-Carbobenzyloxy-L-lysine-*b*-γ-propargyl-L-glutamate). ε-Carbobenzyloxy-L-lysine-NCA (300 mg, 0.98 mmol) was dissolved in 5ml of DMSO in a Schlenk tube. PPI dendrimer 8AM (4.73 mg, 0.006 mmol) was dissolved in 1 ml of DMSO and added to the reaction mixture. The reaction was left to stir at room temperature under a dry nitrogen atmosphere until the NCA had been completely consumed as monitored by SEC and FT-IR. Then (207 mg, 0.98 mmol) of γ-PLG-NCA dissolved in 1 ml of DMSO was added to the solution. The reaction was left overnight to stir until the second monomer was completely consumed. The reaction mixture was precipitated into diethyl ether and dried under vacuum to obtain a white viscous solid (349 mg, 68% yield).

2.2.10 Glycosylation of star shaped poly(γ-benzyl-L-glutamate-*b*-γ-propargyl-L-glutamate). Similar procedure as above was followed (56% yield).

2.2.11 Glycosylation of star shaped poly(ε-Carbobenzyloxy-L-lysine-*b*-γ-propargyl-L-glutamate). Similar procedure as above was followed (49% yield).

2.3 Results and Discussion

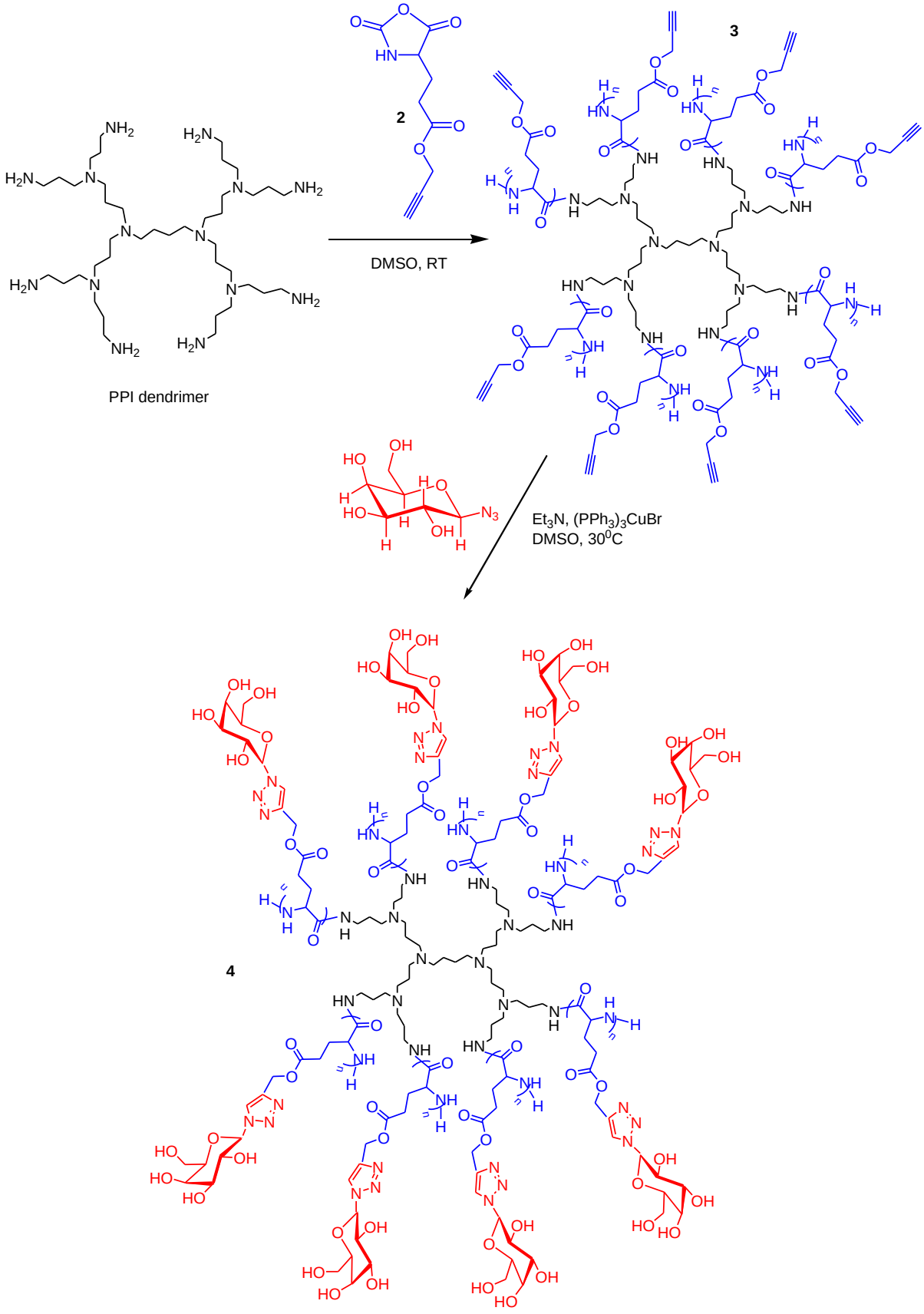
Synthesis of NCA. The synthesis of PLG NCA was carried out as shown in Scheme 2.1, following a modified procedure published by Hammond[19], who first synthesized this NCA monomer, bearing a terminal alkyne group accessible to click chemistry reactions. The synthetic procedure involved a two step process: γ-PLG-HCl (1) was obtained by the addition of glutamic acid to propargyl alcohol, mediated by trimethylsilyl chloride followed by reaction with triphosegene to form the γ-PLG-NCA monomer (2). The removal of the excess of propargyl alcohol is challenging but critical in this process as it could interfere with the NCA forming step. To avoid the presence of free propargyl alcohol, the product was extensively washed with ethanol to yield a white powder (dark grey powder when propargyl alcohol has not been removed). The structure of **2** was confirmed by ^{1}H NMR and FT-IR analysis. The ^{1}H NMR signal at 2.38 ppm (Figure 2.1) is due to the presence of the alkyne group, which also can be confirmed by the presence of a band at 2128 cm^{-1} in the FTIR spectrum. Additionally, FTIR spectra (Figure 2.2, bottom) shows the presence of a C=O group at 1736 cm^{-1} due to the glutamic acid ester in PLG-NCA and characteristic signals of the anhydride groups at 1836 cm^{-1} and 1801 cm^{-1}.



Scheme 2.1. Synthesis of γ-PLG-NCA

Ring opening polymerization. The synthesis of a large number of well defined polypeptides derived from natural or unnatural amino acids has been reported over the last years, using ROP techniques[20]. Lack of control over PDI and polymer architecture were overcome by the development of several polymerization approaches to achieve controlled NCA ROP[21,22]. The choice of the proper solvent is critical in order to obtain highly soluble and defined polypeptides. In our case , the polymerization was initially performed in chloroform, using benzylamine as initiator to obtain a linear polymer which would be used as a model for further experiments. It was found that the obtained polymer was not soluble in chloroform (precipitation occurred 4 h after the beginning of the reaction). It was hypothesized that a more polar (aprotic) solvent would better solubilise the polypeptide and the reaction was repeated in DMSO. This proved successful and the product was characterized and the structure confirmed by ^{1}H-NMR spectroscopy. For that reason, DMSO was used for the polymerization with the 8-arm PPI dendrimer initiator using NCA/dendrimer feed ratios of

120, 240 and 480 to obtain star polypeptides with 15, 30 and 60 units per arm respectively. Polymerization reaction took place at room temperature and the product was characterized by ^{1}H NMR spectroscopy, SEC and FT-IR spectroscopy to confirm the success of the reaction. ^{1}H NMR spectra confirmed the structure of PPLG star polymer showing characteristic peaks of the PPI dendrimer between 3.0-3.3 ppm and of the PPLG triple bond proton at 2.25 ppm Figure 2.1). From the integrated peak areas of the protons of PPI dendrimer and the signals of the protons next to peptidic bond (peak b, middle spectrum) the degree of polymerization was calculated and proved to be in agreement with the initial feed ratio. FTIR spectra suggests an α-helical conformation due to the amide peaks at 1654 cm^{-1} and 1544 cm^{-1} (Figure 2.2, middle). In addition, the triple bond is also visible at 2132 cm^{-1}. While GPC measurements in DMF revealed mostly monomodal peaks for the polypeptides a strong peak broadening was observed most likely due to the low solubility of the polypeptides. Reliable molecular weight information was thus not available. It can be speculated that the low solubility is a consequence of the hydrophobicity of the polypeptide side chain. It was indeed observed that the solubility significantly improved after "clicking" on the sugar moiety, which allowed reliable molecular weight analysis.



Scheme 2.2. Reaction scheme of the synthesis of glycosylated star-polypeptides

Glycosylation (''click'') reaction. Click reaction techniques were firstly described by Sharpless and include the 1,3-dipolar cycloaddition reaction between an alkyne moiety and an azide group to form a triazole ring.[23] The above methods reveived great attention over the last few years due to their highly reaction efficiency, their mild reaction conditions and the formation of only few byproducts, which can easily be removed. In this project, the glycosylation reaction was carried out using azido functionalized galactose, which was "clicked" to the triple bond of the PPLG unit of the polypeptide, with the subsequent formation of a triazole ring. The Cu catalyst that was used for the reaction was removed by treatment with Amberlite Resin and excessively dialysis. After the completion of the reaction, immediate solubility of the polymer in water was observed. Spectroscopic evidence for the successful glycosylation was obtained from ^{1}H NMR spectra (Figure 2.1) revealing the characteristic proton peaks of the triazole ring (f), polypeptide backbone (b) and galactose (s1-s6). ^{1}H NMR spectra also proved the quantitative glycosylation due to the absence of the triple bond peak. Likewise, in the FT-IR spectra (Figure 2.3) the dissapearance of the triple bond band at 2132 cm^{-1} after the reaction with azido sugar can be observed suggesting near-quantitative conversion in agreement with literature reports[24,25]. FT-IR spectra provides a further evidence for the secondary structure of all the glycopolypeptides, with amide peaks at 1650 cm^{-1} and 1542 cm^{-1} characteristic of a helical structure. The N-H stretching of PLG-NCA, PPI-PPLG and PPI-PPLG-gal was observed at 3273 cm^{-1}, 3301 cm^{-1} and 3292 cm^{-1}, respectively. The theoretical molecular weights of the glycosylated polypeptides were calculated according to the molecular weights of star PPI-PPLG polypeptides assuming that 100% glycosylation occurred.

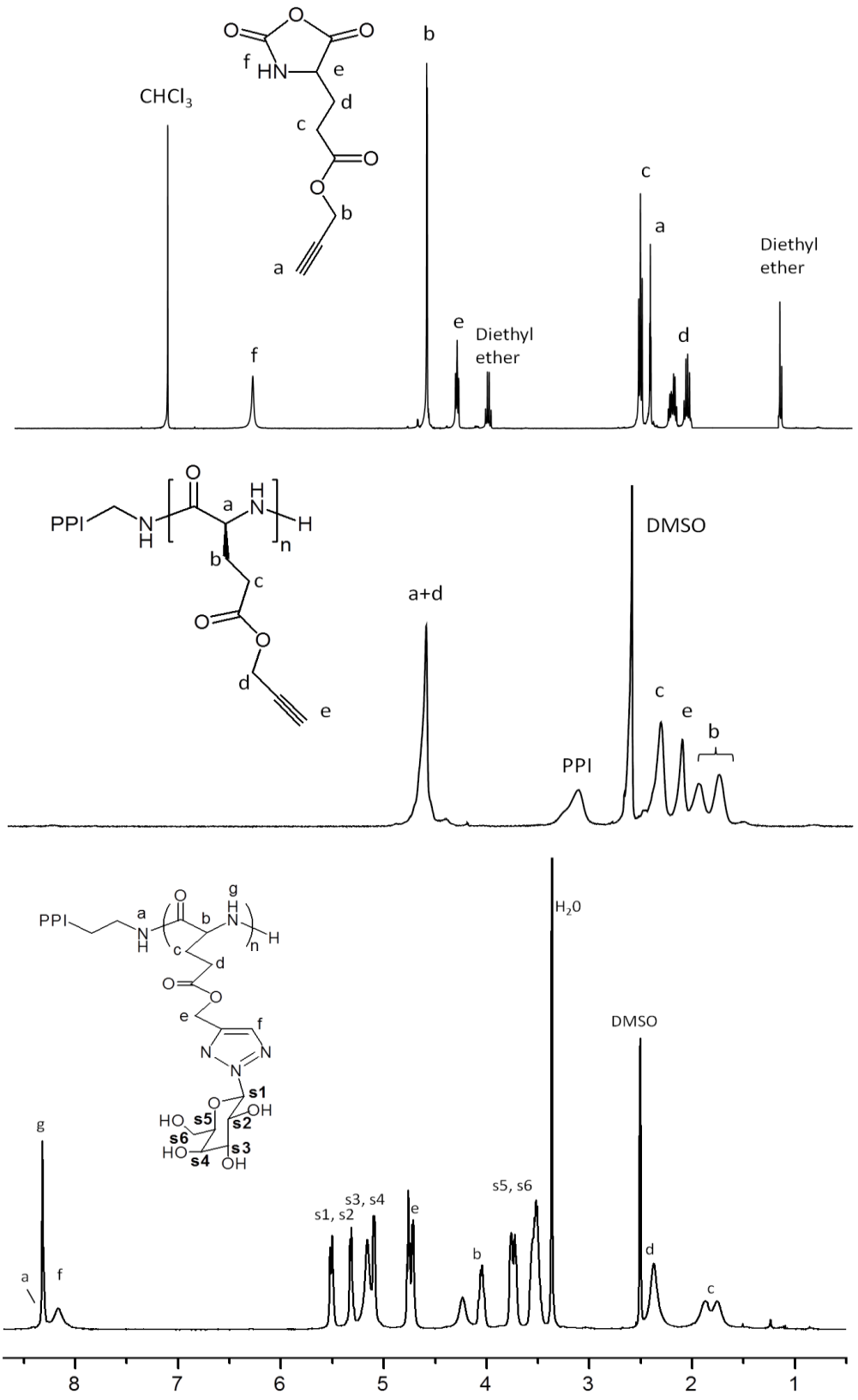


Figure 2.1. ^{1}H-NMR spectra of γ-PLG-NCA (**2**) (top) in d-chloroform, PPI-PPLG (**3**) in d-trifluoroacetic acid (d-TFA) (middle) and PPI-PPLG-galactose (**4**) in d-DMSO (bottom).

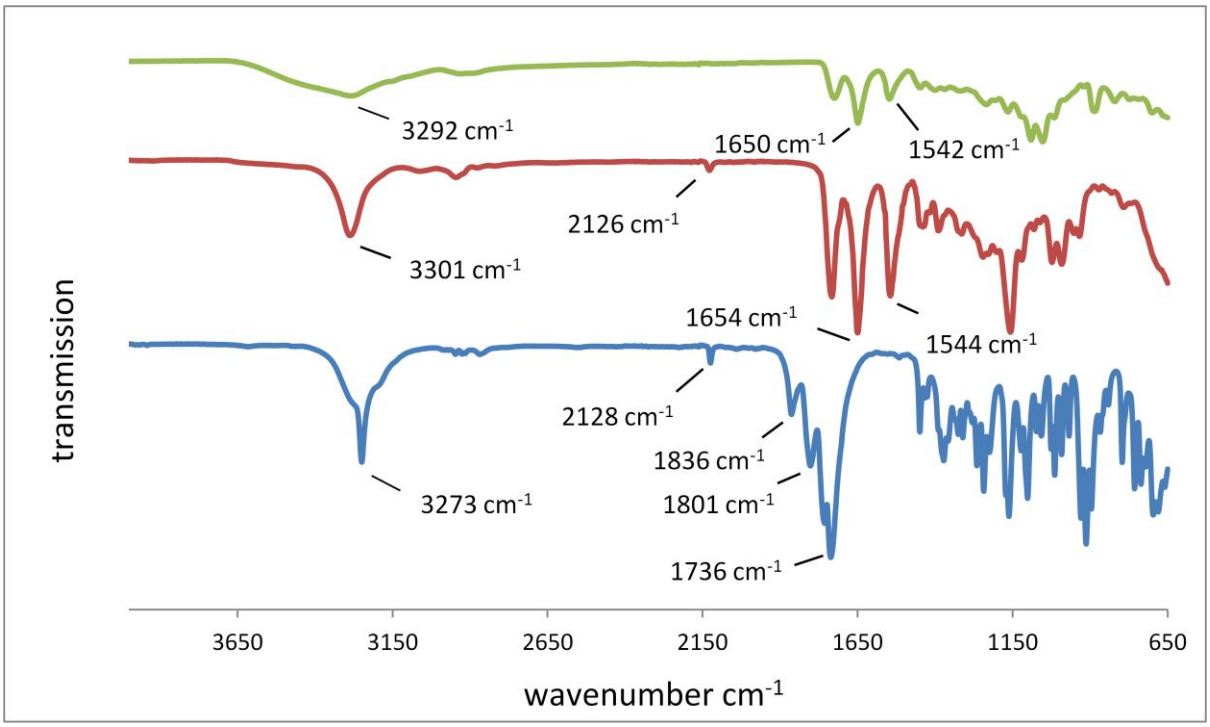


Figure 2.2. FT-IR spectra of PLG-NCA (**2**) (bottom), star PPI-PPLG (**3**) (middle) and star PPI-PPLG-galactose (**4**) (top)

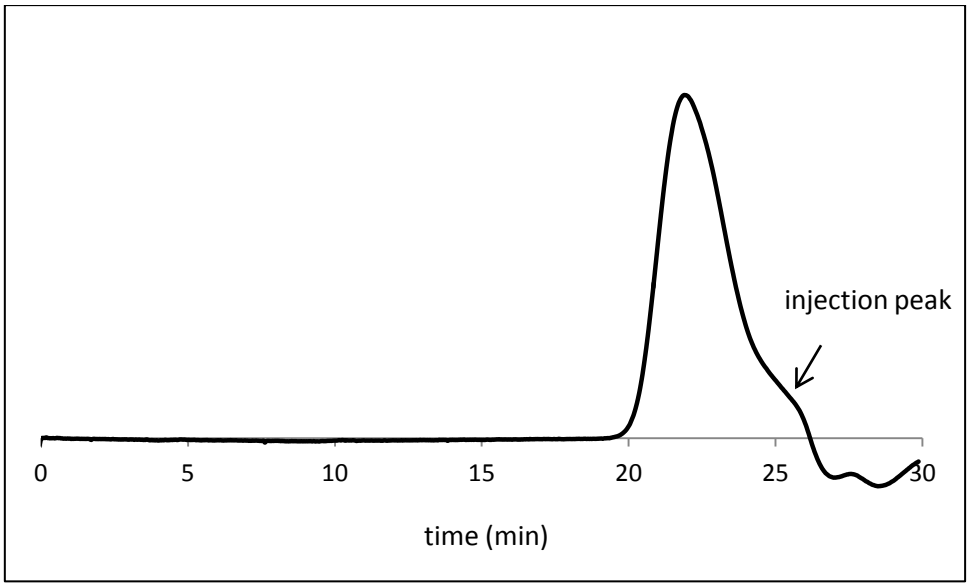


Figure 2.3. SEC traces of PPI-PPLG$_{15}$ (PDI=1.055), dn/dc value not available

Table 2.1. Star-shaped polypeptides initiated by 8 arm PPI dendrimer.[a)] calculated assuming initiation from all amino groups and quantitative conversion: [c(polymer)/c(dendrimer) x M(polymer)] + M(dendrimer). Entries 4-7 indicate block polypeptides.

Entry	polypeptides	NCA/ NH_2	NCA/ dendrimer	M_n (g mol^{-1}) NMR	M_n (g mol^{-1}) (theoretical)[a]	Conformation (FTIR)
1	**PPI-PPLG$_{15}$**	15	120	20800	20600	α- helix
2	**PPI-PPLG$_{30}$**	30	240	40800	38300	α - helix
3	**PPI-PPLG$_{60}$**	60	480	80900	73700	α - helix
4	**PPI-PBLG$_{20}$-PPLG$_{20}$**	40	320	62500	65400	α - helix
5	**PPI-PBLG$_{10}$-PPLG$_{30}$**	40	320	58400	52900	α - helix
6	**PPI-PLL$_{20}$-PPLG$_{20}$**	40	320	71600	74700	α - helix
7	**PPI-PLL$_{10}$-PPLG$_{30}$**	40	320	62900	66300	α - helix

Lectin binding experiments. Lectins are proteins that have the ability to selectively bind to sugar molecules in a highly specific and non-covalent way. Demonstration of glycopolypeptides binding to these cell receptor mimics is an efficient method to evaluate the potential of glycopolypeptides for biological interaction. The lectin selected for the binding assay was the *Ricinus Communis Agglutinin* lectin (*RCA_{120}*). *RCA_{120}* is a galactoside sugar specific lectin, which validated its use for recognition of the lactobionic acid grafted onto the L-PBLG glycopolypeptide[26].

The glycopolypeptides (entries 1-3, Table 2.1) were solubilized in deionised water in concentrations of 0.5 mg/ml and 2 mg/ml and a solution of *RCA_{120}* lectin (0.2mg/ml) was added. The two solutions were mixed and the turbidity owing to the precipitation caused by

the binding of the lectin to the sugar was recorded in 5 minute time intervals at a wavelength of 450 nm using a UV/Vis spectrometer.

Immediate precipitation occurred (vial A in the inset in Figure 2.4) for both polypeptide concentrations confirming the spontaneous binding of the glycopolypeptide to the lectin. The solution with higher polypeptide concentration expressed higher turbidity, which remained almost stable over a period of 30 minutes for both samples. As negative controls, a blank sample and a solution of glycopeptides mixed with ConA (mannose binding lectin) were used and resulted in no significant change in the absorbance. These simplistic lectin binding tests only allow a qualitative comparison between samples. In agreement with literature reports, the higher the sugar concentration in the sample, the higher the tubidity[17]. The data demonstrate that lectin binding is spontaneous irrespective of the glycopolypeptide concentration, which might suggest a high accessibility of the sugars.

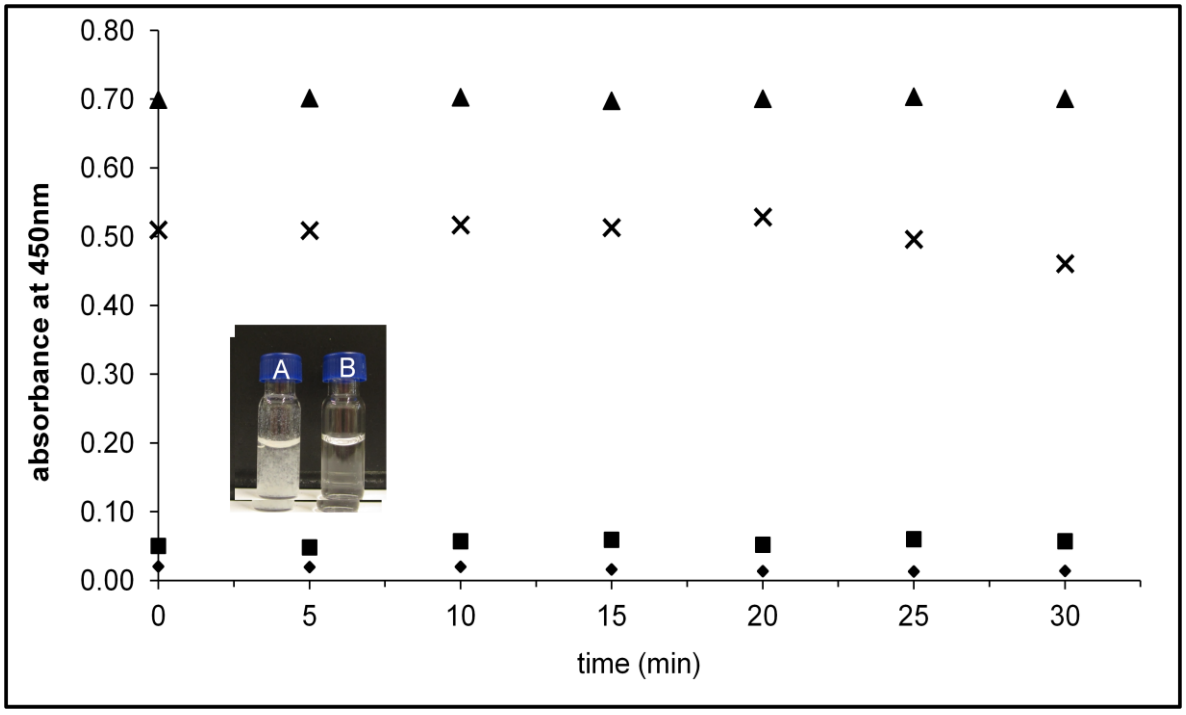
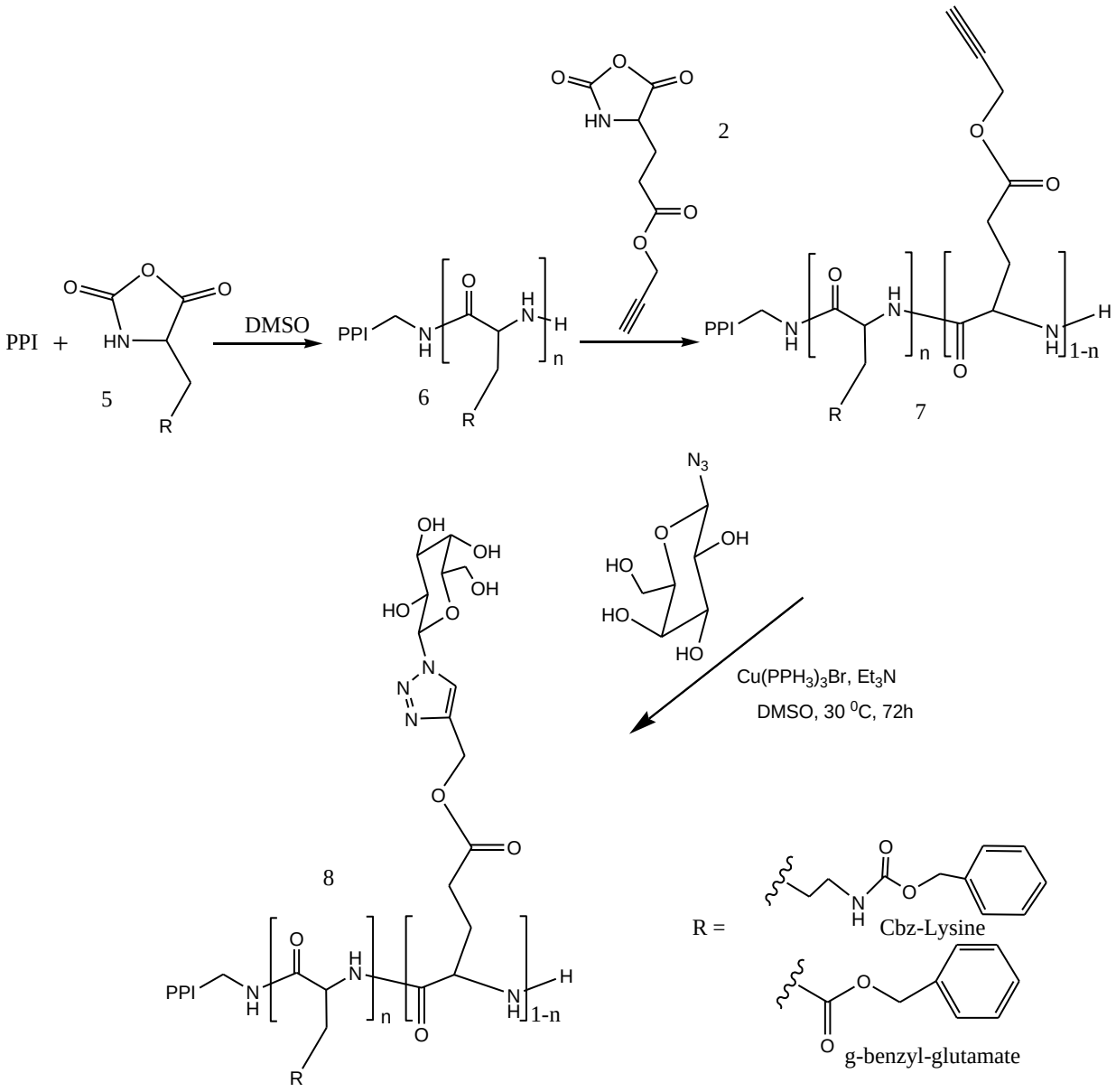


Figure 2.4. (▲) lectin RCA_{120}, PPI-$PPLG_{15}$-gal concentration 2 mg/mL; (x) lectin RCA_{120}, PPI-$PPLG_{15}$-gal concentration 0.5 mg/mL; (■) Concanavalin A, PPI-$PPLG_{15}$-gal concentration 2mg/mL (◆) lectin RCA_{120}, PBS buffer without glycopeptide. A: PPI-$PPLG_{15}$-gal solution at 2 mg/mL with RCA_{120} , B: PPI-$PPLG_{15}$-gal solution at 2 mg/mL with Con A.

Synthesis of block glycopolypeptides. In order to increase the structural diversity and functionality of the star polymers, a second block was added to the star polypeptide structure. The purpose for that was to introduce a second functionality, like pH sensitivity, to the polymers. Two different NCAs were used: γ-benzyl L-glutamate (BLG) NCA and ε-carbobenzyloxy-L-lysine Lysine (Cbz-LYS) NCA. As before, 8-arm PPI dendrimer was used as an initiator to polymerize BLG-NCA or Cbz-LYS NCA to obtain the first block. The reaction was monitored by SEC and FT-IR and when the NCA was consumed, PLG-NCA was added directly to the reaction to form the second block. DMSO was used as a solvent and the reaction took place in room temperature. Monitoring the NCA consumption for the synthesis of the first block is crucial as previous research has show that upon completion of the reaction side reaction occur that can result in the removal of the amine end-group thus preventing macroinitiation of the second NCA[27,28]. ^{1}H NMR data confirmed the success of the reaction, displaying characteristic peaks of polymer backbone and aromatic ring of protected PBLG or Cbz-PLL units. Comparing the signals of the protons of the aromatic ring of protected PBLG or Cbz-PLL with the one of the two two protons next to the triple bond of PPLG, the ratio of the two blocks was calculated and found in good agreement with the theoretical one. A set of block polypeptides was synthesized this way, with the ratio of the two blocks to vary from 1:1 to 1:3 (entries 4-7, Table 2.1), with the second number indicating the PPLG block. The reason for using a higher ratio of PPLG units was to investigate the potential increase of hydrophilicity after clicking the sugar on.



Scheme 2.3. Reaction scheme for the synthesis of glycosylated block star-polypeptides

Glycosylation reaction took place using the same conditions as described above and no interference with the second block was observed. For the characterisation of the glycopolypeptides ^{1}H NMR and FT-IR spectroscopy were used to confirm the success of the reaction, whereas FT-IR revealed α-helical structures before and after glycosylation for all the polymers. All glycopeptides expressed very good solubility in aqueous media. However, selective deprotection of PBLG or Cbz-PLL units could not be achieved in order to obtain carboxylic acid or amine end group functionality. This is due to the labile ester bond in the

PPLG block. Cleavage of the benzyl ester bond by using acidic conditions, led also to the cleavage of the sugar moiety of the block-polypeptides. For that purpose, different protecting groups should be used in order to achieve selective deprotection. An alternative developed in our research group after completion of the work in this chapter is the replacement of the benzyl glutamate by tert.-butyl glutamate as this group can be removed under much milder conditions[29]. Here Fluorenylmethyloxycarbonyl chloride (Fmoc) protected PLL was tested as an alternative first block and initiated the polymerization of PLG-NCA. Fmoc group can be easily removed using a mild base like pyridine. In our case though, the block copolymers that were synthesized were not soluble in any of the common organic solvents, making their characterization and deprotection challanging. Further investigations were made and an alternative popypeptide block was used. The results are described in the next chapter.

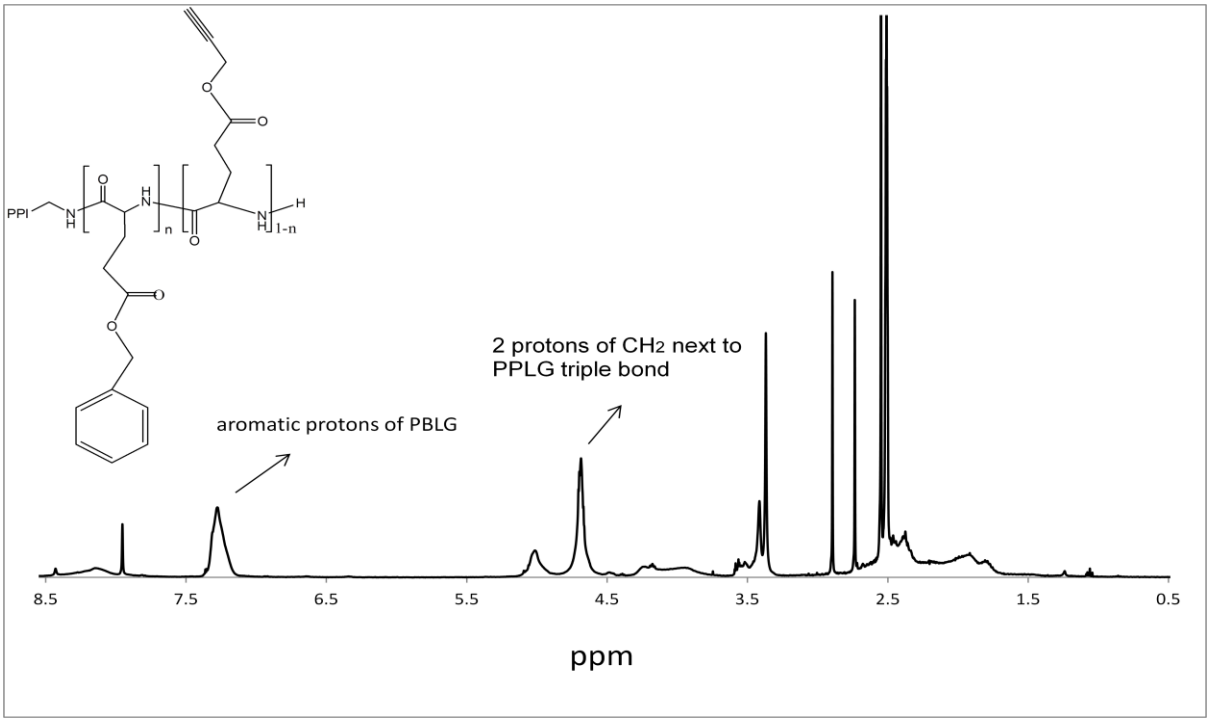


Figure 2.6. ^{1}H-NMR spectrum of PPI-PBLG$_{10}$-PPLG$_{30}$

2.4 Conclusions

In this chapter the successful synthesis of star homo- and block glycopolypeptides was described. The latter structure was envisaged as an amphiphilic micelle-like structure bearing a hydrophobic core and a hydrophilic outer shell, due to the presence of galactose. Using an 8-arm PPI dendrimer a range of homo and copolypeptides with clickable groups were obtained. After conjugation with galactose, the interaction of glycopeptides with biological molecules was tested by lectin-binding assays and it confirmed spontaneous and selective binding irrespective of the glycopolypeptide concentration. This chapter presents a series of model experiments to obtain novel biofunctional star-polymers and forms a platform for further investigation. A drawback of the proposed synthetic strategy is the fact that no selective deprotection of the individual polypeptide blocks could be achieved, which has to be addressed to develop advanced functional polypeptides.

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Chapter 3

Synthesis of 8-arm star glycopolypeptides suitable for bio-recognition and DNA complexation

3.1 Introduction

Over the past years, there has been a great progress in the field of drug delivery. Micelles[1], liposomes[2], particles[3] and DNA nanostructures[4] have been designed for that purpose. In addition, dendrimers have been proven to be suitable drug carriers for both hydrophobic and hydrophilic drugs[5]. Dendrimers are symmetric molecules with a monodisperse and branched architecture with many functional end groups, depending on the dendrimer generation. One of their main advantages is that they can be used to either covalently attach other groups or drugs on their surface or host them as guest molecules in the dendritic core[6]. Furthermore, dendrimers have been reported for imaging purposes due to their well-defined structure and architecture[7]. However, the loading capacity of dendrimers is limited. High molecular weight star-polymers, on the other hand, could potentially carry a higher payload and be designed to carry a combination of drugs[8]. For example, the possibility to design a system suitable for carrying both an anti-cancer drug and a contrast agent is of high importance and could play a key role in theranostics.

Several examples of the drug delivery potential of star polypeptides comprising a dendrimer core and polypeptide periphery have been reported. Using a four-arm amino terminated, disulphide cored PAMAM dendrimer derivative with a diethylene glycol-L-glutamate outer shell, reduction and thermo sensitive micelles and hydrogels were readily prepared.[9] Temperature (LCST at 40°C) and DTT triggered reduction permitted micelle and hydrogel size control. Release of doxorubicin from reduction sensitive micelles was shown to increase the release rate by 50% but the star polypeptide exhibited a more prolonged release profile than a comparative linear analogue. Such an effect highlights the benefit of a star architecture in controlled release applications. In spite of this, the trigger specific drug release was

questionable due to nonspecific release of doxorubicin without DTT limiting the realistic applicability of this material.

The potential of a series of generation 2 and 3 PAMAM dendrimers decorated biodegradable PGA were investigated as drug delivery vehicles.[10] Albeit drug encapsulation was demonstrated, the polymers showed susceptibility to enzymatic degradation, a potential release stimulus, whilst selectively targeting and labelling tumorous cells. The polypeptidic nature of these materials, i.e. glutamic acid, rendered them enzymatically sensitive and the branched architecture prolonged enzymatic degradation in comparison to linear PGA. Furthermore the star architecture resulted in superior functionality arising from the numerous PGA arms and terminal amines present on these arms. Consequently post modification of these materials was readily achieved via the covalent attachment of folic acid, a targeting moiety for cancer cells typically over expressing folate receptors, and a fluorescent moiety for diagnostic purposes.

Our interest is in the development of biocompatible three-dimensional micelle-like structures consisting of a hydrophobic core and a hydrophilic shell. To achieve this, we apply ring-opening polymerization of different NCAs using polypropyleneimine dendrimers (PPI) as initiator. Glycosylation of the polypeptides will be carried out with azide-functionalized galactose via Huisgens cycloadditionError! Bookmark not defined. The galactose units will not only provide hydrophilicity, but are also expected to promote lectin binding. Polypeptides have been proven to be biodegradable and also to have a well defined structure. Modifying the parameters and the properties of these glycopolypeptides, will allows us to obtain structures, which will have biocompatible features and are anticipated for drug delivery purposes.

3.2 Experimental section

3.2.1 Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Diethyl ether was purchased from VWR. DMSO, DMF, ethyl acetate, THF and ethanol were used directly from the bottle under an inert and dry atmosphere. DL-propargylglycine and H-Lys(Z)-OH were purchased by Bachem. Ricinus communis (castor bean) Agglutinin RCA_{120} (10 mg/mL in buffered aqueous solution) and Concanavalin A (Con A, Type IV, lyophilized powder) from Canavalia ensiformis (Jack bean) were purchased from Aldrich and used as received. α-Pinene (98%) and bis(trichloromethyl) carbonate (triphosgene) 99% were purchased from Sigma Aldrich. PPI (polypropylene imine) dendrimer generation 2 was purchased from SyMO-Chem BV (The Netherlands). 1-β-Azido-2,3,4,6-tetraacetyl- D-galactose was synthesized following a literature procedure[11]. All air sensitive compouns were used under a nitrogen atmoshere.

3.2.2 Instrumentation: ^1H NMR spectra were recorded at room temperature with a Bruker Avance 400 (400 MHz); DMSO-d6, $CDCl_3$ and D_2O were used as solvents and signals were referred to the signal of residual protonated solvent signals. TMS was used as an internal standard for DMSO-d6 and $CDCl_3$. ATR-FTIR spectra were collected on a Perkin-Elmer Spectrum 100 in the spectral region of 650-4000 cm^{-1} and were obtained from 4 scans with a resolution of 2 cm^{-1}. A backround measurement was taken before the sample was loaded onto the ATR unit for measurements. SEC analysis using Dimethylacetamide (DMAC) as eluent was carried out using a Shimadzu LC-10AD pump (flow rate 0.8 mL/min) and a WATERS 2414 with a differential refractive index detector (at 35 °C) calibrated with poly(methyl methacrylate) (range 1000 to 2,000,000 g/mol). Two PSS PFGlin-XL (7 μm, 8 x 300 mm) columns at 40 °C were used. Injections were done by a Spark Holland MIDAS injector using

a 50 μL injection volume. Before SEC analysis was performed, the samples were filtered through a 0.2 μm PTFE filter (13 mm, PPhousing, Alltech).

3.2.3 Synthesis of DL-Propargylglycine NCA (1). This synthesis was described before by Huang et al.[12] Briefly, in a three-neck round bottom flask, DL-Propargylglycine (2.3 g, 20.4mmol) and α-pinene (13.92 g, 101.9 mmol) were added and dissolved in 70ml of anhydrous THF. The reaction mixture was heated to 50 °C under nitrogen and then triphosgene (4.1 g, 13.8 mmol) in 20 mL of THF was added dropwise over a period of 1 h. The reaction was further progressed for 4h until the mixture became clear. Afterwards, the mixture was concentrated under reduced pressure and the NCA precipitated by addition of 100 mL of n-heptane. The mixture was placed in freezer overnight, then filtered and the crude product was dissolved in dry THF. Recrystallization took place twice by addition of n-heptane to obtain white crystals (1.98 g, 72% yield). ^{1}H NMR (acetone-d6, δ, ppm): 2.60 (*J* = 3.6, 1H, C≡CH,), 2.81 (*J* = 6.9, 2H, -CH2-C≡), 4.72 (*J* = 3.4, 1H, CH), 7.98 (1H, NH).

3.2.4 Synthesis of ε-Carbobenzyloxy-L-lysine NCA (2). Synthesis was performed following a literature procedure[13]. ε-Carbobenzyloxy-L-lysine (10.3 g, 35.7mmol) and α-pinene (10.1 g, 74.3 mmol) were dissolved in 80ml ethylacetate in a three-neck round bottom flask. The flask was placed in silicon bath (90 °C) and triphosgene (5.7 g, 19.2 mmol) was added slowly after being dissolved in 20 ml ethylacetate. After 3h the solution became clear and all solids disappeared, 2/3 of the ethylacetate was removed by distillation. Then 90 ml of n-heptane was added, left at room temperature (10min) and then in the freezer (1h) to crystallize. Then the mixture was filtered and solid was redissolved in ethylacetate (50 ml), while hea ted. After the solution became clear, it washed with n-heptane, recrystallized twice and dried under vacuum to obtain a white solid (9.8 g, yield 90%). ^{1}H NMR (CDCl3, d,

ppm): 1.53 (*J* = 6.4, 4H, C-CH_2-CH_2-C), 1.8-2.2 (*J* = 3.6, 2H, CH_2CHCO), 3,23 (*J* = 3.4, 2H, CH_2NH), 4.83 (1H, NHC=O), 5.08 (*J* = 1.2, 1H, NHCO), 5.21 (*J* = 3.5, 2H, CH_2O), 7.48 (*J* = 7.8, 5H, aromatic ring).

3.2.5 Synthesis of star shaped Poly(ε-Carbobenzyloxy-L-lysine-*b*-DL-propargylglycine) (3). The NCA monomer of ε-Carbobenzyloxy-L-lysine (495 mg, 1.61 mmol) was dissolved in 5 ml of DMSO in a Schlenk tube. A solution of PPI dendrimer 8AM (15.61 mg, 0.02 mmol) in 1ml of dry DMSO was added after the NCA was dissolved. The reaction left to stir at room temperature under a dry nitrogen atmosphere, until the NCA had been completely consumed as monitored by SEC and FTIR. After 4h, 112.43 mg (0.81 mmol) of DL-propargylglycine NCA dissolved in 1 ml of DMSO as the second monomer was added to the solution using PPI-PLL as the macroinitiator. The reaction was stirred at room temperature for 24h untill the second monomer was completely consumed. The reaction mixture was precipitated into diethyl ether, centrifuged and dried under vacuum to obtain a pale yellow solid (399 mg, 64% yield).

3.2.6 Synthesis of star shaped Poly(ε-Carbobenzyloxy-L-lysine-*r*-DL-propargylglycine). The same procedure as above was followed, with the difference that the two NCAs are dissolved in DMSO at the same time and then the PPI dendrimer was added to obtain a random copolymer (yield 80%).

3.2.7 Glycosylation of Poly(ε-Carbobenzyloxy-L-lysine-*b*-DL-propargylglycine). Star shaped Poly(ε-Carbobenzyloxy-L-lysine-b-DL-propargylglycine) (150 mg, 0.24 mmol of alkyne units, 1-Azido-1-deoxy-β-D-galactopyranoside (**4**) (73.8 mg, 0.36 mmol, 1.5 equivalent to alkyne) and triethylamine (17 μl, 0.12 mmol, 0.5 equivalent to alkyne) were

dissolved in 5 ml of anhydrous DMSO in a Schlenk tube. The mixture left to stir under nitrogen atmosphere for 30 min. $(PPh_3)_3CuBr$ (22.2 mg, 0.024mmol, 0.1 equivalent to alkyne) was then added in the solution and bubbled under nitrogen for another 30 min. Then, the Schlenk tube was placed in oil bath at 30 °C for 72h. Then Amberlite Resin 120 was added to the mixture and left to stir for 24h to remove the biggest part of the Cu catalyst. After filtration to remove the resin, the solution was added dropwise to an excess of THF solution, redissolved in DMSO, precipitated twice in THF and dialyzed against water for 72h. The polymer then was lyophilized to obtain white solid (35% yield).

3.2.8 Benzyl Ester Hydrolysis of Glycosylated Poly(ε-Carbobenzyloxy-L-lysine-*b*-DL-propargylglycine). The glycosylated copolymer (50 mg, 0.156 mmol of protected units) was dissolved in 2 ml of trifluoroacetic acid (TFA). A 6-fold excess with respect to carbobenzyloxy-L-lysine of a 33% of HBr in acetic acid (0.164 mL) was added. After 16 h, the solution was added dropwise to diethyl ehter, redissolved in DMF, precipitated twice in diethyl ether and dialyzed in water for 3 days. The polymer was then lyophilized to obtain **6** in white solid form yielded 34%.

3.2.9 Enzyme linked lectin assay (ELLA) on NUNC MaxiSorp plates. The enzyme lectin assay was made following a literature procedure[14]. Immobilization was performed by adding 50 µl of the glycopeptide to be tested, at a concentration of 5 µg/ml in PBS per well and incubate overnight at 4 °C. These wells were used to monitor the cross-activity of lectins, detect antibodies, with plate surfaces that would generate backround signals. Then the glycoprotein solution was removed by inversion and blocked for 2h with 200 µl 0.5% polyvinyl alcohol (PVA) in PBS. The wells were washed four times with 20 mMTris (pH 7.6), 150 mMNaCl, and 0.05% Tween 20 supplemented with 1 mM $CaCl_2$, 1 mM $MnCl_2$, and

1 mM $MgCl_2$ (TBST). Then 50 ml of lectin was added and incubated for 1h, followed by a four time washing with TBST to remove the unbound lectin, followed by the addition of 50 µl of 1:10000 diluted antibody (anti-biotin – Sigma A0185-1VL) in TBST and left to react for 1 hour. Plates were subsequently washed four times with 200 µl TBST and 1 more time with 200 µl PBS. 90 µl of TMB [add 1 TMB Tablet (Sigma T3405) to 10 ml citrate buffer pH 5.5. Add 2 µl 30% H_2O_2 per tablet directly before use] solution was added. Plates were left to develop for 10 min and then 50µl 10% H_2SO_4 was added to stop the reaction. The absorbance was measured at 450 nm.

3.2.10 Complexation with pDNA. The ratio of star-shaped polypeptide to pDNA was calculated according to the molar ratio of the amino group in the polypeptide to the phosphate group in pDNA, which is the N : P ratio. To prepare the polyplexes, solutions of the star-shaped polypeptides were prepared in tris HCl buffer (pH 7.4) and 10mM NaCl and were mixed with pDNA at various N : P rations. The mixtures were incubated at room temperature for 30 min to complete the polyplex formation.

3.2.11 Gel retardation assay. the pDNA polyplexes were mixed with a 6× loading dye (Promega G190A-Blue/orange 6×) and analysed by running 1% (w/v) agarose gel electrophoresis at 100 V for 1 h in TBE buffer (Tris–borate–EDTA buffer, Gibco, Biosciences, Ireland) with 0.5 µg/mL ethidium bromide. Visualisation was obtained using UV transillumination (G. Box, Syngene, UK).

3.3 Results and discussion

The first who described the synthesis and polymerization of propargyl glycine NCA (1, scheme 3.1) were Schlögel and Pelousek in 1960[15]. The polypeptides displayed low solubility in the most common solvents and fast gelling occurred leading to the synthesis of low molecular weight polypeptides. The low solubility of poly(DL-propargylglycine) was also confirmed by a study that was made in our group by Huang et al[12]. To avoid precipitation due to the low solubility of long chains of poly(DL-propargylglycine) we aimed at the synthesis of star polypeptides with low molecular weights.

The ring opening polymerization of NCAs initiated by dendrimers was reported by us and others[16]. Using PPI dendrimers the controlled synthesis of star-shaped polypeptides was demonstrated, bearing a dendritic core and a polypeptide shell[17]. Following this approach and varying the monomer feed ratio, a set of random and block copolymers was synthesized with different size, arm length and molar mass. The initiator was PPI denrimer of generation 2 bearing 8 arms and the two monomers that were used for the above synthesis were DL-propargylglycine NCA (**1**) and ε-carbobenzyloxy-L-lysine NCA (**2**). The monomer ratios aimed for the two polypeptides on the polymer chain were varied from 1:1, 2:1 and 1:2 respectively, in order to get different hydrophobic to hydrophilic ratios after sugar conjugation. In the case of random co-polypeptides both monomers (**1**) and (**2**) were dissolved in DMSO at the same time and then the PPI initiator was added. In this case, the two monomers were random attached on the polypeptide chain which would result in random "clicked" sugar molecules along the polymer arms assuming no sterical restrictions. In the case of block co-polypeptides, Cbz-Lysine-NCA (**2**) was used as a first block. After dissolving in DMSO and upon the addition of PPI initiator the reaction was left to stir at room temperature for 2 hours and then samples of 0.2 ml were taken every 30 minutes to

measure FT-IR and GPC data in order to monitor the progress of the reaction. After 5 hours it was found that all Cbz-Lysine NCA was consumed and then the second monomer (**1**) was added. The reaction was left to complete overnight. In this case all propargylglycine units are gathered close to the shell of the star polymers meaning that after clicking the sugar all the hydrophilic moieties will be close and on the surface of the star polymer, creating a micelle-like structure.

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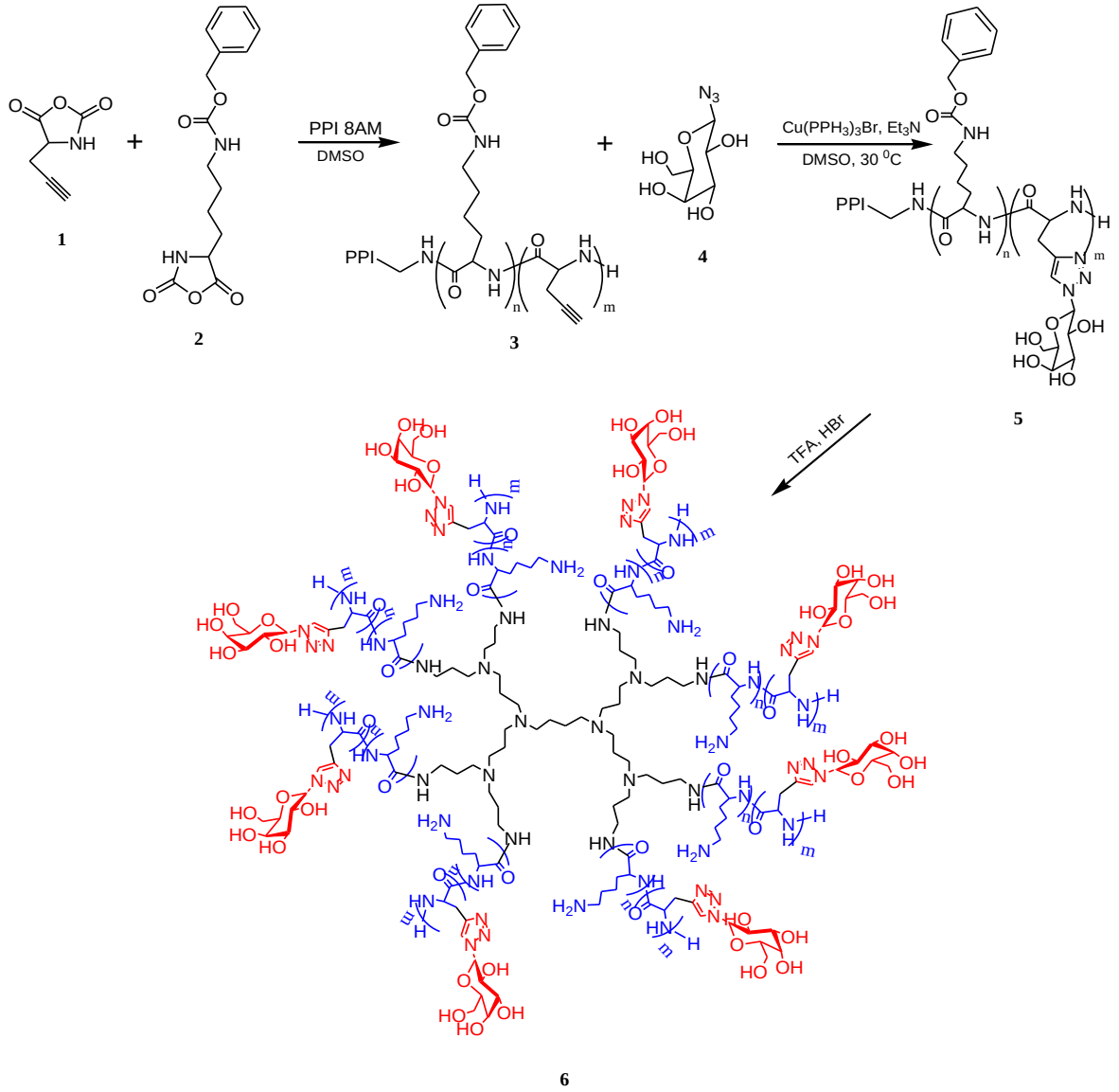


Scheme 3.1. Reaction scheme of the synthesis of star-shaped glycopeptides.

The successful synthesis of all star polypeptides was confirmed by ^{1}H NMR analysis (Figure 3.1). Comparing the number of protons of the aromatic ring (peak i) of the protected lysine with the protons of the triple bond (peak m) of the propargyl-glycine, it was confirmed that the composition of all star copolymers were in agreement with the initially monomer feed ratios for both random and block copolymers. From the data it can, however, not been concluded whether the monomers of the statistical copolymerisation are truly random. Very little is known about NCA copolymerisation parameters in general and for this monomer pair in particular. While there is thus a possibility that a slight monomer gradient is present within the individual arms, the assumption of a random or statistical arrangement is reasonable. For the block copolymerisation a statistical structure can be excluded due to the sequential addition of monomers. Both polymer structures thus constitute two different monomer arrangements, even in the case of a slight monomer gradient for the random copolymer.

The following steps included the glycosylation and deprotection of Cbz-Lysine units. The glycosylation of all random and block star polypeptides (entries 1-6, Table 3.1) was done with azido functionalized galactose, which was clicked on the alkyne group of PAG units through the formation of a triazole ring (Scheme 3.1). Only the star glycopolypeptides with higher ratio of propargylglycine units (entries 9, 12 Table 3.1) showed good solubility in aqueous media, in contrary to the others (entries 7, 8, 10, 11, Table 3.1) which were partially soluble in water. Deprotection of Cbz-Lysine units was carried under acidic conditions using HBr and TFA as a solvent, which led to very good solubility in water for all glycopolypeptides. NMR spectroscopy revealed that the acidic conditions did not have any effect on the sugar molecules, which were still present on the hydrolized star-polymers. More specifically, the NMR spectrum in Figure 3.1 shows the successful deprotection of the lysine block (disappearance of peak at 7.2-7.3 ppm) as well as the glycosylation of poly(ε-carbobenzyloxy-L-lysine-*b*-DL-propargylglycine) (Figure 3.1 bottom), confirmed by the

proton peak of the triazole ring at 8 ppm (peak h) and the proton peaks of the sugar moiety in the area of 3.5-4.0 ppm (peak s2-s6) as it was found also in literature[18]. Moreover, the dissapearence of any triple bond proton peaks indicates quantitative conversion to the glycosylated product, which can be also confirmed by the dissapearance of the triple bond band at 2134 cm^{-1} in the FT-IR spectra (Figure not shown) in agreement with previous reports[18,19]. The dendrimer peaks in the NMR spectrum either overlap with the proton peaks of the lysine block (Figure 3.1 top) or they are not visible at all (Figure 3.1, bottom) due to the fact that the hydrophobic dendritic core is not accessible to the NMR solvent D_2O. As a result, the calculation of molecular weight through NMR is impossible and also the initiation from all the primary amino groups cannot be confirmed, although, as it has been shown before[16], the simultaneously increase of molecular weights as dendrimer generation increases, indicates a high initiation efficiency.

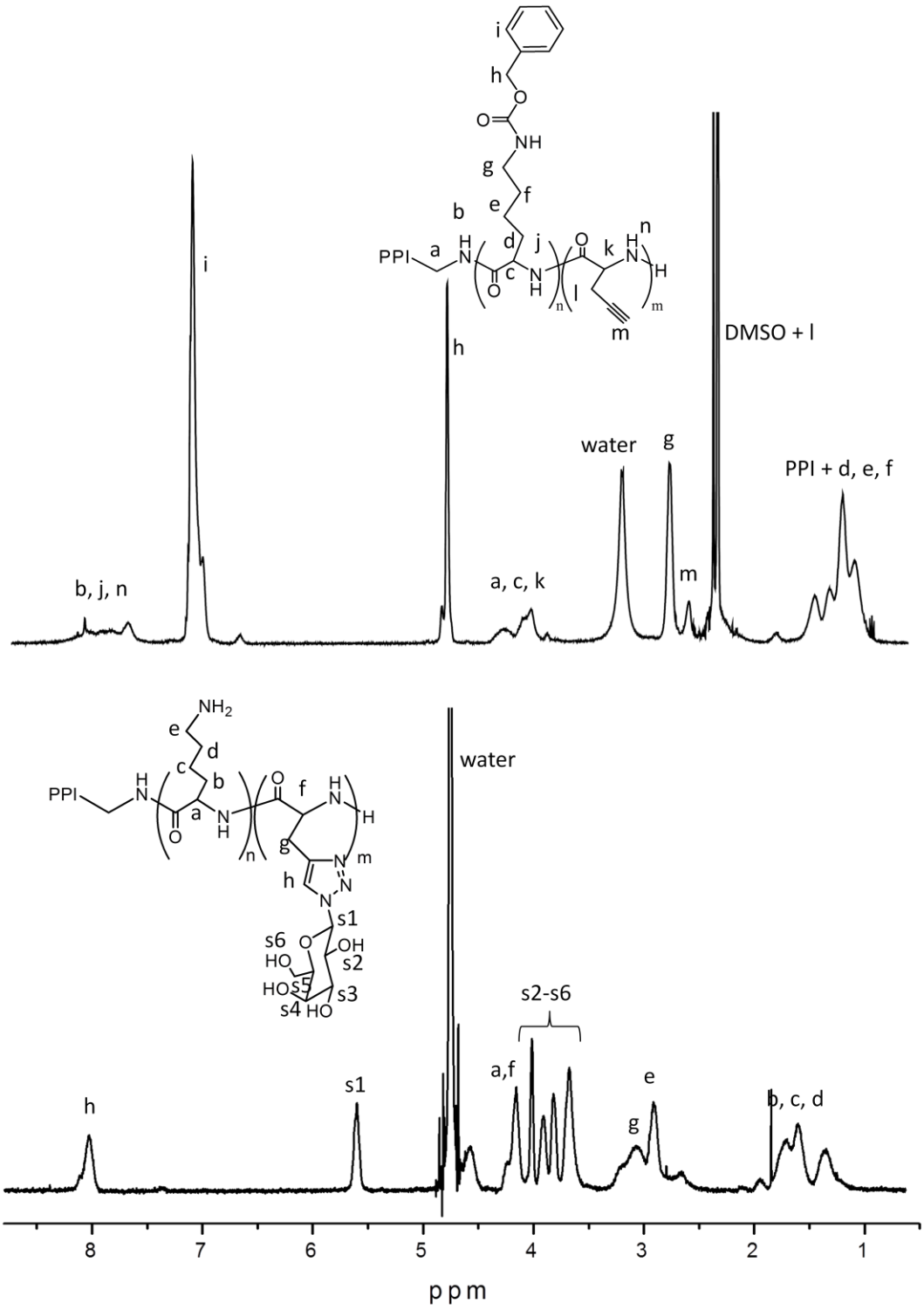


Figure 3.1 ^{1}H NMR spectra of star Poly(ε-Carbobenzyloxy-L-lysine-b-DL-propargylglycine) (**3**) in DMSO (top) and glycosylated star Poly(ε-Carbobenzyloxy-L-lysine-b-DL-propargylglycine**)** (**6**) after hydrolysis with TFA/HBr in D_2O (bottom).

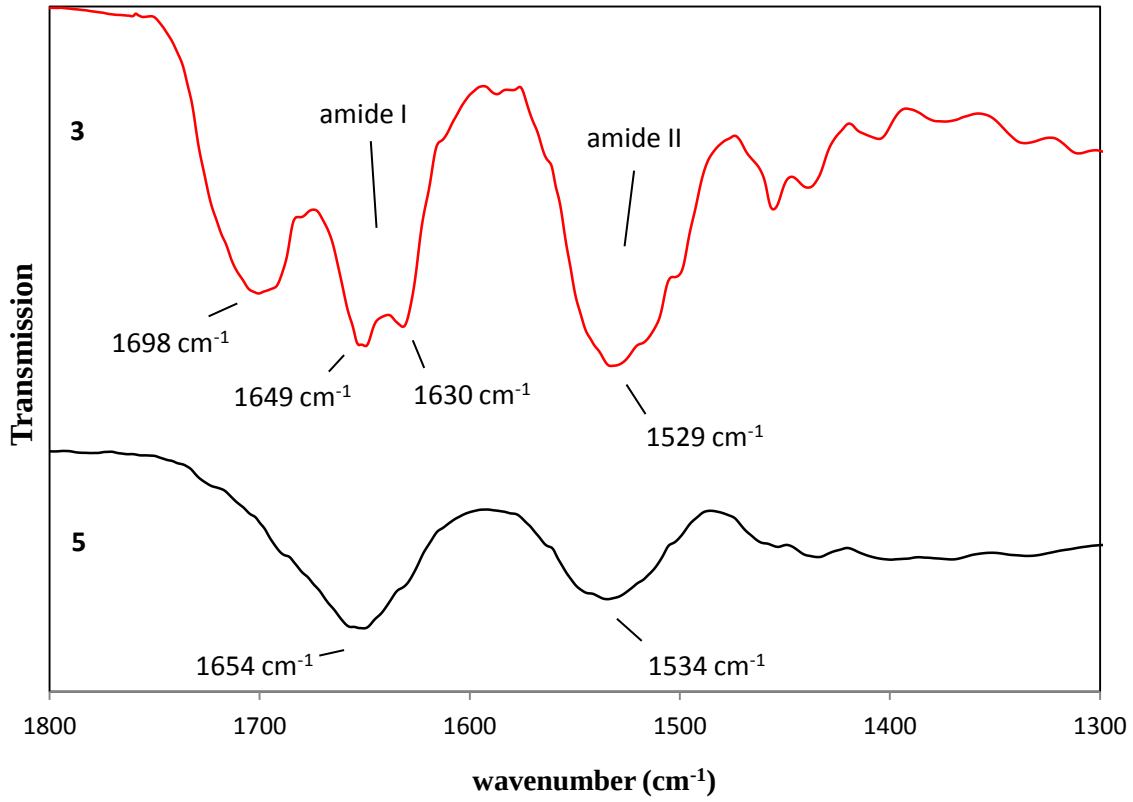


Figure 3.2 FTIR spectra of star poly(ε-carbobenzyloxy-L-lysine-*b*-DL-propargylglycine) (**3**) (top) and glycosylated star poly(ε-carbobenzyloxy-L-lysine-*b*-DL-propargylglycine**)** (**5**) (bottom).

The secondary structure of the protected star polymers in the solid state was determined by FTIR spectroscopy. All of them exhibited a dominant β-sheet conformation, which is evident from the two amide I (C=O stretching) peaks at 1698 cm^{-1} and 1639 cm^{-1} and the amide II (N-H bending) peak at 1529 cm^{-1}. The remaining peaks indicate also the presence of some random coil elements. After glycosylation the polypeptide adopted an α-helical secondary structure irrespective of their composition and structural arrangements with characteristic bands observed at 1654 cm^{-1} and 1534 cm^{-1} (Figure 3.2). The above data are in good agreement with a study published by Henkel et al. describing the IR data of peptides from solid phase synthesis[20].

Table 3.1. Star polypeptides before and after glycosylation initiated by PPI dendrimer generation 2. Numbers refer to units of each monomer per dendrimer arm. Molecular weights are calculated through Size Exclusion Chromatography (SEC) and are compared with the theoretical ones.

Entry	polypeptide	NCA/ NH_2	NCA/ dendrimer	M_n (g mol^{-1}) SEC (DMAC)	M_n (g mol^{-1}) (theoretical)	PDI (SEC)	FT-IR
1	**PPI-PLYS$_7$-r-PAG$_7$**[(a)]	14	112	17700	20700	2.3	β sheet – random coil
2	**PPI-PLYS$_{14}$-r-PAG$_7$**[(a)]	21	168	31700	35300	2.1	β sheet – random coil
3	**PPI-PLYS$_7$-r-PAG$_{14}$**[(a)]	21	168	27700	26000	2.2	β sheet – random coil
4	**PPI-PLYS$_{10}$-b-PAG$_5$**[(a)]	15	120	25200	25400	2.6	β sheet – random coil
5	**PPI-PLYS$_5$-b-PAG$_5$**[(a)]	10	80	17500	14900	2.8	β sheet – random coil
6	**PPI-PLYS$_5$-b-PAG$_{10}$**[(a)]	15	120	20100	18700	2.1	β sheet – random coil
7	**PPI-PLYS$_7$-r-PAG$_7$-gal**	14	112	29400	32100	1.2	α - helix
8	**PPI-PLYS$_{14}$-r-PAG$_7$-gal**	21	168	42200	46800	1.5	α - helix
9	**PPI-PLYS$_7$-r-PAG$_{14}$-gal**	21	168	31700	48900	1.1	α - helix
10	**PPI-PLYS$_{10}$-b-PAG$_5$-gal**	15	120	34700	33600	1.3	α - helix
11	**PPI-PLYS$_5$-b-PAG$_5$-gal**	10	80	27600	23100	1.4	α - helix
12	**PPI-PLYS$_5$-b-PAG$_{10}$-gal**	15	120	34300	35100	1.5	α - helix

[(a)] Solubility of unglycosylated polypeptides is low in the most common organic solvents, leading to unreliable GPC data.

The molecular weight of the polypeptides was calculated from SEC, using poly(methyl methacrylate) standards and dimethylacetamide (DMAC) as the mobile phase. The polydispersities of the non-glycosylated polypeptides were found to be quite high (2.1-2.8) and in some cases traces were biomodal. Hadijichristides et al.[21] described that bimodal peaks can be caused by linear polymers formed as a "by-product" during initiation from PPI dendrimers through the tertiary amines of the dendritic core. However, it is more likely that signal broadening and bimodality are due to the fact that the polymers containing propargylglycine showed very low solubility in most of the common solvents including DMAC. This is supported by the fact that after glycosylation with galactose the solubility improved and polydispersities were significantly lower (1.2-1.5) leading to monomodal SEC traces in almost all cases. Some tailing at the lower molecular weight end of the SEC traces can still be observed. Whether this is due to structural imperfections or column interactions cannot be distinguished without further studies. Nevertheless, the theoretical molecular weights of the polypeptides before and after glycosylation were in reasonable agreement with the ones calculated from SEC (Table 3.1).

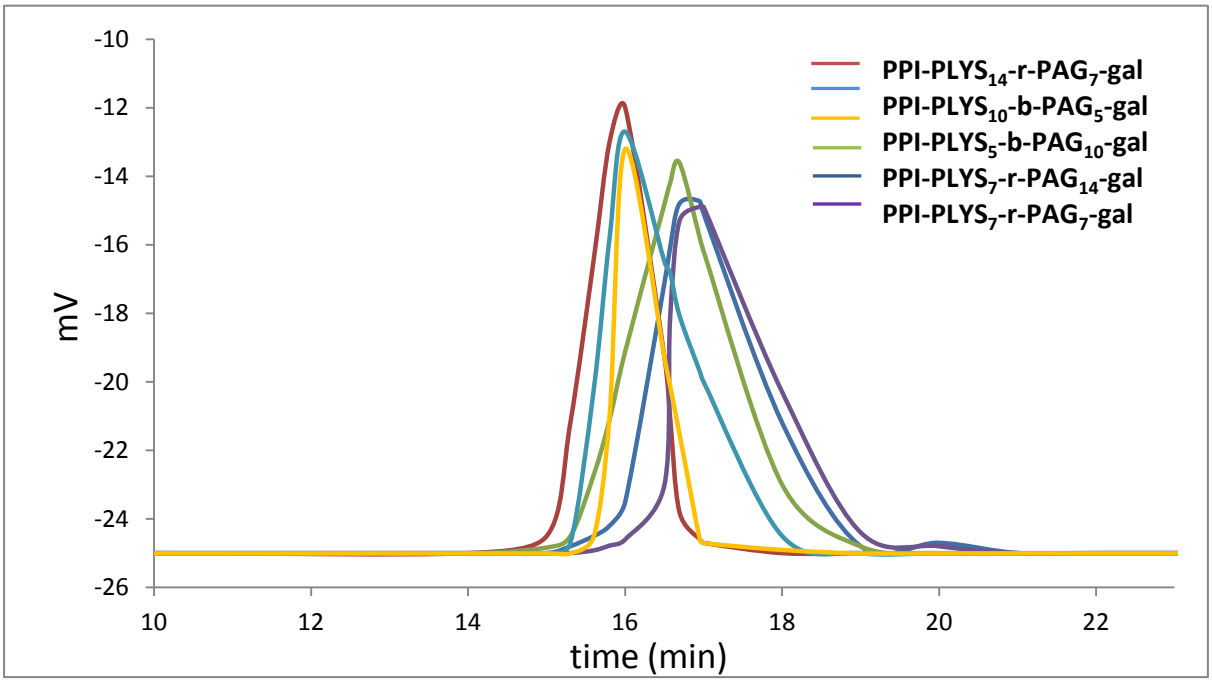


Figure 3.3. SEC (Size Exclusion Chromatography) traces (DMAC, PMMA standards) of all glycosylated protected star polypeptides

3.3.1 Enzyme linked lectin assay (ELLA) for biorecognition

ELLA was used to investigate the ability of the glycopeptides to interact with biological systems. To achieve this, the binding of the glycopeptides to different lectins was systematically studied. Lectins are proteins of non-immune origin, that bind selectively to specific carbohydrates. A large number of lectins from different sources (plants, animals) has been characterized and due to their interactions with sugars, they play an important role in translating the glycocode[22,23]. Three lectins were used to detect the bio-ability of the glycopeptides: i) ECL (*Erythrina cristagalli lectin*), ii) RCA (*Ricinus communis Agglutinin*) and iii) NPL (*Biotinylated Narcissus Pseudonarcissus Lectin*). ECL and RCA are lectins specifically binding to galactose moities, whereas NPL is a mannose binding lectin. The experimental procedure that was followed was described by Thompson et al[24]. All

glycopeptides were incubated in 96 well plates after interacting with the aforementioned lectins and their absorbance was measured at 450 nm. As a negative control, the absorbance of PBS buffer was measured before and after mixing with the different lectins and it was found to be between 0 and 0.07, revealing that no interaction occurred. Both galactose binding lectin (ECL, RCA) solutions with the glycopeptides showed significantly increased absorbance confirming the sugar-lectin conjugation. On the other hand, the glycopeptide solutions with the mannose binding lectin NPL showed low absorbance (0.05-0.10) comparable with the one of PBS buffer, leading to the conclusion that no interaction took place. Another observation that can be made is that star polypeptides with same amount of propargylglycine units per arm have similar absorbance. This can be seen in Figure 3.4 for the 5-5 and 10-5 (block copolymers) samples as well as for the 7-7 and 14-7 (random copolymers) samples, with the two numbers indicating the lysine units and propargylglycine/galactose units per arm, respectively. In the case of random copolymers the sample with the double units of propargylglycine/galactose per arm (sample 7-14) showed comparably higher absorbance than the other two, especially in the case of RCA lectin, explained by the fact that theoretically it is bearing twice the amount of galactose units than the other two. Interestingly, the lectin binding results of the other two random copolymers is very similar despite the fact that the ratio of lysine to propargylglycine/galactose is different (14-7 and 7-7). It seems that the result is simply determined by the total number of galactose, which is identical, rather than the ratio of the two comonomers. The two block copolymers with 5 galactose units per arm (10-5 and 5-5) both show a relative higher binding than the random copolymers with 7 galactose units and are almost as high as the copolymer with 14 galactose units. This suggest that the block copolymer arrangement with the galactose units at the periphery facilitates more efficient lectin binding possibly due better accessibility. Surprisingly, the block copolymer sample 5-10 had a reproducibly lower absorbance.

Whether this is due to structural imperfections or a saturation effect in the binding cannot be concluded from the data and should be further investigated.

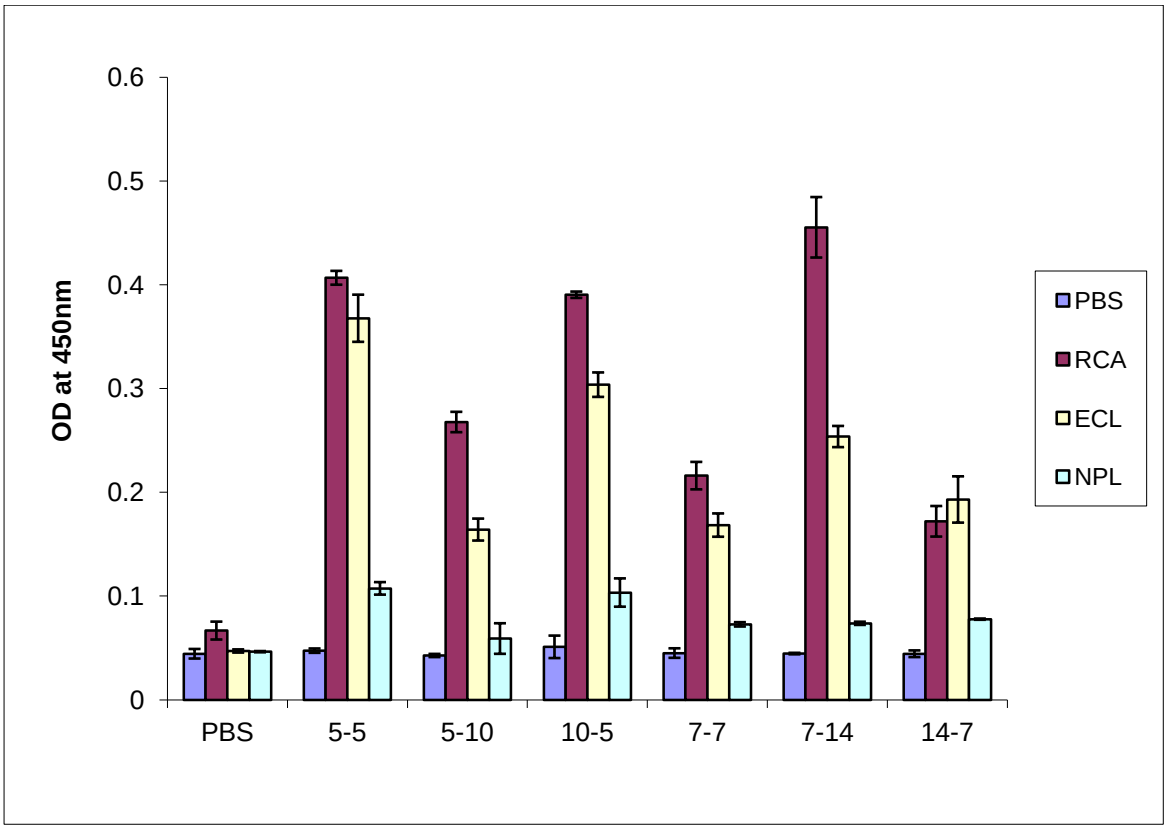
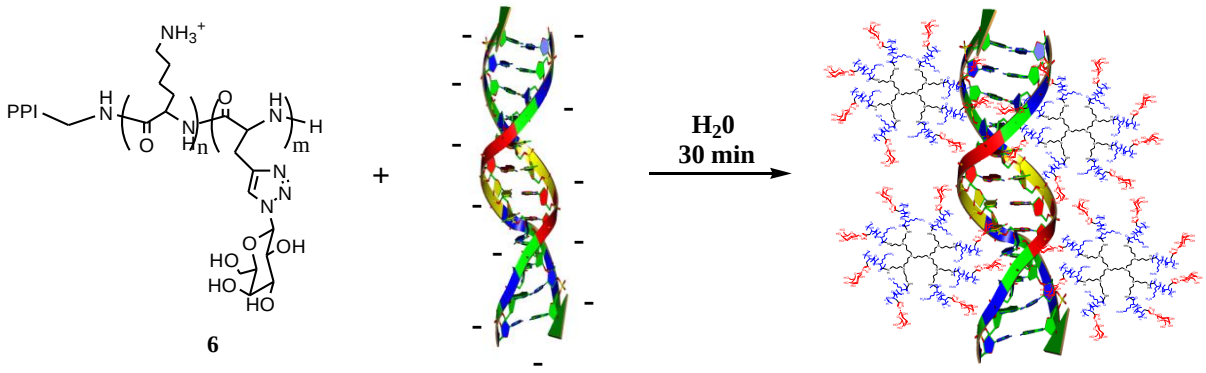


Figure 3.4. Absorbance at 450 nm of glycopeptides solutions after reacting with different lectins [ECL (*Erythrina cristagalli lectin*), RCA (*Ricinus communis Agglutinin*), NPL (*Biotinylated Narcissus Pseudonarcissus Lectin*)]. PBS buffer was used as a negative control. The numbers below the columns indicate the units of deprotected lysine and propargylglysine/galactose per dendrimer arm, respectively. (5-5, 5-10, 10-5: block copolymers, 7-7, 7-14, 14-7: random copolymers)

3.3.2 pDNA complexation

The complexation between plasmid DNA and the glycopeptides is based on their opposite charges. DNA has a polyanionic charge, which can be neutralized by the positive charge of

the NH_3^+ component of the lysine block of the glycopeptide. The deprotected star glycopolypeptide $PLYS_5$-b-PAG_{10}-galactose and pDNA were mixed in different N/P ratios to achieve 100% complexation. ζ-potential was measured before and after complexation and it was found that an N/P ratio above 10 was required to form cationic polyplexes, proving a full conjugation. Dynamic light Scattering (DLS) studies showed an increase in the size to more than 150 nm after the complexation with pDNA. The desirable size, in order to achieve complete introduction of pDNA into cells is <200 nm. Bigger particles will be rejected by the reticuloendothelial system (RES)[25]. In some cases the DLS measurements resulted in traces with high polydispersities, due to the fact that the polypeptides can form aggregates in aqueous media. Considering the size difference between the star polymers and the DNA one cannot assume loading of the DNA into the star polymers but rather complexation and charge compensation by attachment of the polymers to the DNA as illustrated in Scheme 2.2. In this scenario bridging between complexes is possible, which could explain broadening of the DLS peaks. Further optimization and experiments for example with smaller siRNA should be carried out.



Scheme 3.2. DNA complexation with star-glycopeptide bearing positively charged deprotected lysine moieties.

3.3.3 Gel retardation assay of pDNA polyplexes

The gel retardation assay is a sensitive technique for studying protein-DNA interactions. This method relies on the stability of DNA polyplexes with proteins, when they are subjected to polyacrylamide gel electrophoresis. DNA itself migrates quickly through the gel matrix, whereas protein-DNA polyplexes show slower mobility. The assay was carried out at various N/P ratios (1, 2, 3, 5, 10). It was concluded that the $PLYS_5$-b-PAG_{10}-galactose block glycopolypeptide was fully complexed with pDNA at an N/P ratio of 10, something which was also confirmed by a cationic charge measured by ζ-potential (Figure 3.5). As negative and positive controls pDNA and PEI/pDNA respectively were used.

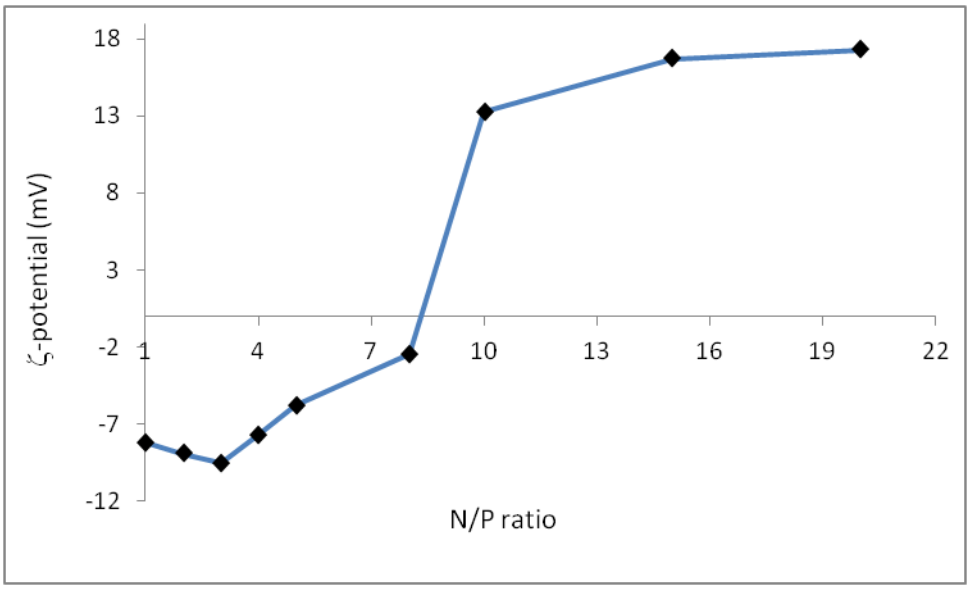


Figure 3.5. ζ-potential of PPI-$PLYS_5$-b-PAG_{10}-galactose/pDNA polyplexes over a range of N/P ratios.

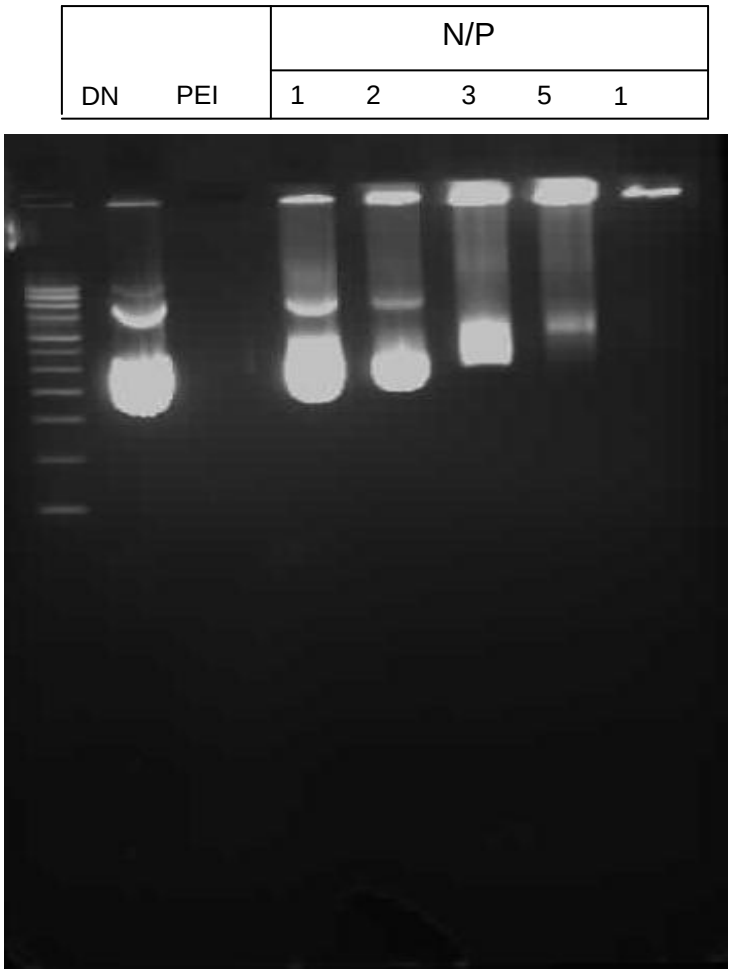


Figure 3.6. 1% agarose gel electrophoresis of block copolymer PPI-LYS_5-b-PAG_5-galactose complexed with pDNA at different N/P rations.

3.4 Conclusions

In this chapter, the successful synthesis of novel, well-defined, star copolypeptides is described. The polymer structure was systematically varied to comprise random and block arrangements of lysine and galactosylated propargylglycine in different compositions. Enzyme linked lectin assays proved the ability of the glycopolypetides for specific lectin binding. It was found that for the random arrangement the binding efficiency was mainly determined by the total amount of galactose in the polymer. For the block copolymers, where

the galactose is located at the polymer periphery relatively higher binding efficiency as compared to the random arrangement was found. It was hypothesized that this is due to better accessibility of the galactose and thus a consequence of the polymer architecture. However, more detailed studies are required to confirm this. Furthermore, the capability of one of the glycopeptides to complex with plasmid DNA was investigated and confirmed at an N/P ratio of 10 as was proved by gel retardation studies and ζ-potential measurements. This work provides evidence for the importance of the structural arrangements in the design of polymer with lectin binding properties. For applications, it would be required to further modify the polymers to add better loading properties and investigate the pharmacological performance.

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Chapter 4

Thermoresponsive glycopolypetides with temperature controlled selective lectin binding properties

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Abstract

Linear polypeptide poly(γ-propargyl-L-glutamate) (PPLG) was synthesized by the ring opening polymerization (ROP) of the corresponding N-carboxy anhydride (NCA). Azide-functional galactose and 1-azido-2-(2-methoxyethoxy)ethane (mEO_2) were co-clicked on the polypeptide at different ratios to introduce selective binding and thermoresponsive properties. Selective binding was demonstrated by turbidity assays with Ricinus communis Agglutinin (RCA_{120}) lectin. Cloud point measurements confirmed the cloud point temperature (T_{cp}) of the polymers increasing with the ratio of mEO_2 to galactose. Moreover, lectin binding experiments at temperatures above and below T_{cp} suggest that binding is suppressed above T_{cp}. This opens opportunities to design functional materials with temperature controlled biological response.

4.1 Introduction

Stimuli-responsive polymers have been intensively investigated including materials responding to temperature, light, pH, and biological cues such as enzymes.[1-4] A promising application for thermoresponsive polymers is in drug delivery due to the fact that the temperature of infected tissue (e.g. tumors) is slightly elevated.[5-7] One challenge of this strategy is to fine tune the material's response temperature so that the phase transition triggered release of the payload occurs in a very narrow temperature window precisely at the desired temperature. Another challenge arises from the non-specificity of most thermoresponsive materials, which demands the systemic application of higher dosages. A strategy to overcome this is the incorporation of targeting molecules in the thermoresponsive polymer. Carbohydrates (glycans) are promising targeting candidates since the discovery that they regulate numerous biological processes such as cell communication and selective binding of other biological species through highly specific glycan-lectin binding.[8-10] We are interested in exploring ways to manipulate this specific binding event with the long-term goal to design smart materials in which lectin binding can be triggered by an external stimulus.

To the best of our knowledge only two instances of the combination of thermo- and lectin-responsive polymers have been reported. In a first example Schubert described the synthesis of thermoresponsive glycopolymers by atom transfer radical copolymerization (ATRP) of N-allylacrylamide (NAllAm) and N-isopropylacrylamide (NiPAm).[11] The pendant allyl groups were subsequently utilized for conjugation with thiol-functional glucose and galactose via thiol-ene chemistry. Turbidity measurements confirmed that the copolymers bearing protected sugar moieties had a lower cloud point temperature (T_{cp}) than those bearing deprotected sugars. Biorecognition of the polymers was studied by turbidity assays using

Concanavalin (ConA) and Ricinus Communis Agglutinin lectin (RCA_{120}), albeit the influence of the temperature on the lectin binding was not investigated.

In a second example Zhu described the preparation of the hydrophilic block glycopolymer poly(N-isopropylacrylamide-*co*-6-Ovinyladipoyl-D-glucose)-*b*-poly(N-isopropylacrylamide) using reversible addition-fragmentation chain transfer (RAFT) copolymerization of NIPAm and 6-O-vinyladipoyl-D-glucose (OVAG).[12] This amphiphilic block copolymer forms micelles in water, which upon heating above the T_{cp} significantly increased in size due to a coil-to-globule transition of the glycopolymer block. Interestingly, Dynamic Light Scattering (DLS) measurements suggested an influence of the temperature on the lectin binding of the micelles, which was better below the T_{cp}. This was explained by the enhanced accessibility of the glycans in the random coil structure below T_{cp} as compared to their accessibility when the glycopolymer block adopts a globular structure in the micelle above the T_{cp}.

Both of the above polymers are polyacrylic in nature and lack biodegradability. An interesting alternative class of polymers is polypeptides obtained from the ring-opening polymerization of amino acid N-carboxyanhydrides (NCA). Progress in NCA polymerization has opened opportunities to synthesize a wealth of well-defined polypeptide structures.[13-15] As a result, polypeptides promise potential in biomedical fields such as drug delivery[16-21], tissue engineering[22] or imaging[23]. The synthesis of glycosylated polypeptides has been achieved using direct polymerization of glycosylated NCA or post-modification by conventional chemistry as well as click chemistry.[24-31] On the other hand, a range of thermoresponsive polypeptides have been described in the literature.[21,32-35] Notably, Hammond described the ring opening polymerization of poly(γ-propargyl-L-glutamate) (PPLG) to obtain a polypeptide platform on which they attached different oligo(ethylene glycols) through copper catalyzed click reaction in order to induce thermoresponsiveness.[36] In an extension of this work, the same authors grafted both 1-azido-2-(2-methoxyethoxy)

ethane (mEO_2) and N-(2-azidoethyl)-N-isopropylpropan-2-amine (diisopropylamine) on the PPLG backbone to achieve both thermal and pH responsiveness.[37]

In this work we present the synthesis of a polypeptide with combined lectin recognition and thermoresponsive properties. The polymers are obtained by ring-opening polymerization of γ-propargyl L-glutamate NCA and conjugated with mEO_2 and galactose through copper catalyzed click chemistry. Varying the ratios of the two units on the polymer backbone, the hydrophilicity as well as the T_{cp} could be modified. Moreover, we demonstrate that the lectin binding can be thermally controlled.

4.2 Experimental Section

4.2.1 Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Diethyl ether was purchased from VWR. DMSO, ethyl acetate and ethanol were used directly from the bottle under an inert and dry atmosphere. 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose was synthesized following a literature procedure[38]. 1-Azido-2-(2-methoxyethoxy)ethane (mEO_2) was synthesized using a similar procedure as the one reported by Hammond.[37] All chemicals were used without any purification unless otherwise noted.

4.2.2 Methods. ^{1}H-NMR spectra were recorded at room temperature with a Bruker Avance 400 (400 MHz), DMSO-d_6, $CDCl_3$ and D_2O were used as solvents and signals were referred to the signal of residual protonated solvent signals (D_2O). TMS was used as an internal standard for DMSO-d_6 and $CDCl_3$. ATR-FTIR spectra were collected on a Perkin-Elmer Spectrum 100 in the spectral region of 650-4000 cm^{-1} and were obtained from 4 scans with a resolution of 2 cm^{-1}. A backround measurement was taken before the sample was loaded onto

the ATR unit for measurements. CD-spectroscopy was performed on a Jasco J-815 spectrometer with 0.0050 mM solution of the polypeptide in demineralized water. Mean residue ellipticities were calculated from the CD spectra following a literature procedure[39] using the equation $[\Theta]_{MRW} = (\Theta \times M_{MRW})/(10 \times c \times l)$, Θ: experimental ellipticity in mdeg, M_{MRW}: molecular weight in g/mol, c: concentration in mg/mmL, l: path length 0.5 cm. M_{MRW} used for calculations: 19925 g/mol (mEO_2), 20217 g/mol (10% galactose), 20305 g/mol (13% galactose), 20627 g/mol (24% galactose), 21096 g/mol (40% galactose). Helicities were calculated at λ = 222 nm using $f\alpha = (-[\Theta 222]_{MRW} + 3000)/39{,}000$. All turbidity assays were carried out on an Perkin Elmer Lambda900 UV-VIS instrument using UV quartz cuvettes. Assays with lectin were monitored at 450 nm in PBS buffer solution, while assays without lectin were monitored at 500 nm in deionised (DI) water.

4.3.3 Synthesis of γ–propargyl L-glutamate hydrochloride. The preparation of γ-PLG-HCL was carried out following a modified procedure reported by Hammond and coworkers.[36] To a solution of L-glutamic acid (10 g, 68 mmol) suspended in propargyl alcohol (300 mL, 5.2 mol), 17 mL of chlorotrimethylsilane was added drop-wise under nitrogen. The solution was stirred at 40 ºC overnight and then the crude product was precipitated into diethyl ether. To avoid the presence of free propargyl alcohol, the product was extensively washed with ether, filtered, recrystallized from ethanol and dried under vacuum to obtain a white powder (dark grey powder when propargyl alcohol has not been removed). Yield: 13.3 g (60 mmol, 88%). ^{1}H NMR (D_2O, δ ppm): 2.06 (2H, $CHCH_2$), 2.53 (2H, $CO\text{-}CH_2$), 2.84 (1H, C≡CH), 3.80 (1H, $CHCH_2CH_2$), 4.59 (2H, $OCH_2C{\equiv}CH$).

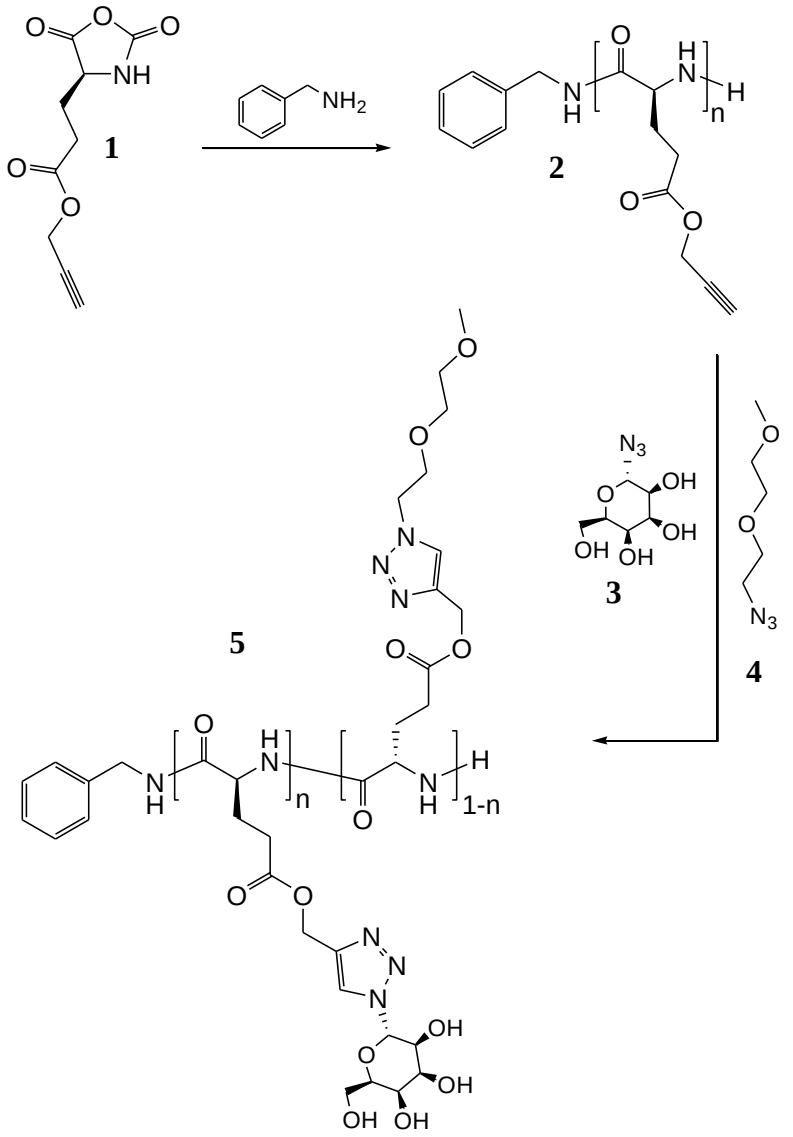
4.3.4 Synthesis of N-carboxyanhydride of γ-propargyl L-glutamate (PLG-NCA). γ–propargyl L-glutamate hydrochloride (3.6 g, 16 mmol) was suspended in dry ethyl acetate

(120 mL) and the solution was heated to reflux under nitrogen. Triphosgene (2.2 g, 7 mmol) was added and the reaction was left to reflux for 5 more hours. The reaction solution was left to cool to room temperature and was filtered to remove any unreacted γ–propargyl L-glutamate hydrochloride. Then it was cooled to 4 ºC and washed with 120 mL of water, 120 mL of saturated sodium bicarbonate and 120 mL of brine all at 4 ºC. The organic phase was then dried over anhydrous magnesium sulfate, filtered and evaporated to give a viscous oil. Yield: 2.1 g (9 mmol, 63%). ^{1}H NMR ($CDCl_3$, δ ppm): 2.22 (2H, $CHCH_2$), 2.38 (1H, C≡CH), 2.66 (2H, $COCH_2$), 4.47 (1H, $CHCH_2CH_2$), 4.74 (2H, $OCH_2C{\equiv}CH$), 6.57 (1H, NH).

4.3.5 Synthesis of poly(γ-propargyl L-glutamate) (PPLG). PLG-NCA (620 mg, 2.78 mmol) was dissolved in 10 mL of DMSO, added to a Schlenk tube and left under nitrogen for 30 min. Then, 5.24 mg (0.049 mmol; ratio 60:1) of benzylamine dissolved in DMSO (2 mL) was added to the reaction solution. The reaction mixture was left to stir overnight at room temperature. The polymer was precipitated into diethyl ether, washed with an excess of diethyl ether and collected by centrifugation to fully remove the DMSO. The product was dried overnight under vacuum. Yield: 357 mg (71%).

4.3.6 Conjugation of PPLG with mEO_2 and galactose. The following procedure describes a representative example for the ratio mEO_2:galactose 9:1. The conjugation onto the backbone was carried out at a feed ratio of alkyne/azide/CuBr/N,N,N',N',N''-pentamethyldiethylenetriamine (PMDETA) of 1:1.2:0.1:0.1. PPLG (85 mg, 0.5 mmol of alkyne repeat units), mEO_2 (76,8 mg, 0.54 mmol), azido-galactose (12.3 mg, 0.06 mmol) and PMDETA (10.5 μl, 0.05 mmol) were all dissolved in DMF (8 ml). The CuBr catalyst (7.23 mg, 0.05 mmol) was added to the degassed solution and the reaction was left to stir at room temperature for 3 h. Then the reaction solution was precipitated in cold diethyl ether,

dissolved in distilled water and treated with Amberlite Resin 120 for 2 hours to obtain a clear solution. The Amberlite beads were removed by filtration and the polymer solution was dialyzed against water acidified with HCl for 24 h and then against distilled water for another 24 h. The polymer then was lyophilized to obtain a white solid. Yield: 154 mg (83%). Using the same procedure, a library of the same polymer bearing different ratios of both azides was synthesized.



Scheme 4.1. Synthesis of poly(γ-propargyl-L-glutamate) (PPLG, **2**) and subsequent conjugation by a co-click reaction with mEO_2 (**4**) and azidogalactose (**3**).

4.3 Results and Discussion

The polymerization of γ-propargyl-L-glutamate (PLG-NCA, **1**) was carried out at room temperature in DMSO using benzylamine as the initiator at a monomer to initiator ratio of 60 (Scheme 4.1). ^{1}H-NMR spectra confirmed the structure of poly(γ-propargyl-L-glutamate) (PPLG, **2)** showing characteristic signals of the alkyne peak **g** at 3.4 ppm as well as the proton peak **d** at 2.1 ppm. From the integrated peak areas of the aromatic protons of the benzylamine initiator (7.2 - 7.4 ppm (**a**) and the signals of the combined peaks (**c**, **b**), the average degree of polymerisation P_n was calculated. It was found to be in good agreement with the initial feed ratio (60:1). Size exclusion chromatography confirmed a unimodal molar mass distribution with a polydispersity of 1.1. The characteristic alkyne band at 2129 cm^{-1} could clearly be identified in the FT-IR spectrum of the PPLG (Figure 4.2). Moreover, the spectrum confirmed the α-helical conformation with characteristic amide bands at 1648 and 1545 cm^{-1}.

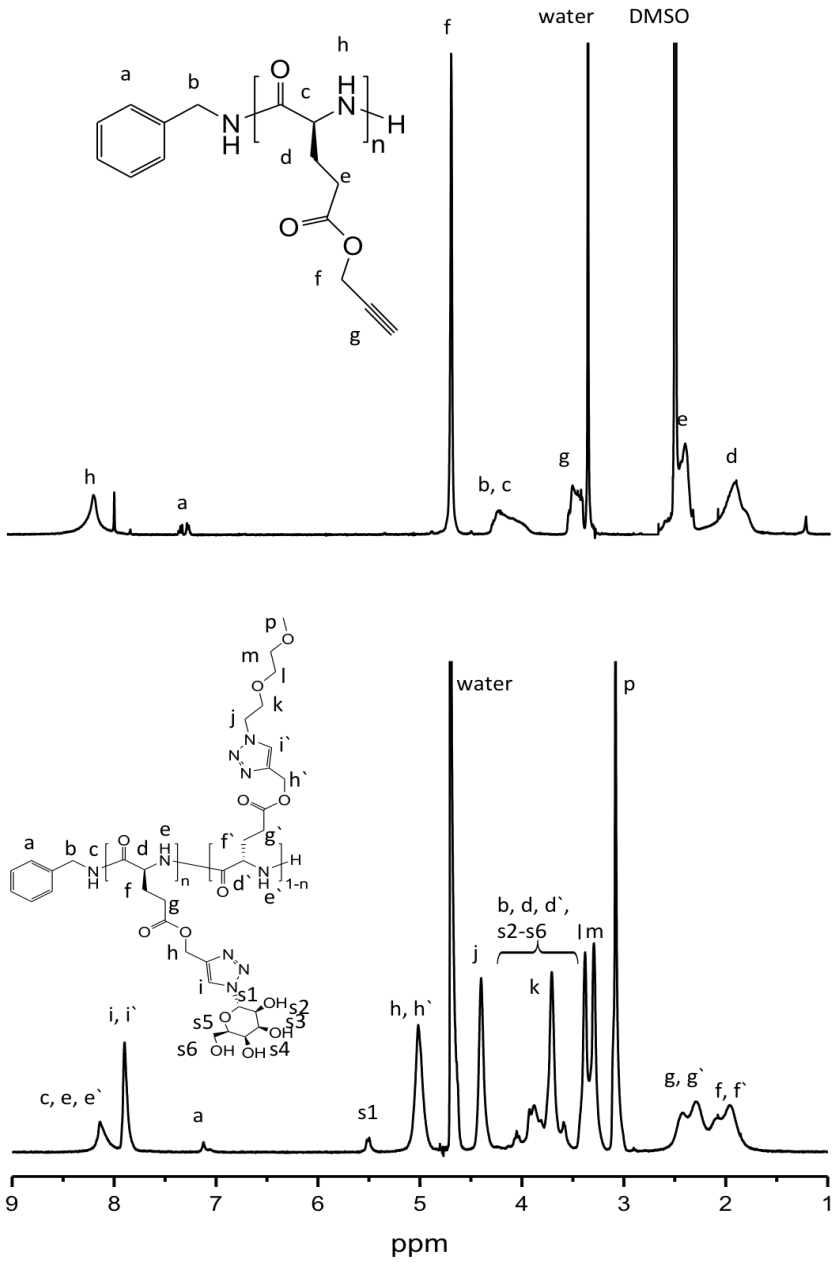


Figure 4.1. ^{1}H NMR spectra of $PPLG_{60}$ (2) in DMSO-d_6 and mEO and galactose conjugated polymer (**5**, entry 2 Table 3.1) in D_2O.

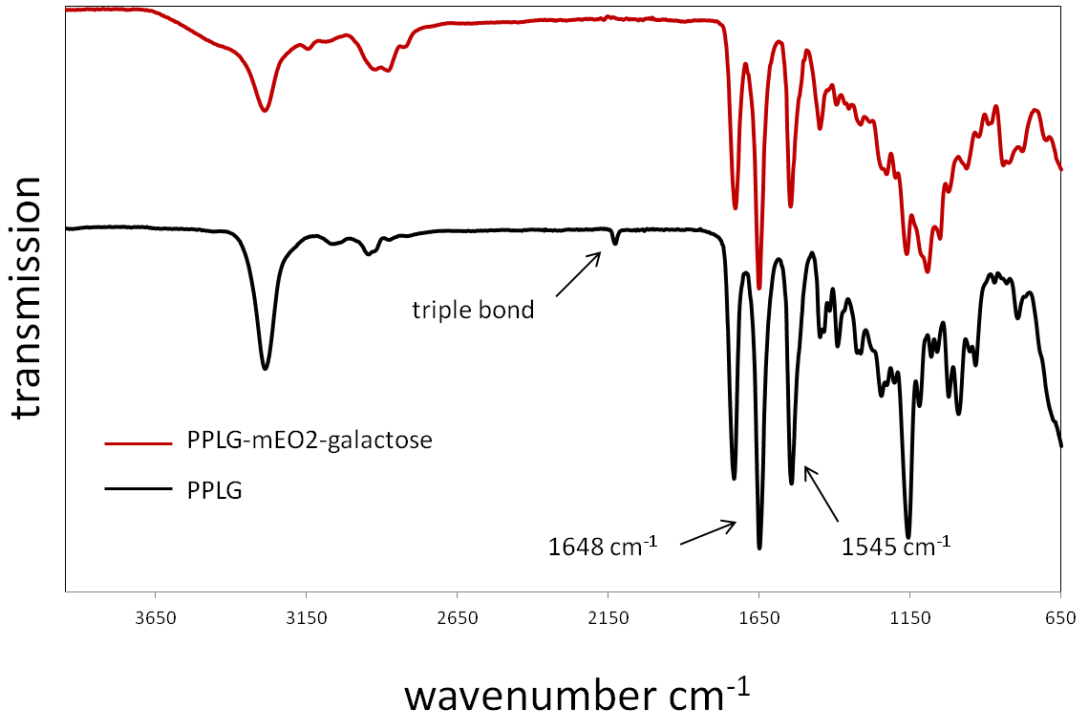


Figure 4.2. FT-IR spectra of PPLG (2, bottom) and PPLG-mEO$_2$-galactose (5, top), indicating the successful polymer conjugation by 'click' chemistry and the presence of a helical conformation.

For the subsequent click reaction different ratios of mEO$_2$ azide (**4**) and 1-β-azido-2,3,4,6-tetraacetyl-D-galactose (**3**) were used with the aim to produce statistical copolymers comprising lectin binding and thermoresponsive substituents (Table 4.1). A polymer bearing 100% mEO$_2$ was synthesized (Table 4.1, entry 1) as well as a series of copolymers with increasing amounts of galactose up to 40% (Table 4.1 entries 2-5). In all cases a slight excess of azides was used with respect to the polypeptide alkyne groups (1.2:1). Colorless polymers were obtained after removal of the copper catalyst by treatment with Amberlite Resin and subsequent dialysis. Quantitative reaction was confirmed by the disappearance of the characteristic alkyne absorbance at 2129 cm^{-1} in the FT-IR spectra upon click reaction (Figure 4.2). Further evidence for the successful click reaction was obtained from the presence of the triazole proton signals at 7.9 ppm in the ^{1}H-NMR spectra (**i, i`** in Figure 4.1).

^{1}H-NMR analysis also provided information on the substitution ratio of mEO_2 and galatose on the PPLG by comparing the characteristic peak areas of galactose (**s1)** and of the mEO_2 methyl group (**p)**. The results confirmed that substitution ratios were in good agreement with the actual feed ratio (Table 4.1). While FTIR spectra verified a α-helical structure for all substituted polymers in the solid state (Figure 4.2), Circular Dichroism (CD) was used to investigate the secondary structures of the copolymers in water. As can be seen in Figure 3, the spectra display two distinctive peak minima at 208 and 222 nm characteristic of a helical conformation. The helicities show a dependence on the substitution of the copolymer. For the fully mEO_2 substituted polymer a helicity of 51.1 % was calculated while the addition of 10 % galactose reduces the helicity to 36.9 %. With the addition of more galactose the helicity increases to 55.6 %. This is in contrast to the recently reported decrease of helicity with increasing degree of amino sugars directly attached to poly(glutamic acid).[30,40] Without a further systematic study it can only be speculated that this is due to the different side chain structures of both systems.

Table 4.1. Summary of PPLG (P_n = 61, M_n = 10300g/mol) substituted with different ratios of mEO_2 and galactose.

Entry	Feed ratio mEO_2/galactose	Ratio mEO_2/galactose[(a)]	Cloud point (ºC)	Helicity (%)
1	100/0	100/0	14	51.1
2	90/10	90/10	42	36.9
3	85/15	87/13	55	46.0
4	75/25	76/24	78	53.4
5	65/35	60/40	-	55.6

(a) calculated from ^{1}H NMR spectra.

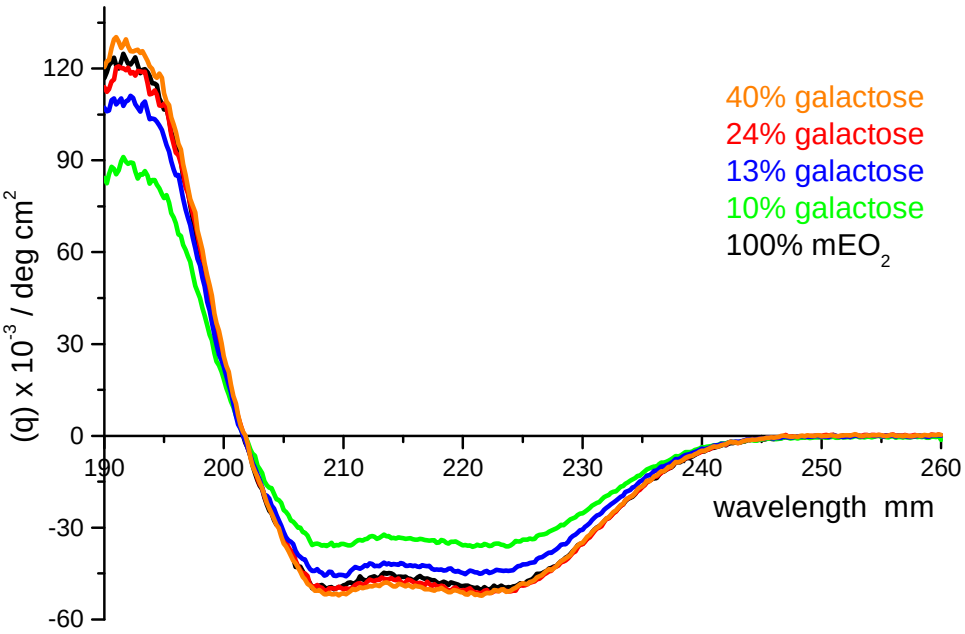


Figure 4.3. Circular dichroism (CD) spectra of polypeptides substituted with different ratios of galactose and mEO_2

Lectins are proteins, which non-covalently bind to carbohydrates with high sugar selectivity. Lectin binding tests were performed with all synthesized polypeptides using Ricinus communis Agglutinin (RCA_{120}) lectin, which specifically binds to galactose and Concanavalin (ConA) lectin, which is a mannose binding lectin. A positive binding result was obtained with all galactose-containing polypeptides upon mixing with RCA_{120} as evident from precipitation. No precipitation was observed when the same polymers were mixed with ConA and for the sample containing 100% mEO_2, as expected (inset Figure 4.4). Moreover, the change in solution absorbance upon addition of the glycopeptide to the RCA_{120} lectin was investigated at lectin concentrations of 0,5 mg/ml and 3 mg/ml in buffer solution. For both concentrations the absorbance remained stable throughout the 30 min. of the experiment and increased approximately linearly with the percentage of galactose in the polypeptide (Figure 4.4).

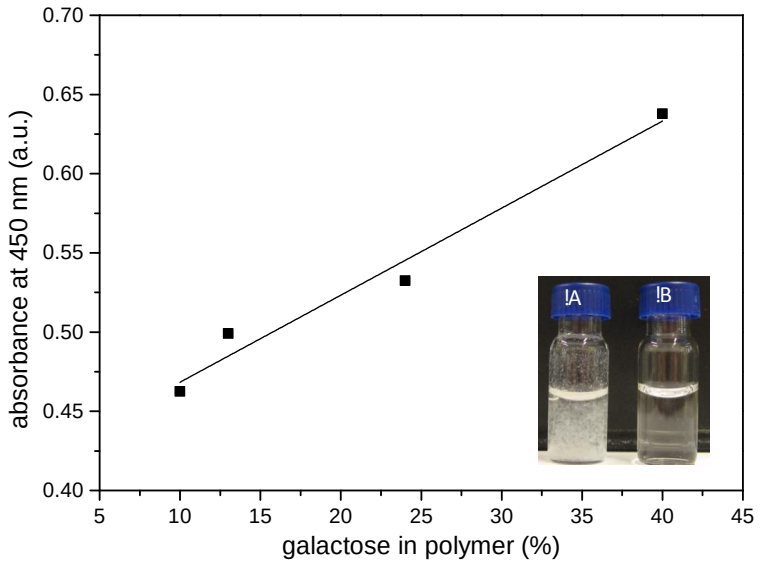


Figure 4.4. Turbidity measurements (450 nm) of copolymers with various degree of galactose substitution at 3 mg/ml with lectin RCA_{120} (line added to guide the eye) in PBS buffer. The inset shows the polymer substituted with 40% galactose in the presence of RCA_{120} (vial A) and ConA (vial B).

The thermoresponsiveness of the glycopolypeptides was investigated by temperature-controlled turbidity measurements. More specifically, 10 mg/ml solutions of the polypeptides in water were prepared and their absorbance was measured at a temperature ranging from 2 ºC to 95 °C at a wavelength of 500 nm. A linear correlation was found between the Cloud Points (CP) and the ratio of mEO_2 to galactose for the polypeptides, i.e. the higher the content of galactose, the higher the CP was (Figure 4.6, Table 4.1). For instance, when the polypeptides were substituted with 100% mEO_2, CP was found to be at 14 ºC. The increase of galactose moieties and the simultaneous decrease of mEO_2 in the polypeptides resulted in an increase in the CP, which reached 78 ºC for the 24% galactose substituted polymer. The CP of the 40% substituted galactose polypeptide was above 100 ºC and could not be determined. The process is fully reversible und the solutions clear upon cooling and become

cloudy again upon heating above the T_{cp}, which was confirmed by the full recovery of the absorbance throughout these heating and cooling cycles.

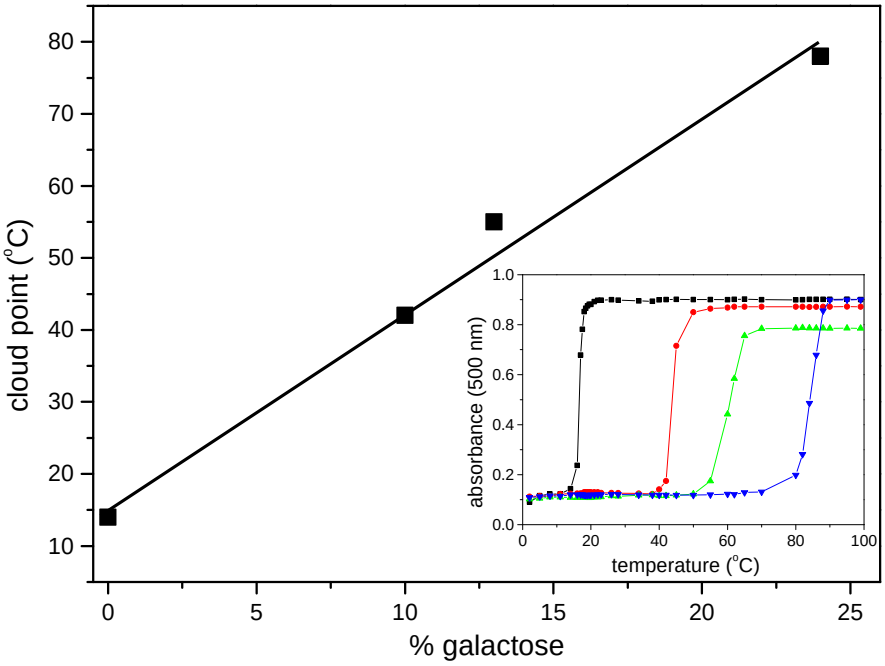


Figure 4.5. Cloud points as a function of the galactose content in the copolymer in DI water (line added to guide the eye). The inset shows the original absorbance measurement at 500 nm for 0 (■), 10 (●), 13 (▲) and 24 % (▼) galactose.

A series of experiments was carried out to investigated whether lectin binding could be thermally controlled through the T_{cp} of the polymers. Solutions of the polypeptide with 90% mEO_2 and 10% galactose (entry 2, Table 4.1) were prepared at concentrations of 8 mg/ml and 2 mg/ml. A RCA_{120} (5 mg/ml) solution was then added to the polypeptide solutions and the absorbance measured as a function of temperature. As depicted in Figure 4.6, when the 8 mg/mL sample was heated above the T_{cp} of 42 °C a sharp increase in turbidity (absorbance) was observed. When RCA_{120} was added to this sample below the T_{cp} the lectin induced aggregation also resulted in a high turbidity. Heating this sample above T_{cp} further increased the turbidity to the same level observed for the sample without lectin. Evidence that this increase in turbidity is primarily caused by the T_{cp} effect rather than lectin induced

aggregation was obtained from the same experiment at lower polymer concentration. At a polymer concentration of 2 mg/mL (Figure 4.6) the T_{cp} effect can be neglected as evident from the minor increase in turbidity upon passing the T_{cp}. On the other hand, lectin addition causes a significant increase in turbidity. When heated above the T_{cp} the turbidity dropped to the level of the sample without lectin. A further control experiment confirmed the absence of any turbidity of a pure lectin solution over the investigated temperature range. The results suggest that no glycan-lectin binding can occur above T_{cp} due the temperature-induced increase of polymer hydrophobicity and chain collapse/aggregation. All described transitions are fully reversible as tested by running three temperature cycles. This system thus presents an example of thermally controlled lectin binding.

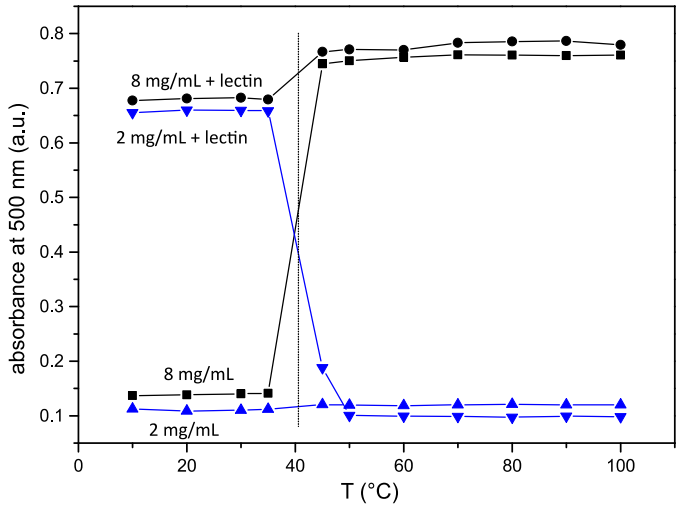


Figure 4.6: Turbidity measurements in PBS buffer (500 nm) of polypeptide with 10% galactose (entry 2, table 4.1) as a function of temperature in the absence and presence of RCA_{120} lectin (5 mg/mL). Dotted line indicates the T_{cp}. Black lines (●, ■): 8 mg/mL; blue lines (▲,▼): 2 mg/mL

4.4 Conclusions

The successful synthesis of well-defined thermoresponsive glycopolypeptides via ring opening polymerization and click chemistry was demonstrated. All polymers had very good solubility in aqueous media and adapted α-helical conformations. Moreover, the ability of the polymers to interact with biological systems was confirmed by lectin binding tests. Additionally, all glycopolypeptides with galactose content up to 24 % displayed a reversible T_{cp} behavior, which could be controlled by changing the ratio of mEO_2 and galactose moieties grafted on the polymeric chain. Turbidity experiments suggest that the lectin binding can be controlled by the solution temperature. No interaction with lectin was observed above T_{cp}, whereas at lower temperatures, the polypeptides expressed distinct binding properties.

4.5 References

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Chapter 5

Thermoresponsive star shaped glycopolypeptides: the influence of temperature on lectin binding

5.1 Introduction

Great progress has been displayed over the last years in the synthesis of stimuli responsive polymers, involving materials with thermal, pH[1], light[2] or redox sensitivity. Moreover, there are cases when two or more stimuli-responses are combined in the same molecule, leading to promising multi-functional structures[3,4,5]. This has for example been demonstrated for drug delivery meterials, Specifically, Liu et al. described the synthesis of star glycopolypeptides, which could self-assemble into reduction- and thermo-sensitive micelles and hydrogels [6]. DOX loaded micelles displayed a reduction-sensitive drug release, as study that opens a new path for on-demand drug delivery. Especially, glycopolypeptides, which express stimuli-response characteristics, are of interest due to their biocompatibility[7], good solubility in aqueous media and their ability to mimic natural glyco-proteins[8]. Only a few studies have been reported involving thermosensitive glycopolymers and their interaction with lectins[9,10,11]. The aforementioned glycopolymers exhibited a lower critical solution temperature (LCST), at which they underwent a phase transition from a water soluble state to an insoluble one. Our group described the synthesis of a linear polypeptide poly(γ-propargyl-L-glutamate) (PPLG) by the ring opening polymerization (ROP) of the corresponding N-carboxy anhydride (NCA), bearing galactose and 1-azido-2-(2-methoxyethoxy)ethane (mEO_2) moieties to introduce selective binding and thermoresponsive properties respectively. Lectin binding experiments at temperatures above and below cloud point temperature T_{cp} suggested that binding is suppressed above T_{cp}. This opens opportunities to design functional materials with temperature controlled biological response[12].

All the above examples refer to linear glycopolymers, which either remain linear or adopt a micellar structure in aqueous media. Not much research has been carried out for the synthesis of branched or star glycopolymers, which combine thermoresponsive and lectin binding

properties. To our knowledge, there is only one case illustrating the above concept. Du Prez et al. described the three-step synthesis of hyperbranched glycopolymers consisting of a thermos-responsive poly(N-isopropylacrylamide) [poly(NIPAM)] skeleton, combined with mannose units. Cu(0) mediated controlled radical polymerization was performed using a branched AB_2 type thiolactone initiator to polymerize linear NIPAM which was crosslinked via disulfide bonds. The cloud point (CP) of the branched glycopolymer was determined by tubidity measurements and was found to be higher than the one of linear poly(NIPAM). Also, the polymer structure changed from coil (below CP) to globular (above CP). Turbidity measurements showed a faster lectin binding above the cloud point, which can be explained by the fact that globular structures above CP, resulted in higher concentration of mannose units to the external part of the globules, making them more accessible to ConA lectin.

Synthetic polymers exhibit a lower degree of structural similarities with biopolymers than polymers synthesized from natural resources like polypeptides, which can be synthesized by the ring opening polymerization of amino acid N-carboxyanhydrides (NCAs)[13,14]. Moreover, our group has demonstrated the successful synthesis of star-polypeptides with well defined structures using poly(propyleneimine) PPI dendrimers as initiators and their potential as drug and gene delivery scaffolds[15,16]. Varying the functional groups on dendrimers' surface we can create a library of materials with different properties and applications or apply an external stimulus like lectin binding[17,18] or thermo-responsiveness[19]. Chen et al. described the synthesis of novel thermo-responsive polypeptides by clicking 1-(2-methoxyethoxy)-2-azidoethane (MEO_2–N_3) or 1-(2-(2-methoxyethoxy)ethoxy)-2-azidoethane (MEO_3–N_3) to poly-(γ-propargyl-L-glutamate) (PPLG)[20], demonstrating a correlation between LCST and chain length of polypeptide backbone.

Based on the latter work, in this chapter we aimed at the synthesis of star polypeptides, which displayed both lectin-binding and thermoresponsive abilities. To achieve that we polymerized

γ-propargyl L-glutamate NCA using a second generation PPI dendrimer as initiator followed by conjugation with mEO_2 and azido-galactose moieties via copper catalyzed click reaction. The bio-recognition ability of the star polypeptides was investigated by turbidity measurements as well as its temperature dependence. Moreover, a correlation study with linear polymers of similar molecular weights and substitution ratios was conducted.

5.2 Experimental Section

5.2.1 Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Diethyl ether was purchased from VWR. DMSO, ethyl acetate and ethanol were used directly from the bottle under an inert and dry atmosphere. PPI (polypropylene imine) dendrimer generation 2 was purchased from SyMO-Chem BV (The Netherlands). 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose was synthesized following a literature procedure[21]. 1-Azido-2-(2-methoxyethoxy)ethane (mEO_2) was synthesized using a similar procedure as the one reported by Hammond[22]. All chemicals were used without any purification unless otherwise noted.

5.2.2 Methods. ^{1}H-NMR spectra were recorded at room temperature with a Bruker Avance 400 (400 MHz), DMSO-d_6, $CDCl_3$ and D_2O were used as solvents and signals were referred to the signal of residual protonated solvent signals (D_2O). TMS was used as an internal standard for DMSO-d_6 and $CDCl_3$. ATR-FTIR spectra were collected on a Perkin-Elmer Spectrum 100 in the spectral region of 650-4000 cm^{-1} and were obtained from 4 scans with a resolution of 2 cm^{-1}. A backround measurement was taken before the sample was loaded onto the ATR unit for measurements. CD-spectroscopy was performed on a Jasco J-815

spectrometer with 0.0050 mM solution of the polypeptide in demineralized water. Mean residue ellipticities were calculated from the CD spectra following a literature procedure[23] using the equation $[\Theta]_{MRW} = (\Theta \times M_{MRW})/(10 \times c \times l)$, Θ: experimental ellipticity in mdeg, M_{MRW}: molecular weight in g/mol, c: concentration in mg/mmL, l: path length 0.5 cm. Helicities were calculated at λ = 222 nm using $f\alpha = (-[\Theta 222]_{MRW} + 3000)/39{,}000$. All turbidity assays were carried out on an Perkin Elmer Lambda900 UV-VIS instrument using UV quartz cuvettes. Assays with lectin were monitored at 450 nm in PBS buffer solution, while assays without lectin were monitored at 500 nm in deionised (DI) water.

5.2.3. Synthesis of γ–propargyl L-glutamate hydrochloride. The synthesis of γ-PLG-HCL was carried out following a modified procedure reported by Hammond and coworkers[24]. To a solution of L-glutamic acid (8.6 g, 58.5 mmol) suspended in propargyl alcohol (300 mL, 5.2 mol), 14.85 mL of chlorotrimethylsilane (117mmol) was added drop-wise under nitrogen. The solution was stirred at 40 ºC overnight and then the crude product was precipitated into diethyl ether. To avoid the presence of free propargyl alcohol, the product was extensively washed with ether, filtered, recrystallized from ethanol and dried under vacuum to obtain a white powder. Yield: 9.3 g (41 mmol, 72%). ^{1}H NMR (D_2O, δ ppm): 2.07 (2H, $CHCH_2$), 2.51 (1H, C≡CH), 2.82 (2H, $COCH_2$), 3.80 (1H, $CHCH_2CH_2$), 4.57 (2H, $OCH_2C{\equiv}CH$).

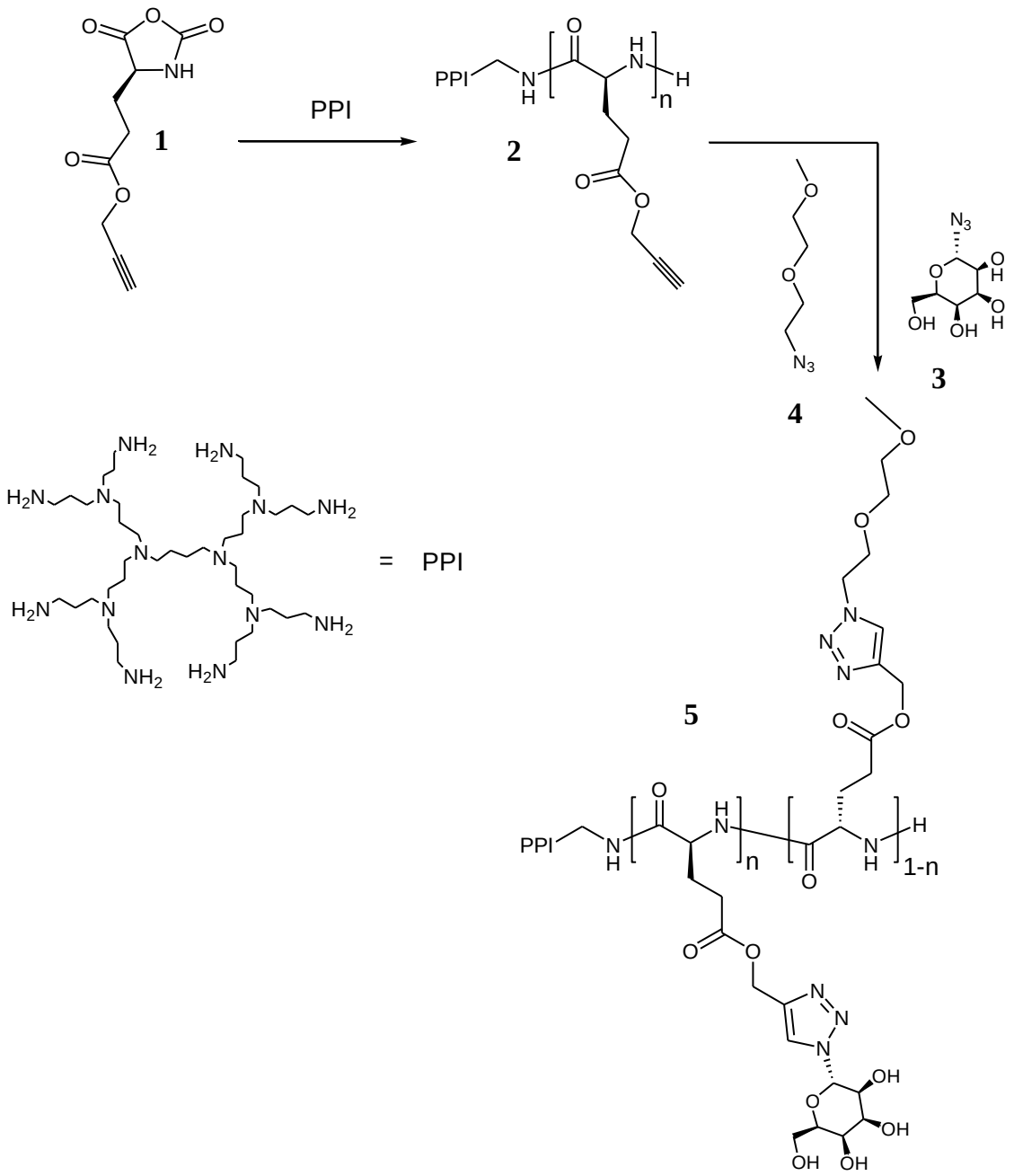
5.2.4. Synthesis of N-carboxyanhydride of γ-propargyl L-glutamate (PLG-NCA). γ–propargyl L-glutamate hydrochloride (4 g, 17.8 mmol) was suspended in dry ethyl acetate (120 mL) and the solution was heated to reflux under nitrogen. Triphosgene (2.6 g, 8.3 mmol) was added and the reaction was left to reflux for 4 more hours. The reaction solution was left to cool to room temperature and was filtered to remove any unreacted γ–propargyl L-glutamate hydrochloride. Then it was cooled to 4 ºC and washed with 120 mL of water, 120

mL of saturated sodium bicarbonate and 120 mL of brine all at 4 ºC. The organic phase was then dried over anhydrous magnesium sulfate, filtered and evaporated to give a viscous oil. Yield: 2.8 g (9 mmol, 76.5%). ^{1}H NMR ($CDCl_3$, δ ppm): 2.22 (1H, $CHCH_2CH_2$), 2.39 (1H, C≡CH), 2.68 (2H, $COCH_2$), 4.47 (1H, $CHCH_2CH_2$), 4.75 (2H, $OCH_2C{\equiv}CH$), 6.58 (1H, NH).

5.2.5 Synthesis of star shaped poly(γ-propargyl L-glutamate) (PPLG). γ-PLG-NCA (400 mg, 1.90 mmol) was dissolved in 8 mL of DMSO, added to a Schlenk tube and left under nitrogen for 30 min. Then, 36.59 mg (0.047 mmol; ratio 40:1) of PPI dendrimer 8AM was dissolved in DMSO (2 mL) and added to the reaction solution. The reaction mixture was left to stir overnight at room temperature under vacuum. The polymer was precipitated into diethyl ether, washed with an excess of diethyl ether and collected by centrifugation. The product was dried overnight under vacuum. Yield: 286 mg (79%).

5.2.6 Conjugation of star-shaped PPLG with mEO_2 and galactose. The following procedure describes a representative example for the ratio mEO_2:galactose 8:2. The conjugation onto the backbone was carried out at a feed ratio of alkyne/azide/CuBr/N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA) of 1:1.3:0.1:0.1. PPLG (55 mg, 0.29 mmol of alkyne repeat units), mEO_2 (43 mg, 0.30 mmol), azido-galactose (15.5 mg, 0.076 mmol) and PMDETA (6.1 μl, 0.029 mmol) were all dissolved in DMF (6 ml). The CuBr catalyst (4.16 mg, 0.029 mmol) was added to the degassed solution and the reaction was left to stir at room temperature for 6 h. The reaction solution was precipitated in cold diethyl ether, dissolved in distilled water and treated with Amberlite Resin 120 for 2 hours to obtain a clear solution. The Amberlite beads were removed by filtration and the polymer solution was dialyzed against water acidified with HCl for 24 h and then against

distilled water for another 24 h. The polymer then was lyophilized to obtain a white solid. Yield: 72 mg (73%).



Scheme 5.1. Synthesis of star-shaped poly(γ-propargyl-L-glutamate) (PPI-PPLG, **2**) followed by co-click reaction with mEO_2 (**4**) and azidogalactose (**3**).

5.3 Results and discussion

The synthesis of star shaped polypeptides (PPI-PPLG, **2**) was achieved using a second generation PPI dendrimer (8 arms) as initiator. To overcome solubility problems, DMSO was used as a solvent for the reaction. The feed ratio of monomer/initiator was 40:1 in order to obtain dendrimers with 5 units per arm. The presence of the alkyne terminal group was confirmed by the signal at 2.2 ppm in ^{1}H-NMR spectra as well as by the $C \equiv C$ stretching at 2130 cm^{-1} in FT-IR spectra. From the integrated peak areas of the protons adjacent to amine groups of the dendritic initiator (**b+d**, 3ppm) and the signals of the protons next to peptidic bond (peak **f)**, the degree of polymerization was determined and found to be in good agreement with the initial feed ratio. Noteably, even after extensive washing with ether, remains of DMSO were traced in the NMR spectra of the polypeptide. FT-IR spectra confirmed the presence of β-sheet conformation with characteristic amide bands at 1692, 1627 and 1533 cm^{-1} as well as traces of α-helix due to the amide band at 1652 cm^{-1}.

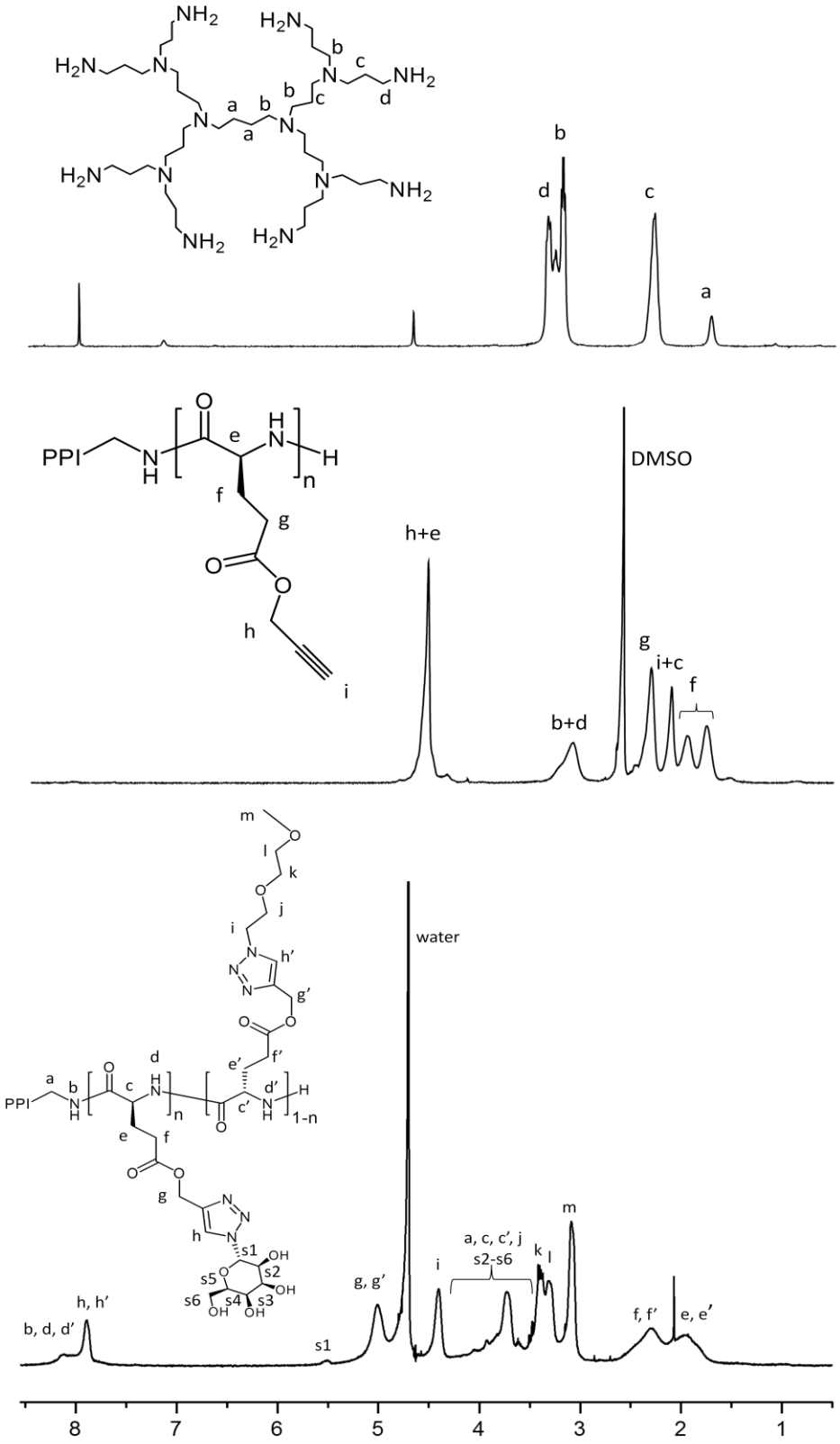


Figure 5.1. [1]H NMR spectra of PPI dendrimer (top) in d-trifluoroacetic acid (d-TFA), star polypeptide PPI-PPLG (middle) in d-TFA and mEO_2 and azido galactose conjugated star polypeptide (bottom) in D_2O.

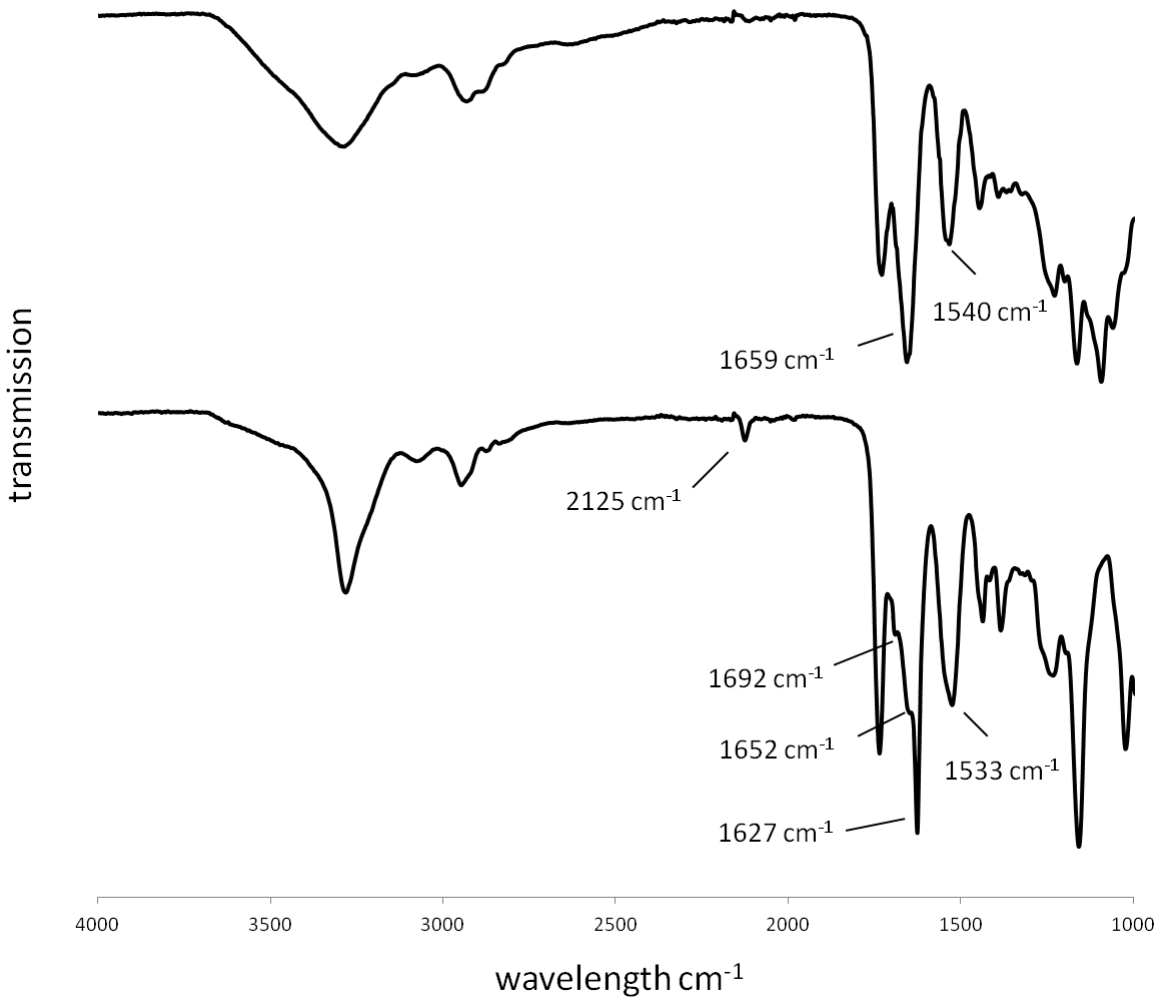


Figure 5.2. FT-IR spectra of PPI-PPLG (bottom) and PPLG-mEO_2-galactose (top). indicating the successful polymer conjugation by 'click' chemistry and the transition from a β-sheet (bottom), to an α-helix conformation (top)

In order to obtain both lectin binding and thermo-responsive star polypeptides, mEO_2 (4) and 1-β-azido-2,3,4,6-tetraacetyl-D-galactose (3) were subsequently conjugated with the alkyne groups on the polypeptide chain. A library of statistical copolymers was synthesized varying the ratio of the two azides i.e. the ratio of azido-galactose was increased from 0% to 47%. Due to the dendrimers dense interior part, the polypeptide units close to the dendritic core, were less accessible to the two azides (3+4). For that reason the reaction time was extended

to 6 hours. Furthermore, an excess of azides was used compared to the polypeptide alkyne groups (1.3:1) to achieve complete reaction. All polymers showed very good solubility in water. The success of the reaction was confirmed by the formation of the triazole ring, with characteristic proton signals at 7.8 ppm (peaks **h, h'**) and also the appearance of galactose and mEO_2 peaks as it can be seen in the ^{1}H-NMR spectra (Figure 5.1). Moreover, the characteristic alkyne absorbance at 2125 cm^{-1} in the FT-IR disappeared indicating quantitative click reaction. FTIR spectra also demonstrated the presence of α-helical conformation for all substituted polymers in the solid state, with characteristic bands at 1659 and 1540 cm^{-1}. Moreover, the ratio of the substitution on the PPI-PPLG was calculated comparing the characteristic peak areas of galactose (**s1**) and of the mEO_2 methyl group (**m**) in the NMR spectra, whereas the dendrimer peaks are not visible. Circular Dichroism (CD) also revealed traces of helical structure displaying two distinctive peak minima at 208 and 222 nm. Helicity percentage was dependent on the galactose ratio on the polypeptide chain, i.e. helicity decreased when more galactose was attached (Table 5.1). Interestingly, for the 100% mEO_2 sample helicity dropped down to 2%, a result that shows that sugar has the main effect on the helicity maybe due to possible hydrogen bonds that may be formed between the sugar molecules, which can affect the structure of the polypeptide. Further investigations, though, have to be conducted in order to fully understand and explain these data.

Table 5.1. Summary of star shaped PPI-PPLG (P_n = 48) substituted with different ratios of mEO_2 and galactose.

Entry	Feed ratio mEO_2/galactose	Ratio mEO_2/galactose[(a)]	Cloud point (°C)	Helicity (%)
1	100/0	100/0	18	2
2	90/10	92/8	30	23.9
3	80/20	80/20	43	16.5
4	70/30	66/34	62	12.3
5	60/40	53/47	85	6.3

(a) calculated from ^{1}H NMR spectra.

Lectin binding

The bio-recognition ability of all star polypeptides was investigated performing lectin binding assays. Lectins are natural proteins that selectively bind to sugar molecules in a non-covalent manner. For that purpose, two different lectins were used: Ricinus communis Agglutinin (RCA_{120}) lectin, which specifically binds to galactose and Concanavalin (ConA) lectin, which is a mannose binding lectin. For this experiment, both lectins (2 mg/ml) as well as all the star-polypeptides (0.5 mg/ml, 2 mg/ml) were dissolved in PBS buffer solution. Upon mixing the polymer solution with the lectin solution, UV absorbance at a specific wavelength was measured for a period of one hour. Successful binding was evident from an increase in the absorbance as well as from precipitation due to the aggregation of lectins. All glycosylated polymers showed a positive binding result after mixing with RCA_{120} lectin. In contrary, no significant increase in the absorbance or precipitation occurred for the polymers that were just dissolved in PBS buffer or mixed with ConA lectin. Moreover, higher concentration of polymer solution led to higher absorbance. Nevertheless, for 0.5 mg/ml as well as 2 mg/ml of

polymer solution, absorbance remained almost stable throughout the 60 min of the experiment.

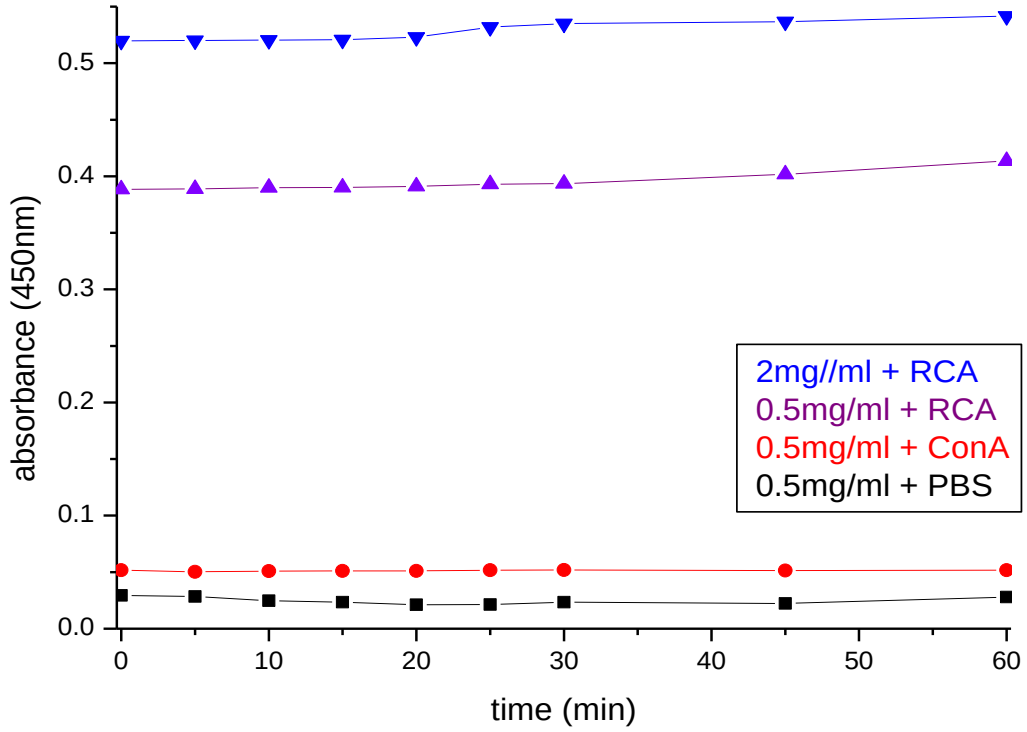


Figure 5.3. Turbidity measurements (450 nm) of star-polymers (entry 3, table 5.1) at 0.5 and 2 mg/ml with lectin RCA_{120} in PBS buffer. As negative controls, the absorbance of 0.5 mg/ml of polymers in PBS buffer and 0.5 mg/ml mixed with ConA lectin was measured at the same wavelength.

Cloud Point measurements

The effect of galactose and mEO_2 ratios on Cloud Point temperatures was investigated by turbidity measurements. For that purpose, 10 mg/ml solutions of all the star-polypeptides were prepared in PBS buffer and their absorbance was measured at temperatures ranging from 5 ºC to 99 ºC at 500 nm wavelength (Figure 5.4). CP was found to be fully dependent on the galactose/mEO_2 ratio. Increase in the amount of galactose bound to the polypeptide led to a higher CP, which can be explained by the fact that since galactose is more hydrophilic

than mEO_2, polypeptides with higher content in sugar are expected to display a higher CP. In our case, the polymer substituted with 100% mEO_2 (i.e. 0% galactose) displayed a CP of 18 ºC, which increased and reached 85 ºC after substitution with 47% with galactose. The whole process proved to be reversible after repeating the heating and cooling cycle three times.

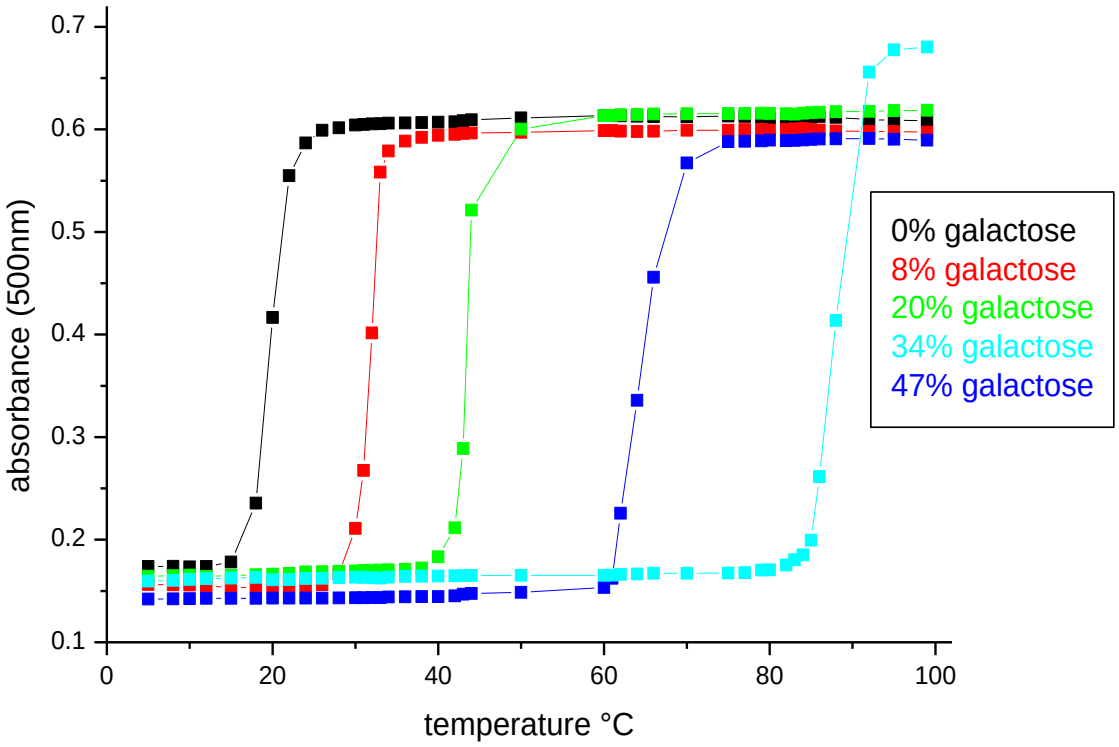


Figure 5.4. Influence of temperature on the absorbance of PPI-PPLG substituted with galactose at 0%, 8%, 20%, 34%, 47% .

Influence of CP on lectin binding

The influence of T_{cp} on lectin binding was investigated through a series of turbidity experiments. A solution of the star-polypeptide with 80% mEO_2 and 20% galactose was prepared at 2 mg/ml concentration and the change in absorbance in relation with temperature was measured at 500 nm. Then, RCA_{120} (3 mg/ml) solution was added to the polypeptide solution and mixed with a pipette. The change of absorbance was again measured for the same wavelength. When the sample without the lectin was measured no significant change in absorbance was observed, indicating that T_{cp} does not have any effect on turbidity due to low solution concentration. On the other hand, upon addition of the lectin solution, an immediate increase in turbidity was observed, as can be seen in Figure 5.5. After heating above the T_{cp} the turbidity decreased and reached the same level of the sample without the lectin. The same procedure was repeated three times and proved to be fully reversible. From the above experiment it could be concluded that no lectin-binding can occur above T_{cp}, possibly due to the increase of star-polypeptides' hydrophobicity, which affects their arrangement in the solution preventing galactose moieties from being 'visible' to lectins. Based on the above observations, this system can be considered as suitable for thermally controlled lectin binding.

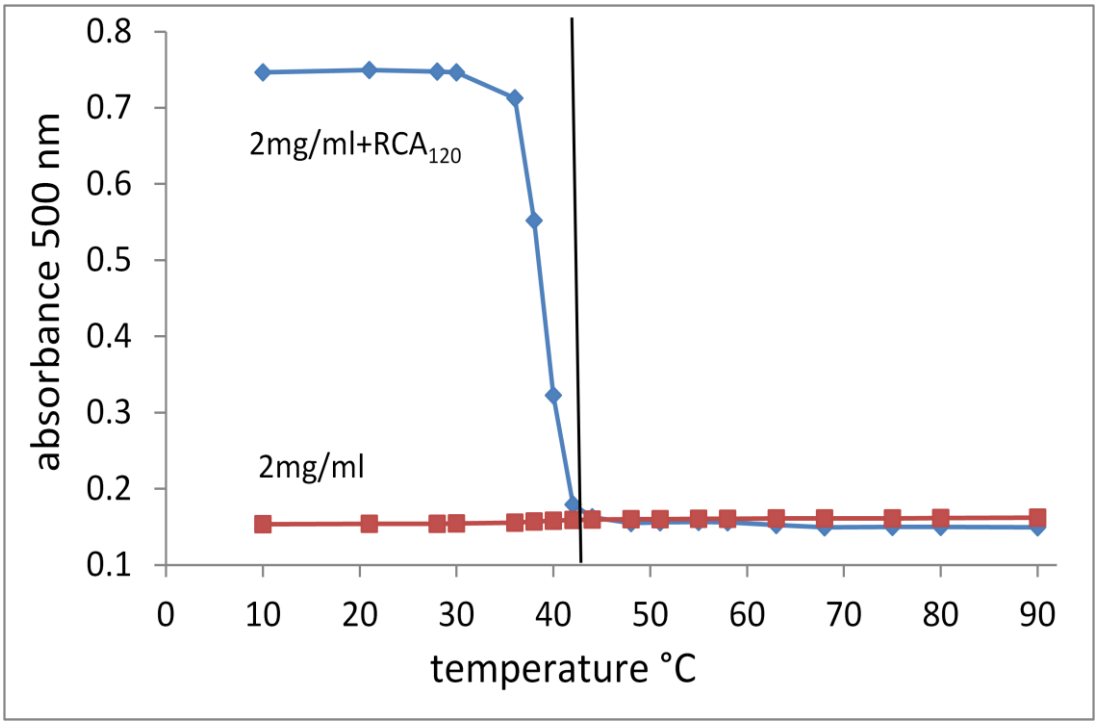


Figure 5.5. Turbidity measurements in PBS buffer (500 nm) of polypeptide with 20% galactose (entry 3, table 4.1) as a function of temperature before and after the addition of RCA_{120} lectin (3 mg/mL). Line is added to indicate to T_{cp}.

Comparison between linear and star-shaped polypeptides

A comparison study between linear (investigated in chapter 4) and star polypeptides was conducted in order to investigate the effect of polypeptide structure on helicity and cloud point. For that purpose, a library of linear and star polypeptides was synthesized, aiming at a substitution ratio of mEO_2 and galactose to be 80/20 respectively (Table 5.2). Entries 1-3 refer to linear polymers, whereas entries 4-5 refer to star polypeptides. Comparing the linear polypeptides it can be concluded that an increase in the number of polypeptide units, leads to an increase in cloud point as well as percentage of helicity. The same can be observed for star polypeptides. The rate of increase for linear polymers though it is much lower compared with the stars. Doubling the number of units in linear polymers (entries 1 and 3 in Table 5.2)

resulted in a slightly higher CP (59 to 71 ºC) in contrary to star polymers (entries 4,5 in Table 5.2) where CP "jumped" from 48 ºC to more than 100 ºC. More interesting is the comparison between linear and star polypeptides with similar number of units (entries 2 and 4). In this case, there is a significant difference in the CP, indicating that linear polymer (entry 2, Table 5.2) expressed better water solubility, which resulted in a higher CP temperature (71 ºC**)** in contrary to the star polymer (entry 4, Table 5.2), which displayed a CP at 43 ºC. This, presumably, can be also confirmed by the difference in helicity, i.e. 49.2% for the linear polymer and 16.5 % for the star one. It can be assumed that the star polypeptides favor a random coil conformation which would decrease their solubility resulting in lower cloud point temperatures. This study can be used as a useful platform for the synthesis of linear and star thermoresponsive glycopolypeptides, whose helicity and CP can be easily tuned by changing the structure or the molecular weights

Table 5.2. Summary of linear and star shaped polypeptides substituted with 80/20 mEO_2 /galactose ratio respectively.

Entry	Polymer	Ratio mEO_2/galactose	Cloud point (ºC)	Helicity (%)
1	L-PPLG60	76/24	78	53.4
2	L-PPLG52	78/22	71	49.2
3	L-PPLG32	82/18	59	17.8
4	PPI-PPLG48	80/20	43	16.5
5	PPI-PPLG96	78/22	>100	33.6

5.4 Conclusions

In this project, the successful synthesis of temperature responsive star-glycopolypeptides was demonstrated. All polymers characterized by ^{1}H-NMR and FT-IR spectroscopy and the results found to be in good agreement with the theoretical values. The ability of the polymers to be recognized by biological systems was confirmed by lectin binding tests. Moreover, all star-polypeptides displayed a reversible T_{cp} behavior, which could be controlled by changing the ratio of mEO_2 and galactose moieties grafted on the polymeric chain. Turbidity experiments showed that the glycan-lectin binding could be controlled by the solution temperature. Moreover, a comparison between linear and star glycopolypeptides revealed the effect of the structure and molecular weight on cloud point and helicity.

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Chapter 6

Summary and outlook

Over the last years, great effort has been made on the synthesis of polypeptides. Synthetic polypeptides are attractive biomimetic materials, which have gained great interest, due to their ability to fold into well-defined secondary structures such as α-helix and β-sheet. Their tendency to undergo helix to coil transition is an essential characteristic for stimuli-responsive materials. Moreover, their biodegradability and biocompatibility features have shown great potential in different aspects of biomedicine such as drug delivery, tissue engineering, biomedical imaging or protein-ligand interacitons.

The aim of this PhD project was to obtain a series of various synthetic polypeptides of linear and star-shaped architecture followed by the subsequent apply of an external stimulus, such lectin-binding and thermo-responsiveness. The synthesis of the polypeptides was achieved by N-carboxyanhydride ring opening polymerization (NCA-ROP) of the corresponding amino-acids. NCA-ROP was proved as an effective route to synthesize well defined polypeptides of high molecular weights on a large scale synthesis.

To achieve the bio-recognition of all the synthesized polypeptides that are described in the research chapters (2-5), their successful conjugation with sugar moieties was performed, through orthogonal "click" chemistry techniques. Their binding to biological model systems was investigated and confirmed by highly specific lectin-binding experiments.

Initially, in Chapter 2 the successful synthesis of star homo- and block glycopolypeptides initiated by second generation PPI dendrimers and their subsequent glycosylation using Huisgen [3+2] cycloaddition reaction was described. γ-Propargyl-L-glutamate NCA was used for that purpose, due to its alkyne groups, which are suitable for 'click' chemistry reaction. To increase the functionality of our system a second block was added to the polymeric chain. The final form of the structure is an equivalent of a micelle bearing a hydrophobic dendritic core and a hydrophilic outer shell, due to the presence of galactose. The interaction of

glycopeptides with biological systems was confirmed by lectin-binding assays. A drawback of these structures is the fact that no selective deprotection of the individual polypeptide blocks can occur and further research was done in Chapter 3 to synthesize star block glycopolypeptides with improved solubility and functionalities.

More specifically, in Chapter 3, the successful synthesis of novel, well-defined, star glycopolypeptides bearing a hydrophobic dendritic core and a hydrophilic shell is described. Similarly, as above, the initiator was PPI denrimer of generation 2 bearing 8 arms and the two monomers that were used for the above synthesis were DL-propargylglycine NCA and ε-carbobenzyloxy-L-lysine NCA. The successful synthesis was confirmed by NMR and FT-IR spectroscopy. Enzyme linked lectin assays (ELLA) proved the selective binding ability of the polymers and especially in the case of random copolymers leading to higher lectin binding by changing the number of the propargylglycine units, which are bound with the sugar moieties. Furthermore, the capability of one of the glycopeptides to complex with plasmid DNA was investigated and confirmed at an N/P ratio of 10 as was proved by gel retardation studies and ζ-potential measurements. Further studies include the pDNA and siRNA complexation with the remaining glycopeptides. The system presented is quite versatile and can be readily modified (i.e. change the length of the polymeric chains, dendrimer generation, introduce new monomer), in order to obtain more functionalities and possibly be useful for different applications.

In chapter 4, the successful synthesis synthesis of a polypeptide with combined lectin recognition and thermoresponsive properties was described. The polymers are obtained by ring-opening polymerization of γ-propargyl L-glutamate NCA and conjugated with mEO_2 and galactose through copper catalyzed click chemistry. All polymers had very good solubility in aqueous media and adapted α-helical conformations. Moreover, the ability of the polymers to interact with biological systems was confirmed by lectin binding tests. Additionally, all

glycopolypeptides with galactose content up to 24 % displayed a reversible T_{cp} behavior, which could be controlled by changing the ratio of mEO_2 and galactose moieties grafted on the polymeric chain. Turbidity experiments suggest that the lectin binding can be controlled by the solution temperature. No interaction with lectin was observed above T_{cp}, whereas at lower temperatures, the polypeptides expressed distinct binding properties.

The same procedure was followed in Chapter 5 for the synthesis of star polypeptides, which also which displayed both lectin-binding and thermoresponsive abilities. To achieve that we polymerized γ-propargyl L-glutamate NCA using a second generation PPI dendrimer as initiator followed by conjugation with mEO_2 and azido-galactose moieties via copper catalyzed click reaction. All polymers characterized by ^{1}H-NMR and FT-IR spectroscopy and the results found to be in good agreement with the theoretical values. The ability of the polymers to be recognized by biological systems was confirmed by lectin binding tests. Moreover, all star-polypeptides displayed a reversible T_{cp} behavior, which could be controlled by changing the ratio of mEO_2 and galactose moieties grafted on the polymeric chain. Turbidity experiments showed that the glycan-lectin binding could be controlled by the solution temperature. Moreover, a comparison between linear and star glycopolypeptides revealed the effect of the structure and molecular weight on cloud point and helicity. The above experiments could be used as a platform for the synthesis of thermoresponsive materials suitable for hosting drug molecules, whose release would be controlled by tuning the temperature.