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SCHOOL OF SCIENCES AND ENGINEERING

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Bachelor Thesis

"Protein-Polymer Bioconjugates via Copper Mediated LivingPolymerization & Rapid Disproportionation Without External Deoxygenation or Oxygen Scavengers"

by

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Abstract

In the recent years, several methods of controlled and living radical polymerization have almost become a routine in polymer laboratories. However, special equipment and specialized personnel are still needed in the majority of cases to achieve the stringent reaction conditions, while protocols remain problematic e.g. for aqueous reaction environments and biomolecular components. Single Electron Transfer-Living Radical Polymerization (SET-LRP) reactions can be achieved according to literature reports in aqueous environment through deoxygenation. During SET-LRP Cu (I) takes part into a disproportionation reaction forming Cu(0) and Cu(II). Very recently, in 2018, literature reports introduced living polymerization reactions without the need of degassing, significantly simplifying polymerization experimental procedures. Taking advantage of these reports, goal of this thesis was the creation of a range of amphiphilic biopolymers via a grafting from polymerization approach utilizing various monomers and bench top experimental protocols. In a spotlight article published by Haddleton and collaborators, the effect of headspace elimination on copper mediate polymerizations, was discussed. In the reactions described within this thesis a simple setup, namely a syringe, was introduced to remove air from the headspace above the reaction mixtures thus eliminating the headspace. Moreover, the thesis was focused on the creation of *Giant Amphiphiles-i.e.* protein-polymer bioconjugates. The characterization of the products was carried out via chromatography such as Gel Permeation Chromatography (GPC) and Polyacrylamide Gel Electrophoresis (PAGE). Nuclear magnetic Resonance (NMR) was used to characterize monomers and initiators while, Transmission Electron Microscopy (TEM) was used to image the morphology of the resulting superstructures. Polymerizations were studied in terms of monomer efficacy, while the optimization for the polymerization of styrene from protein macroinitiators is presented. We believe that the approach presented here will provide a strong basis for further studies and applications of *Giant Amphiphiles*.

Σύνοψη

Τα τελευταία χρόνια οι μέθοδοι ελεγχόμενου ζωντανού ριζικού πολυμερισμού αποτελούν πλέον ρουτίνα σε εργαστήρια μελέτης πολυμερών. Ωστόσο, η χρήση ειδικού εξοπλισμού από εξειδικευμένο προσωπικό είναι ακόμα απαραίτητη στην πλειοψηφία των LRP πολυμερισμών ώστε να είναι δυνατή η επίτευξη των αυστηρών απαιτήσεών τους όσον αφορά τις συνθήκες αντίδρασης (π.χ. απουσία οξυγόνου), ενώ τα πρωτόκολλα παραμένουν προβληματικά σε περιπτώσεις που συμπεριλαμβάνουν βιομόρια ή υδατικό περιβάλλον αντίδρασης. Οι αντιδράσεις SET-LRP (ζωντανός πολυμερισμός ριζών απλής μεταφοράς ηλεκτρονίων) μπορούν να επιτευχθούν σύμφωνα με βιβλιογραφικές αναφορές με απομάκρυνση του οξυγόνου. Κατά τη διάρκεια SET-LRP πολυμερισμών, ο Cu(I) συμμετέχει σε αντίδραση οξειδοαναγωγής σχηματίζοντας Cu(0) και Cu (II). Πολύ πρόσφατα, το 2018,αναφέρθηκαν αντιδράσεις ελεγχόμενου ριζικού πολυμερισμού δίχως ανάγκη απαέρωσης, απλοποιώντας ακόμη παραπάνω την πειραματική διαδικασία. Με αφετηρία και γνώμονα τα παραπάνω δεδομένα, βασικός στόχος της διπλωματικής εργασίας ήταν η δημιουργία μιας γκάμας αμφίφιλων βιοπολυμερών μέσω εμβολιασμού από (grafting *from*) βιοεκκινητές και με χρήση διάφορων μονομερών χρησιμοποιώντας την μέθοδο ελεγχόμενου ριζικού πολυμερισμού SET-LRP και αναπτύσσοντας απλούστερα πειραματικά πρωτόκολλα. Επιπρόσθετα, η βελτιστοποίηση βασίστηκε σε πολύ πρόσφατο άρθρο του Haddleton και συνεργατών του σχετικά με την επίδραση της μείωσης του υπερκείμενου χώρουσε καταλυόμενους από χαλκό πολυμερισμούς. Οι αντιδράσεις που παρουσιάζονται εδώ, πραγματοποιήθηκαν μέσα σε σύριγγα ώστε να είναι δυνατή η απομάκρυνση του αέρα από την αντίδραση, ελαχιστοποιώντας τον υπερκείμενο χώρο. Επιπρόσθετα, η διπλωματική εστιάστηκε στην δημιουργία *Γιγάντιων Αμφίφιλων* βιοπολυμερών. Ο χαρακτηρισμός των προϊόντων έγινε με διάφορες μεθόδους χρωματογραφίας, όπως χρωματογραφία χρωματογραφία μέσω πηκτώματος (GPC) και ηλεκτροφόρηση σε πηκτή πολυακρυλαμιδίου (PAGE), ενώ τα μονομερή και οι εκκινητές ταυτοποιήθηκαν με φασματοσκοπία Πυρηνικού Μαγνητικού Συντονισμού (NMR). Τέλος οι αυτοοργανωμένες υπερμοριακές δομές μελετήθηκαν με ΗλεκτρονικήΜικροσκοπίαΣάρωσης (TEM). Μελετήθηκαν διαφορετικά μονομερή, ενώ βελτιστοποιήθηκαν οι συνθήκες για τον πολυμερισμό στυρενίου από βιοεκκινητή. Πιστεύουμε ότι οι μελέτες που περιλαμβάνονται σε αυτή την εργασία θα αποτελέσουν μία δυνατή βάση για την περεταίρω μελέτη νέων τρόπων σύνθεσης βιοπολυμερών.

Acknowledgement

For the success in accomplishing of the current thesis and my studies, I would like to express my kindest acknowledgements to some special people that it was an honor to work with them.

First of all, i would like to share my kind respect and thanks to my supervisor, Dr. Kelly Velonia, Assistant Professor of the Department of Materials Science and Technology, whose dedication, support and motivation make me learn from her and work in her laboratory and I truly appreciate that she gave me the chance to be a member of her group. Furthermore, I would like to express my gratitude to my supervisor from the Department of Chemistry, Associate Professor Ioulia Smonou for supporting my thesis as a supervisor from my Department of study.

The next person, I want to share my honor is the Post. Doc of the lab, Alexis Theodorou, with whom I had an excellent cooperation, was there whenever I wanted his advice and help and whose ideas were really innovative. Last but not least, my lab partners, Eva Grafanaki, Panagiotis Skordalidis, Sokratis Kalfaoglou, Peter Mandriotis and Maria Papageorgiou, with whom I spend endless hours full of joy and cooperation inside and outside the lab and it was an honor working with them. The last person I want to thank is Evelina Liarou, who helped me organize my project when it was just a newborn.

Contents

Abbreviations

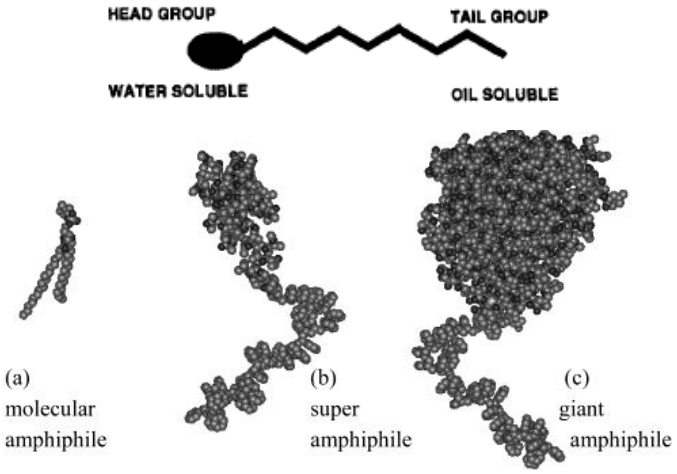
ATRP	AtomTransfer Radical Polymerization
BSA	BovineSerumAlbumin
DMSO	DimethylSulfoxide
EBiB	Ethyl α-bromoisobutyrate
EDTA	Ethylene-diaminetetraaceticacid
GPC	GelPermeationChromatography
HRP	Horseradishperoxidase
LCST	Low Critical Solution Temperature
LRP	LivingRadicalPolymerization
MA	Methyl Acrylate
Me_6TREN	Tris[2-(dimethylamino)ethylamine
MMA	Methyl methacrylate
MWCO	MolecularWeightCut-Off
NIPAAm	N-Isopropylacrylamide
Poly-NIPAAm	Poly-N-Isopropylacrylamide
NMR	Nuclear MagneticResonance
PAGE	PolyacrylamideGelElectrophoresis
PEG	Polyethylene glycol
PET	Photoinduced Electron Transfer
PS	Polystyrene
RAFT	Reversible Addition/Fragmentation Chain
SEM	ScanningElectronMicroscopy
TEM	Transmission Electron Microscopy
THF	Tetrahydrofuran
TLC	ThinLayerChromatography

1.0 Introduction

In the very recent years, increased interest regarding optimization of the stringent synthetic protocols required in living radical polymerizations have specificallyfocused in oxygen elimination procedures such as freeze-pump-thaw and inert gas sparging, which are integral for several methods of radical polymerization such as Atom Transfer Radical Polymerization (ATRP)[(1)(2)] and Reversible Addition/Fragmentation Chain Transfer Polymerization (RAFT).[(3)] As a result, oxygen-tolerant polymerization methods have been reported, including for instance a report by Chapman utilizing enzymes, such as glucose oxidase (GOx), in order to successfully deoxygenate traditional RAFT polymerization mixtures. [(4)(5)] Boyer and co-workers exploited Photoinduced Electron Transfer-RAFT(PET-RAFT) to produce polymeric materials in open reaction vessels by either increasing the concentration of the photocatalyst or employing a reducing agent (for example, ascorbic acid).[(6)(7)(8)]However, the use of photoinduced systems might not be compatible with enzymes or proteins as irradiation could destroy their tertiary structure.[(9)(10)(11)]Furthermore, the reducing agents or enzymes mentioned above could chemically interact with many vinyl monomers. Thus, while these methods may be successful in specific applications, they enhance the complexity of the system limiting the variety of monomers that can be polymerized.[(12)(13)]

The method developed in this Thesis on the other hand, was based on works of Haddleton and collaborators.[(14)(15)] Recently, a copper mediated controlled living polymerization, mediated via a rapid disproportionation reaction involving Cu(I), Cu(0) and Cu(II) in aqueous and polar media was reported. The polymerization efficacy was found to depend on the ligand, Me_6TREN, which forms complexes used as polymerization catalysts. In the study presented here, a water soluble LRP-biomacroinitiator was used and the experimental protocol was optimized to yield amphiphilic bioconjugates by grafting various monomers *from* the biomacroinitiator.

1.1 Theoretical Background



1.1.1 Amphiphilic molecules

Figure1:a)Hypothetical model of an amphiphile consisting of a head polar group, which is water soluble and a tail non-polar group, which is oil soluble. b) Subclasses of amphiphilic molecules.[18]

An amphiphile is the general term used to describe any compound that consists of two distinct components with different affinity for the solvent covalently bonded in the same molecule. As shown in Figure1, amphiphiles consist of a head group with high affinity for polar solvents, particularly water, and one or more tail groups with strong affinity for nonpolar solvents such as hydrocarbons.[16]Amphiphilic molecules can be classified in terms of their molecular weight.[17]Low molecular weight amphiphiles include molecules such as phospholipids. Higher molecular weight amphiphiles, called super amphiphiles, usually involve block copolymers (AB or ABC), which have extensive applications in various fields. Finally, *Giant Amphiphiles*, include protein-hydrophobic polymer bioconjugates.[17][18]

Amphiphilic block copolymers (ABCs) have found extensive use in pharmaceutical applications ranging from sustained-release technologies to gene delivery. Their use in carrying and delivering healing reagents is due to their chemical structure containing a hydrophobic block tethered to a hydrophilic block. Polymeric micelles are most often formed in aqueous solutions at or above a critical value of micelle concentration, adapting the ABCs core/shell structures. When micellization takes place, the hydrophobic core can be loaded with hydrophobic drugs, depending on its core functionalities in order to store release the drug when conditions are favorable.[19]

1.1.2 Giant Amphiphiles

Giant Amphiphiles represent the subclass of amphiphilic molecules with highest molecular weight.[19]An initial synthetic approach for their synthesis entailed a single covalent connection of a protein head-group, to appropriately functionalized hydrophobic polymer such as polystyrene.[18][40][41][42]This first approach was soon replaced with LRP grafting *from* a protein macroinitiator approaches that proved to be more efficient in terms of yields and efficacy.[20][37][43] In many cases, Giant Amphiphiles containing an enzyme as head group retain most of their catalytic activity. Importantly, *Giant Amphiphiles* can self-assemble into nanosized superstructures of various morphologies (Figure 2) adopting structures similar to those reported for low molecular weight surfactants.[18][20][21][22][37][40][41][42][43]

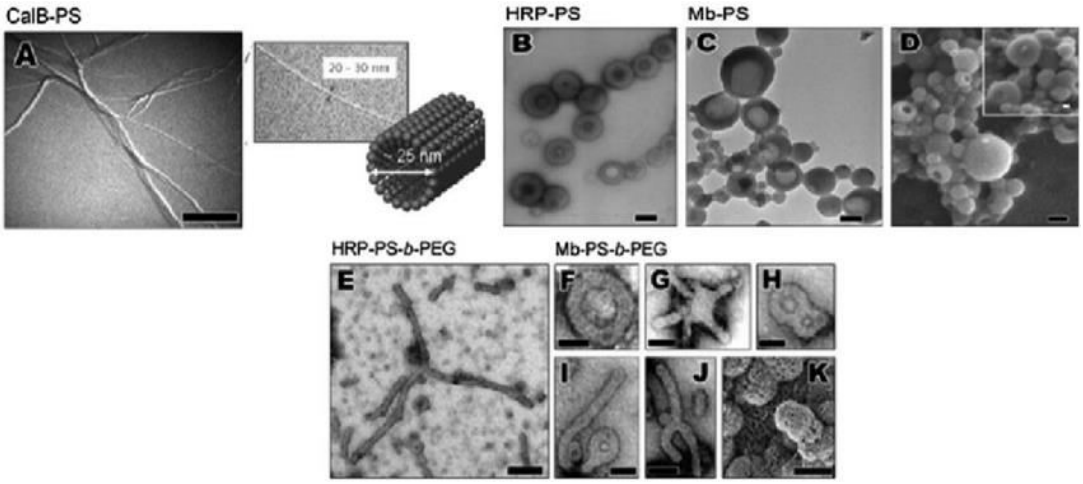


Figure 2: TEM and SEM micrographs of self-assembled protein-polymer Giant Amphiphiles in aqueous media. (a) Micellar rods formed by CALB-PS_{40}[18]) (b) Vesicles formed byHRP-PS_{90} (c, d) Spherical aggregates formed byMb-PS_{90} (e) Assemblies of HRP-PS_{144}-b-PEG_{113} (f-j).[23]

1.1.3 Self-assembly

As previously analyzed, amphiphilic molecules have an affinity for two different types of environments. Having this property, non-soluble parts of amphiphiles achieve through intermolecular interactions to reduce free energy and minimize interactions with the environment and thus self-organize into a preferred orientation.[24] This property of self-organizing is called self-assembling.[25][26][27][28] It usually takes place at interfaces and in solutions, in which by assembling amphiphiles change their interfacial properties and highly boost their compatibility. The first class of amphiphiles that comes up to everyone's mind are lipids and surfactants, which have the ability to self-assemble in aqueous media, water-oil solutions or at hydrophobic surfaces. Self-assembly is expressed through either the formation of

domains of hydrophobic groups (in contact with a nonpolar solvent or surface if it's applicable) or domains of hydrophilic groups (in contact with water, a polar solvent or a hydrophilic surface). In systems containing surfactant, water and oil segregation appears (Figure 3a,b). Surfactant films on the other handcan minimize their energy gap toform discrete aggregates, or can form ''infinite'' aggregates over macroscopic distances (Figure 3c,d). However, special properties arise from super amphiphiles molecules, such as block and graft copolymers involving thermoplastic elastomers, and include self-organization upon temperature stimulus. Amphiphilic molecules are known to form various self-assembled morphologies, most commonly monolayers, bilayers (Figure3a,b), micelles, reversed micelles, vesicles, rod- and sheet-like structures in which the hydrophilic part of the amphiphile lies on one side of asheet-like morphology and the hydrophobic part lies on the other side(Figure 4).[27][28]

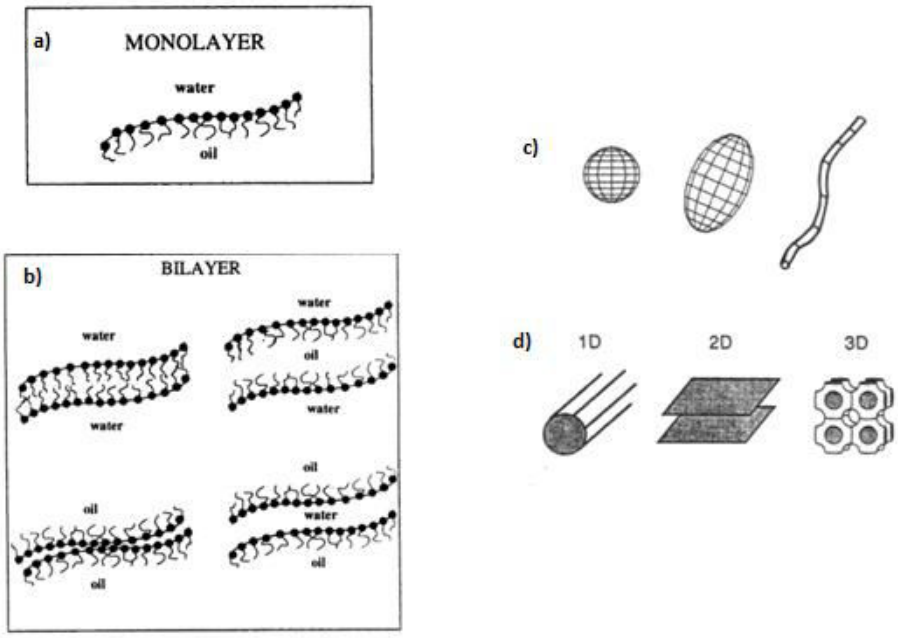


Figure 3 : A) Surfactant monolayer, B) Surfactant bilayers, C-D) Examples of c)discrete and d) continuous surfactant self-assemblies. The latter can extend to (d) 1) cylinders, 2) lamellae, 3) bicontinuous dimensions.[29]

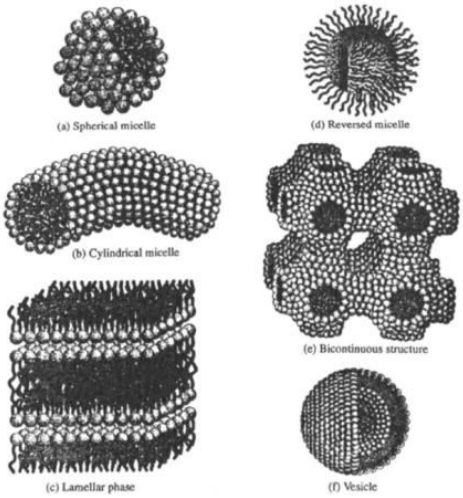


Figure 4:Spherical micelle with a hydrophobic interior and a hydrophilic head-group surface (depicted as spheres) facing water. (b) Cylindrical micelle with a hydrophobic interior and a hydrophilic polar surface facing water. (c) Lamellar phase consisting of surfactant bilayers. (d) Reverse micelle with a water core surrounded by the surfactant polar head-groups.The alkyl chains together with a non-polar solvent construct a continuous medium. (e) Bicontinuous structure with the surfactant molecules assembled into connected films. (f) Vesicle built from bilayers and characterized by two distinct aqueous domains, (core and external medium).[29]

1.1.4 SET-LRP vs ATRP

Single electron transfer living radical polymerization (SET-LRP),[30][31][32] involves a disproportionation reaction. More specifically, Cu(I) is unstable with respect to oxidation and rapidly undergoes disproportionation to Cu(0) and Cu(II) in aqueous and polar media, in order to form copper complexes of various oxidation states with N-ligands. This process interferes in activation and deactivation steps, in the same manner as in ATRP.[1][2][30] In both methods of polymerization, the equilibrium between the active (propagating macroradical $P_n^{\bullet}$) and inactive (halide-terminated propagating macroradical (P_n-X) is established through a rapid activation/deactivation process taking part in various oxidation states of a Cu catalyst (Figure 5). The most often used transition metal is Cu but other metals including Ru, Fe, Mo and Os, have been employed as well.[33] In order for ATRP to proceed, an activated monomer should be present. The activator in ATRP is usually $Cu^{I}X/L$ and the deactivator is $Cu^{II}X_2/L$, where X is O,Cl, Br, and L is an N-containing ligand. In the mechanism described below (Figure5), the activation step is thought to occur through homolytic atom transfer of X from the initiator (P–X) or the dormant polymer halide P_n-X to $Cu^{I}X/L$ via an reduction of the halogen radical to the halide anion generating the initiating radical $P^{\bullet}$ or the propagating macroradical$P_n^{\bullet}$ and oxidation of the catalyst to $Cu^{II}X_2/L$ via an inner sphere electron transfer process. Therefore, the newly created radicals can keep reacting with new monomers, growing the polymeric chains. As far as the deactivation process is concerned, it

involves the reverse procedure, as the halide anion goes back again to the propagating radical regenerating the dormant polymer chain and the reduced $Cu^{I}X/L$ catalyst.[30]

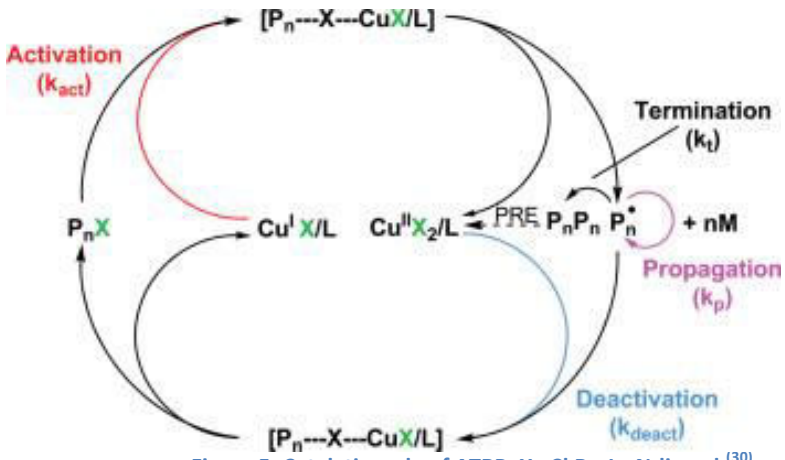


Figure 5: Catalytic cycle of ATRP, X= Cl,Br, L= N-ligand.[30]

The SET-LRP on the other hand, proceeds by both activated and unactivated olefins containing electron withdrawing substituents and where X is Cl, Br, I (Figure 6). The catalysts that take part in SET-LRP are electron-donors such as Cu^{0}, Cu_2O etc. The catalytic cycle for SET-LRP(Figure 6) achieves an equilibrium of $P_n^{\bullet}$ and P_n-X in a very different manner than that of ATRP. Separation of P-X and P_n-X is achieved through an outer sphere electron-transfer process during which Cu^{0} donates a single electron to P_n/P-X producing a radical-anion $[P_n/P\text{-}X]^{\bullet-}$,which degrades under an individual pathway to $P_n^{\bullet}$and X^{-}. Either during the SET procedure or afterwards, the Cu^{I} species becomes associated with the N-ligand and $Cu^{I}X/L$ rapidly disproportionates utilizing solvents such asH_2O, protic, polar aprotic and other polar solvents in the presence of N-containg ligands to regenerate Cu^{0} and generate $Cu^{II}X_2/L$, which is responsible for the outer sphere oxidation of $P/P_n^{\bullet}$ to P/P_n-X and thus deactivation.[30]

The rapid disproportionation of Cu^{I} to Cu^{0} and Cu^{II} occurs because the latter forms more stable complexes with the N-ligand (Me_6TREN) in water than Cu^{I} does (trigonal bipyramidal axial halide geometry with high-binding energies for $[Cu^{II}XMe_6TREN]^{+}$and trigonal pyramidal axial halide geometry with high-binding energies for $[Cu^{I}X\text{-}Me_6TREN]^{+}$.[14]

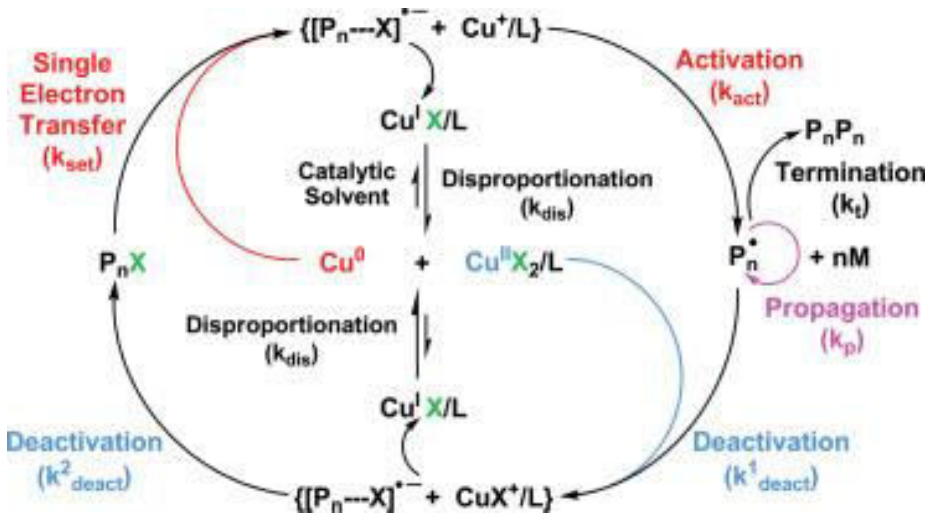
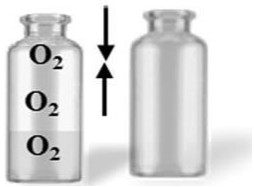


Figure 6: Catalytic cycle of SET-LRP[30]

1.1.5 Head Space Elimination

In order to overcome deoxygenation procedures like freeze-pump-thaw or nitrogen sparging that are often used in copper-mediated controlled radical polymerization processes, such as the classical ATRP, Prof. D. Haddleton and collaborators[14] have eliminated oxygen from the reactionby simply eliminating the headspace in the reaction vessel and achievedcopper-mediated polymerization without external deoxygenation or oxygen scavengers.[14] More specifically, they investigated oxygen consumption using an optical oxygen sensor in systems bearing no headspace, and demonstratedthat the initiator and copper complexes participate in a rapid oxygen consumption creating a ''self-degassing'' system. [14]



Head-space elimination concept -Scheme from E. Liarou.[14]

Purpose of the Thesis

The purpose of the thesis was to take advantage of the improved synthetic protocols offered by the aforementioned research involving copper-mediated polymerization without external deoxygenation or oxygen scavengers,[14] in order to synthesize protein-polymer bioconjugates.

Giant Amphiphiles represent a promising class of hybrid macromolecules.[18][20][22][21][37][40][41][42][43] The most successful to date synthetic protocol for the synthesis of such bioconjugates involved a classic ATRP grafting of monomers *from* protein macroinitiators.[37]Though quantitative, the classical ATRP protocol requires a stringent experimental protocol to ensure absence of oxygen from the reaction medium which requires special equipment, trained personnel and is time consuming. Our goal was to develop a simpler protocol. More specifically, goal of this thesis was to eliminate head-space in a study of a SET-LRP approach toward the synthesis of bioconjugates and optimize the synthetic protocol to afford well defined bioconjugates in high yields and without the need of external deoxygenation or oxygen scavengers (Figure 7).

From the synthetic point of view different monomers were employed in this thesis (Figure 7). In order to experimentally achieve head-space elimination, reactions were performed in:

a) glass-vials with fixed volume of 1.60 mL by adjusting the reaction scale to avoid head space

and

b) plastic syringes in which null head space was easily mechanically achieved.

The initial step of this study involved polymer synthesis, and more specifically poly-NIPAAm (poly-N-Isopropylacrylamide), in order to optimize the head-space elimination protocol and familiarize with the approach. On a subsequent step, several monomers, such as NIPAAm, styrene, methyl acrylate and methyl-methacrylate, were grafted from a Bovine Serum Albumin (BSA) biomacroinitiator. BSA was selected as it is commercially available and possesses one free thiol group at Cys34 residue which is ideal for maleimide coupling chemistry. In order to characterize the biomacromolecules Polyacrylamide Gel Electrophoresis (PAGE) and Gel Permeation Chromatography (GPC) were used. ^{1}H-NMR was employed to confirm the formation of polymers and *Giant Amphiphiles*.

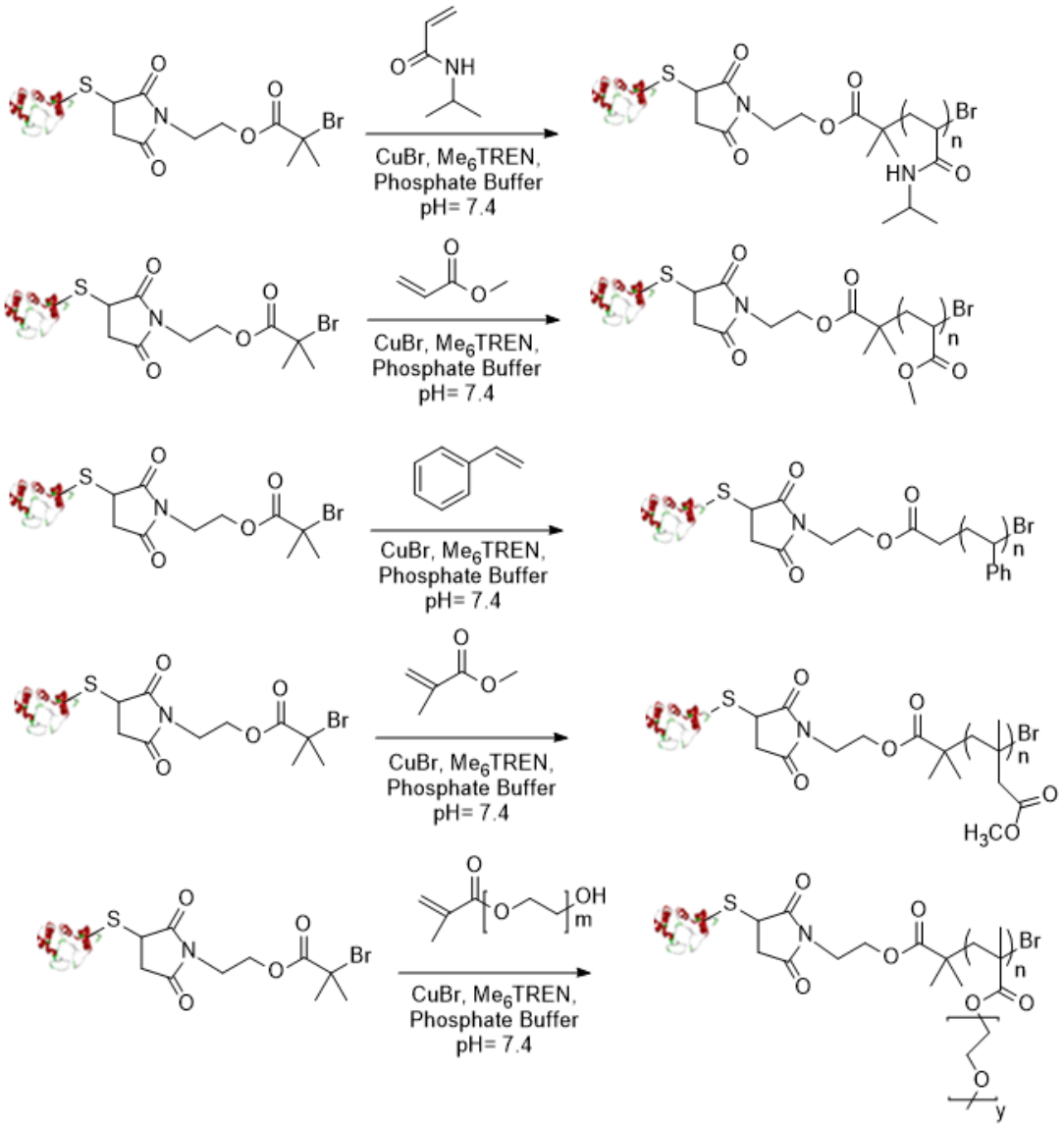


Figure7: Reactions studied in this thesis involved SET-LRP graftingof various monomers *from* a protein (BSA) macroinitiator.

2.0 Results and Discussion

Main purpose of the thesis was to graft a variety of monomers *from* a protein-macroinitiator and synthesize amphiphilic bioconjugates via the SET-LRP method and without the need of deoxygenation or nitrogen sparging, simply by elimination of head-space in the reaction vessels.

We initially studied ethyl α-bromoisobutyrate(EBiB) as initiator in order to optimize a non-deoxygenated reaction system. In this system several monomers, solventsand ligands were studied. BSA was added in the reaction mixtures in order to study its stability under the reaction conditions. Finally, a BSA-macroinitiator was used to optimize polymerization in terms of *Giant Amphiphile* synthesis.

2.1 SET-LRP of NIPAAm in the presence of BSA.

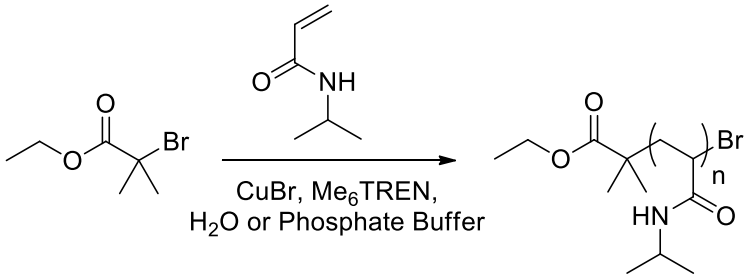


Figure8: SET-LRP polymerization of N-Isopropylacrylamide (NIPAAm) from EBiB.

Reagent Ratios	
NIPAAm	**40**
EBiB	**1**
CuBr	**0.3**
Me_6TREN	**1.25**
$V_{solvent}$(mL)	**1.60**

Table1. Reagent ratios and solvent volume used for the polymerization of NIPAAmfrom EBiB.

SET-LRP polymerization of NIPAAm using EBiB as initiator was initially studied (Figure 8, Table 1). The reaction involved the addition of either distilled H_2O or 20 mM phosphate buffer pH= 7.4 to dissolve the monomer/ligand. Two eppendorf tubes and aglass-vial with V_{max}= 1.60 mL were used. NIPAAm(40 equiv.) was dissolved with the solvent in an eppendorf tube. The initiator EBiB (1 equiv.) and BSA were dissolved in a second eppendorf tube. A 1.6 ml glass vial containing 0.3 equiv. CuBr was placed in ice, solvent and the ligand Me_6TREN (1.25 equiv.) were added, the vial was sealed and the disproportionation reaction was allowed to proceed for 2-3 min under rapid stirring. After a rapid colour change into the characteristic light blue colour of Cu(II) and the formation of solid Cu(0), the solutions pre-prepared in separate eppendorf tubes (namely the monomer and initiator/BSA solution) were

sequentially added to the reaction vial via a syringe. Special care was taken to remove air trapped within the syringe. The reaction mixture was stirred for one hour and formed a light blue solution where solid Cu(0) particles were clearly visible in the product mixture (Figure 9). As an initial test, the product solution was heated and transformed into a thick white opaque material, indicating the presence of poly-NIPAAm. poly-NIPAAm is a temperature responsive polymer and becomes hydrophobic above its LCST and reversibly hydrophilic below the LCST, this behavior was demonstrated by the reversible heating/cooling experiment (Figure 9).[32] [35][36]

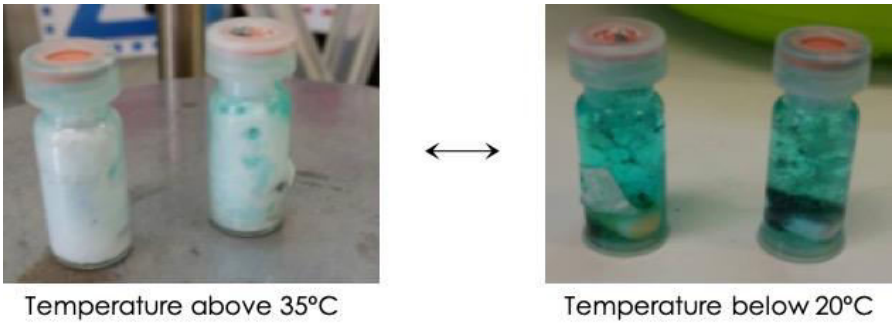
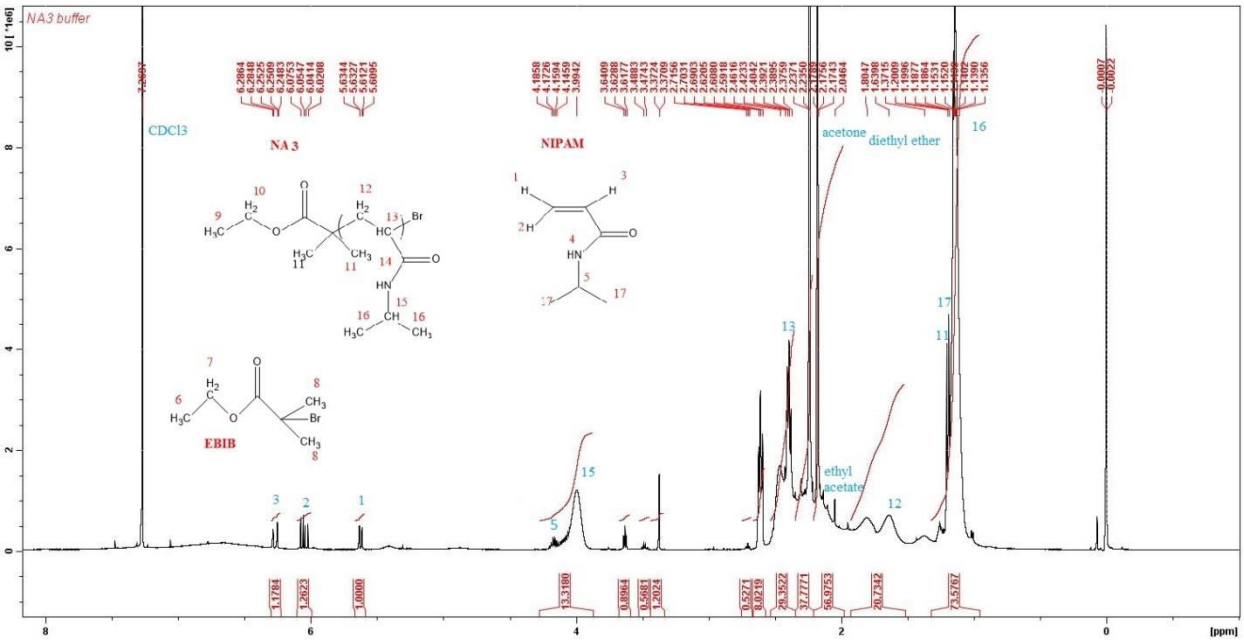


Figure9: Reversible response of the product mixture containing poly-NIPAAm



NMR Spectrum 1: First Reaction of EBiB with NIPAAM with Phosphate Buffer pH= 7.4 used as solvent.

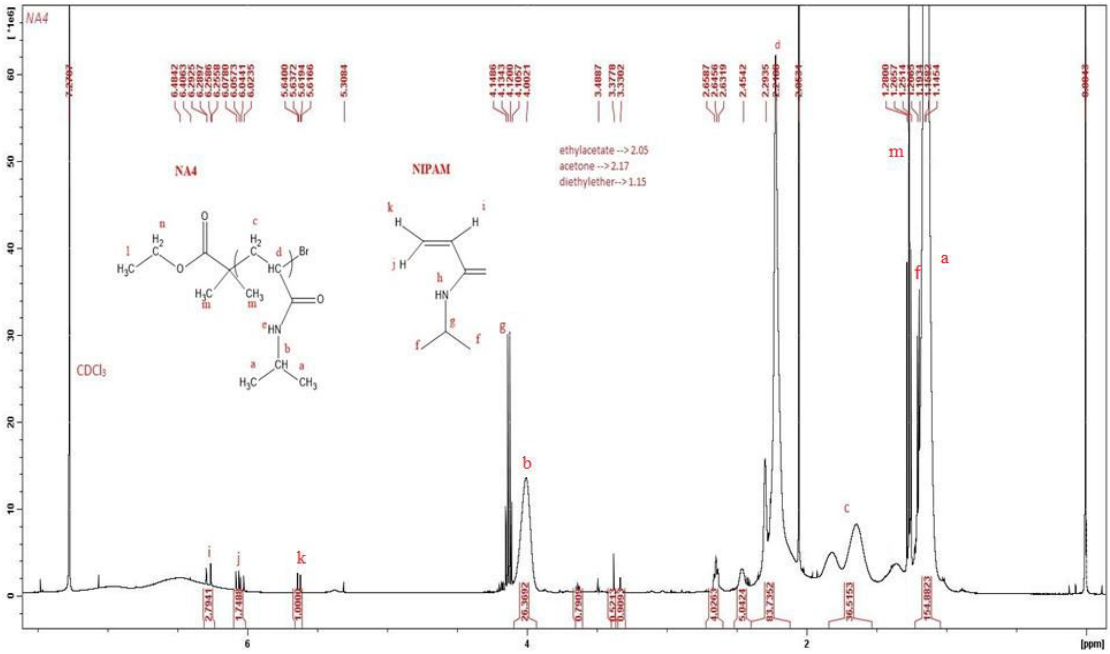
The solvent was removed and the product was characterized by ^{1}H-NMR spectroscopy. The ^{1}H-NMRspectrum revealed two chain peaks with chemical shifts characteristic for the formation of the polymer (NMR Spectrum 1). More specifically the -CH- and -CH_2-of the polymeric main chain at 1.64 and 2.37 ppm respectively and the -CH-and -CH_3 of the pending repeating unit at 3.99 and 1.15 ppm respectively.

Peaks with chemical shifts characteristic for the vinyl NIPAAm monomer(at 5.61, 6.04, and 6.28ppm for the double bond and 4.17 and 1.15ppm for the CH and CH_3), revealed that the reaction was not quantitative. It can be therefore concluded that, the polymerization was successful but not complete as the monomer had not been fully consumed.

	Ratios	
Reagents	**1st reaction**	**2nd reaction**
NIPAAm	40	40
BSA	0.004637	0.004668
EBib	1	1
Me_6TREN	1.25	1.4
CuBr	0.3	0.3
H_2O/Buffer	1.8 mL	1.6 mL

Table 2. Reagent ratios and solvent volume used for the polymerization of NIPAAmfrom EBiB.

In a second attempt to synthesize poly-NIPAAm following the same approach, the total reaction volume was set to match the vial volume at 1.60 mL in order to eliminate head-space (Table 2). The ratios of all reagents were kept constant with the exception of Me_6TREN which was used at a slightly increased ratio of 1.40 (from 1.25). The reaction was again performed using either distilled water or 20 mM phosphate buffer as medium to solubilize the reagents. However, the reaction in which NIPAAm was dissolved in phosphate buffer was not successful as during the injection of the initiator solution, the mixture rapidly thickened and turned solid and therefore stirring was not possible. This behavior was a matter of particular concern and it was probably attributed to a poly-NIPAAm solubility response at temperatures above its LCST (32 °C).



NMR Spectrum 2:SET-LRP polymerization of N-Isopropylacrylamide (NIPAAm) from EBiB (distilled water added).

The ^{1}H-NMR spectrum of the product of the second reaction (distilled water added), revealed peaks with chemical shifts characteristic for the vinyl NIPAAm monomer (at 5.62, 6.02, and 6.26ppm for the double bond and at 4.12 and 1.19 ppm for the alkyl and methyl (-CH- and -CH_3) protons respectively) indicating that the monomer had not been fully consumed. The product poly-NIPAAm was also observed via the characteristic chemical shifts.[35][36]As mentioned above, the -CH- and -CH_2- on the polymeric main chain were observed at 1.64 and 2.37 ppm respectively and the -CH- and -CH_3 of the pending repeating unit at 3.99 and 1.15 ppm.

2.2 Synthesis of poly-methyl-acrylate in the presence of BSA.

Following the synthetic approach used for poly-NIPAAm, the synthesis of poly-methacrylate was also attempted. Alterations on the protocol involved changing disproportionation reagent ratios into In:Cu(I):Me_6TREN 1:0.4:0.4 according to a paper published by the group of Haddleton concerning aqueous copper-mediated living polymerization. [14][15] The solvent added was nanopure water, as there was no noteworthy difference between water and phosphate buffer in the previous reactions. Moreover, the use of a syringe exit-needle during the disproportionation process was introducedin order to allow gases to exhaust. The reaction was stirred fast during disproportionation and left to gently stir for one hour upon the addition of the monomer/ligand and initiator solutions. The resulting polymer was purified from the unreacted monomer and copper salts with repeated precipitations from dichloromethane.

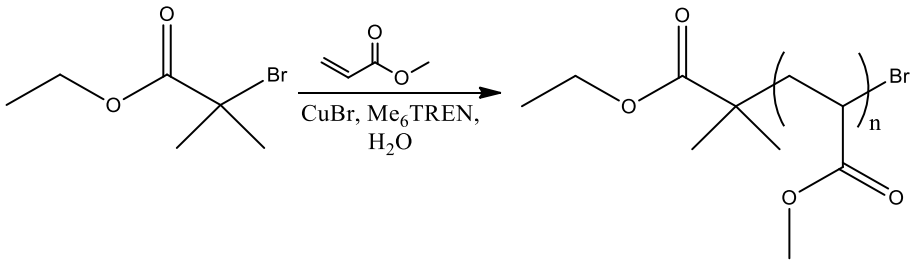
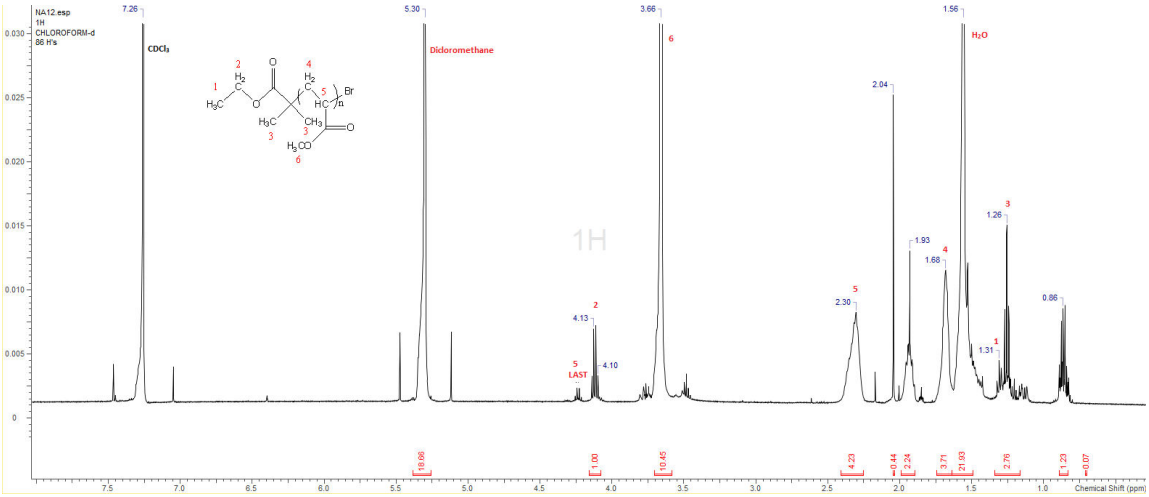


Figure10. SET-LRP polymerization of methyl-acrylate from EBiB.

Reagents	Ratios
Methyl acrylate	40
BSA	0.004637
EBib	1
Me_6TREN	0.4
CuBr	0.4
H_2O	1.32 mL

Table 3. Reagent ratios and solvent volume used for the SET-LRP of methyl-acrylate.

The product was characterized with ^{1}H-NMR spectroscopy(NMR Spectrum 3). The absence of peaks corresponding to the monomer was encouraging. The polymer was identified through the characteristic chemical shifts of its protons at 1.24 ppm(methyl),1.68 ppm (CH_2 main chain),2.30 ppm (CH main chain), 3.66 ppm (ester methyl group),4.13 ppm (CH_2 end group), 4.24 ppm (CH_3 end group).[39]



NMR Spectrum 3: SET-LRP polymerization of methyl-acrylate from EBiB in distilled water.

Since SET-LRP polymerization was successful for both monomers and well-studied by the Haddleton group, we decided to focus on the goal of this thesis, *i.e.* grafting monomers from a protein macroinitiator and optimizing this new reaction. We used BSA functionalized with an LRP initiator (BSA-In) to study this reaction since the BSA-In (shown in Figure 11) it is a well-studied initiator for ATRP polymerizations.[37] In order to achieve efficient grafting *from* BSA, the reaction conditions were altered according to previous experience of the laboratory with classical ATRP.[37]

2.3 Grafting of NIPAAm from BSA-macroinitiator: SET-LRP synthesis of BSA-poly-NIPAAm

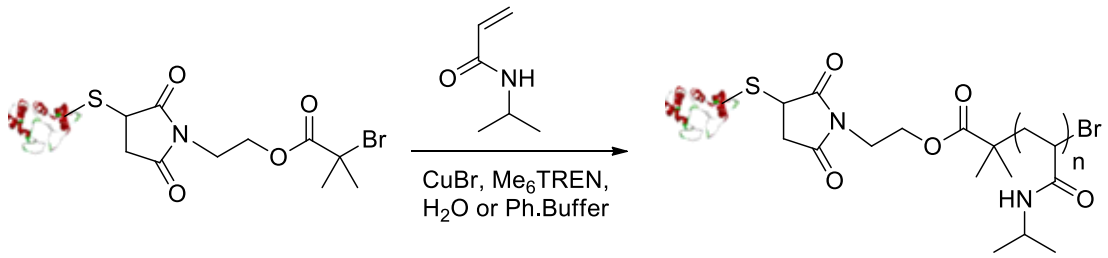


Figure11:SET-LRP polymerization of NIPAAm from BSA-macroinitiator.

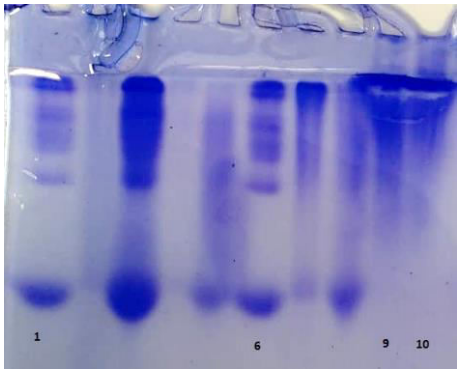
Reagents	Ratios
NIPAAm	400
BSA-In	1
Me_6TREN	60
CuBr	60
H_2O/Buffer	0.23 mL

Table 4. Reagent ratios and solvent volume used for the polymerization of NIPAAm from BSA-In.

As mentioned above, the main purpose of this thesis was to graft polymers from a protein-macroinitiator in order to synthesize amphiphilic bioconjugates via SET-LRP and without the need for deoxygenation. To achieve this, NIPAAm (400 equiv.) and BSA-macroinitiator (1.40 mL of a 0.35mM solution in phosphate buffer, 1 equiv.) were placed in an eppendorf tube. For the disproportionation, CuBr (60 equiv.) was dissolved in the solvent and the ligand Me_6TREN (60 equiv.) was added. Both water and20 mM phosphate buffer pH=7.4,were tested as solvents. The total volume of the aqueous solvent was decreased to 0.23 mL.

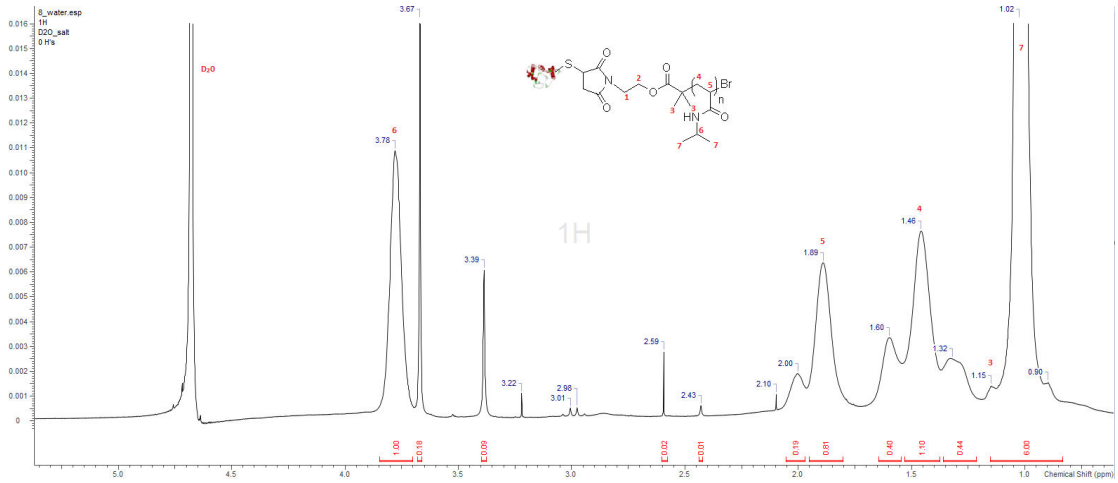
The polymerization was as previously performed, with fast stirring for 2-3 minutes during disproportionation (in the presence of a syringe-exit needle at the top of the vial). The solution containing the monomer and the initiator was subsequently added and the reaction was allowed to stir for one hour.

For the purification of the bioconjugate, the reaction mixture was extensively dialyzed against 5mM and 20 mM phosphate buffer, pH7.4 containing ~2 mM EDTA in order to complex Cu(II), using regenerated cellulose dialysis membranes with MWCO 10 kDa. The enriched product was characterized with PAGE electrophoresis (Lanes 9 and 10, Electrophoresis 1). The observation of a new dark blue band in the stacking gel well migrating slowly throughout the stacking gel and the frontline of the resolving gel, indicated the formation of the product.[37] More importantly, the complete absence of macroinitiator on the product lane indicated a quantitative reaction (in respect to BSA-In).GPC chromatography was not used, BSA-pNIPAAm was know to adsorb on the column from previous experiments.



Electrophoresis 1: Electrophoretic behavior of BSA-poly(NIPAAm) synthesized in two independent reactions: Lane 1- Native BSA, Lane 6- Native BSA, Lane 9-BSA-poly(NIPAAm)(water added), Lane 10-BSA-poly(NIPAAm)(phosphate buffer added).

A significant result was obtained via NMR spectroscopy (NMR Spectrum4) A small percentage of deuterated water was added in the reaction mixture and the sample was measured without further purification. The formation of the biopolymer was verified through the peaks with characteristic chemical shifts (at 1.02, 1.46, 1.89 and 3.78 ppm) in the ^{1}H-NMR spectrum of the biopolymer. It has to be noted that NMR spectroscopy provides new means for protein-polymer bioconjugate characterization.



NMR Spectrum4: BSA-pNiPAAm in H_2O/D_2O.

2.4 Grafting of poly-NIPAAm from a protected maleimide initiator via SET-LRP

In an effort to optimize the reaction conditions and minimize at the same time oxygen concentration in the reaction vessel, the synthetic approach was significantly altered. At the same time, the reagent ratios in the disproportionation process were also altered. Instead of using a vial as reaction vessel, a plastic syringe was used (Figure 12). This served two different purposes:

a) it increased the compatibility of the vessel with the biomolecule and, at the same time

b) allowed to eliminate head-space in the reaction vessel.

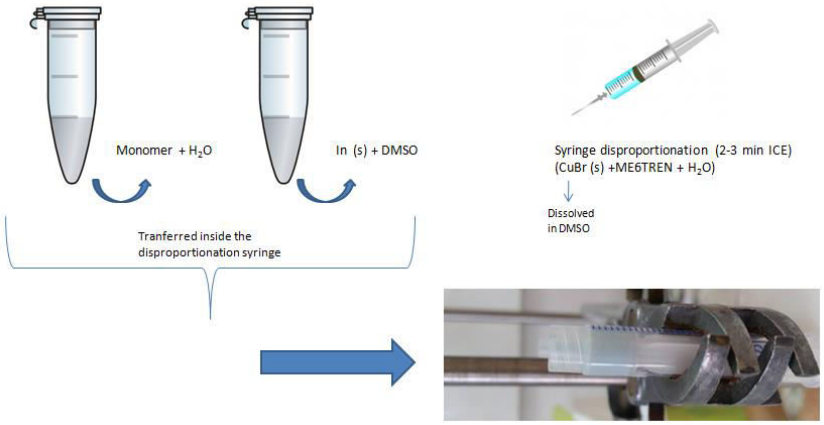


Figure12. Schematic representation of the polymerization protocol involving a syringe as the reaction vessel.

The first polymerization performed using this altered reaction protocol targeted the synthesis of poly-NIPAAm(50 equiv.) from a protected maleimide initiator (1, Figure 13). In this protocol, the solvent volume was decreased to 0.5 mL (Table 5).

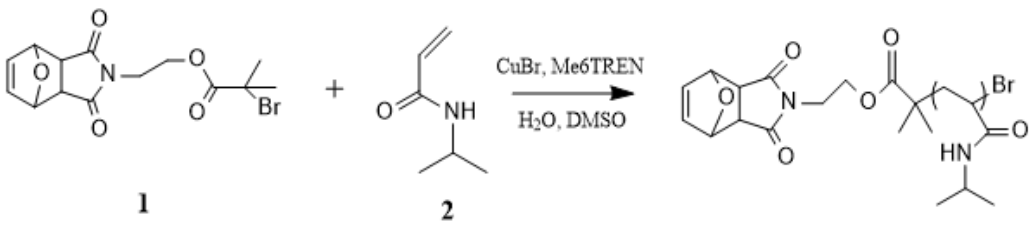


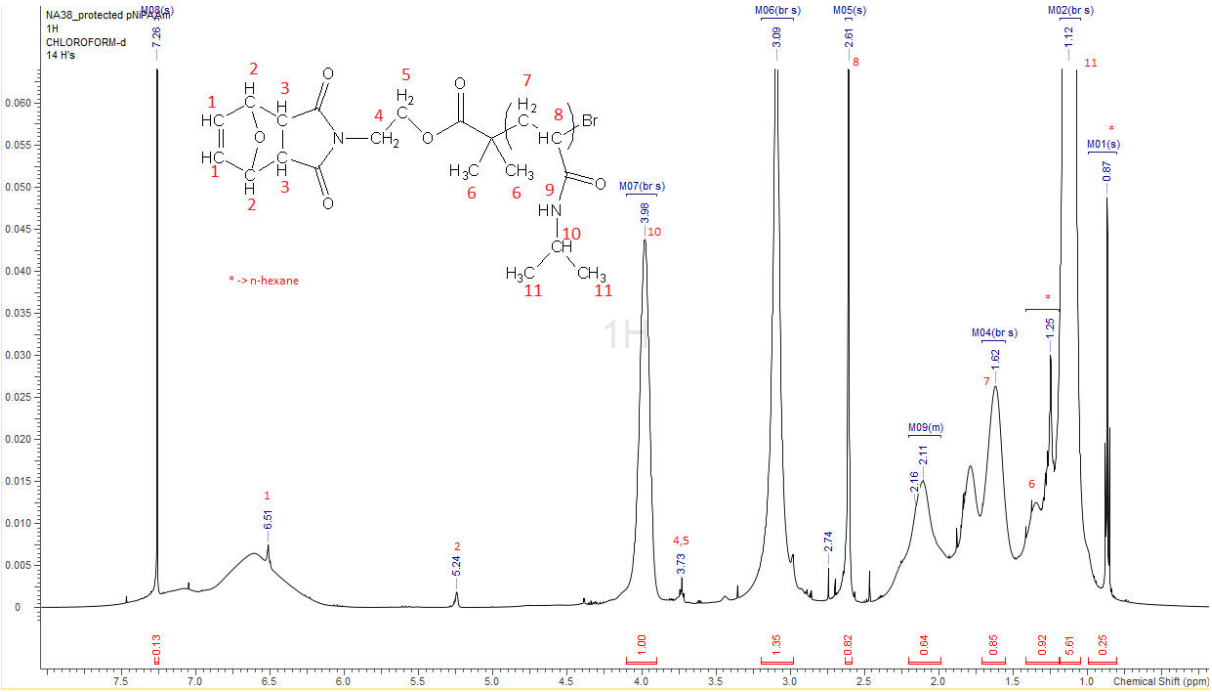
Figure13. SET-LRP polymerization of NIPAAm from protected maleimide LRP initiator (1).

Reagents	Ratios
NIPAAm	50
In	1
Me_6TREN	0.4
CuBr	0.4
H_2O	0.5 mL

Table 5. Reagent ratios and solvent volume used for the polymerization of NIPAAm from the protected LRP initiator 1.

The reaction was left to proceed overnight and the resulting polymer was purified from unreacted monomer and copper salts by repeated dissolution in THF and precipitation from hexane.[38]

After purification, the product was analyzed with ^{1}H-NMR spectroscopy (NMRSpectrum 5).The expected peaks with characteristic chemical shifts for poly-NIPAAm were observed on the spectrum and more specifically: the -CH- and -CH_2-of the polymeric main chain at 1.64 and 2.37 ppm respectively and the -CH- and -CH_3 of the pending repeating unit at 3.99 and 1.15 ppm respectively. Interestingly, the protected end-group protons at5.24 ppm for the double bond and 6.51 ppm for the bridge ether protons) verified the success of the reaction and allowed for a rough calculation of the molecular weight of the polymer.



NMRSpectrum 5: SET-LRP polymerization of NIPAAm fromthe protected maleimideLRP initiator 1.

2.5 Grafting of methacrylate*from* BSA-macroinitiator via SET-LRP:Synthesis of BSA-poly-methylacrylate

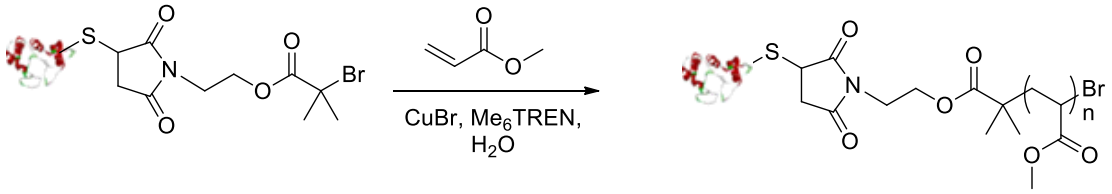


Figure 14.SET-LRP polymerization of methacrylate from BSA-macroinitiator.

Reagents	Ratios
NIPAAm	4000
BSA-In	1
Me_6TREN	40
CuBr	40
H_2O	0.23 mL

Table 6. Reagent ratios and solvent volume used for the polymerization of methacrylate from BSA-In.

Given the successful results of the SET-LRP grafting approach used for BSA-polyNIPAAm, it was imperative to expand this approach to other monomers such as methyl-acrylate. For this reaction, MA (4000 equiv.) and the BSA-macroinitiator (1.40 mL, 0.35 mM, 1 equiv.) were initially place in an eppendorf tube. CuBr (40 equiv.) was added in a vial equipped with an exit-needle and dissolved with water. The ligand Me_6TREN (40 equiv.) was addedto this mixture to initiate disproportionation held at 0 ºC for 2-3 minutes under rapid stirring. The volume of the solvent was adjusted at 0.23 mL in order to avoid headspace in the reaction vial.

For the purification of the bioconjugate, the mixture was extensively dialyzed against 20 mM phosphate buffer pH7.4, 2mM EDTA (to complex Cu(II)) and5mM phosphate buffer, pH7.4), using regenerated cellulose dialysis membranes with MWCO 10 kDa. After purification, the product was characterized with PAGE (2). However, no new band could be observed for the product. The disappearance of the band corresponding to the biomacroinitiator in the lane of the product mixture was nevertheless promising for the formation of a new product.

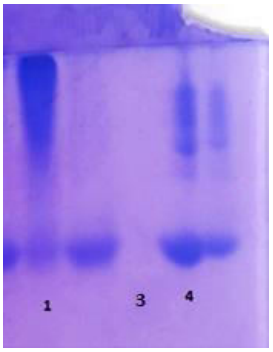
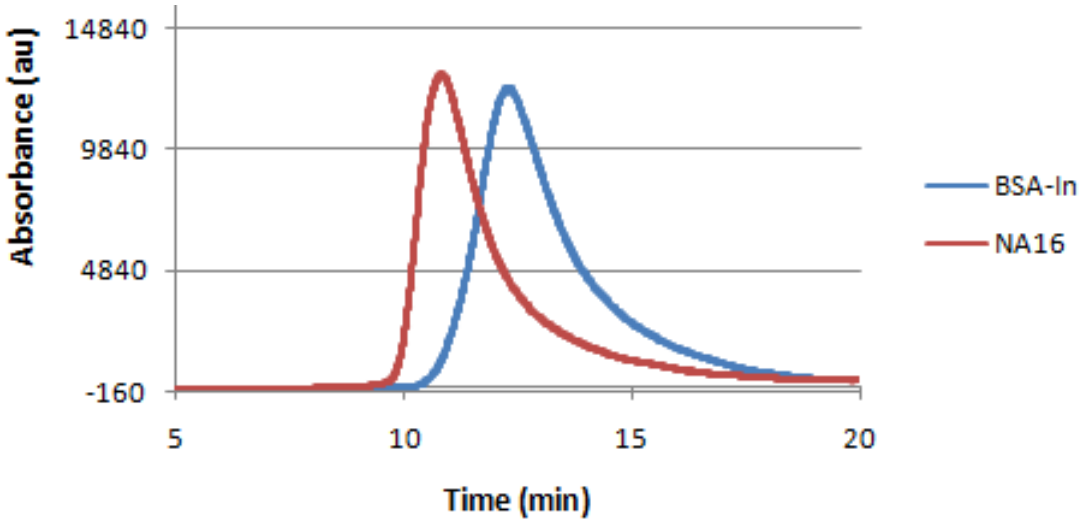


Figure15: Electrophoretic behavior of BSA-poly(MA) ,Lane 3 BSA-poly(MA), Lane 4- Native BSA.

Next step in the effort to characterize the product was a study with GPC chromatography.(GPC Chromatogram 1), which verified the formation of a new bioconjugate with faster elution time than that of the biomacroinitiator.[37] GPC also verified the absence of the macroinitiator form the reaction product mixture.



GPC Chromatogram 1: Chromatogram of BSA-macroinitiatorandBSA-poly-MA (measured at 254 nm).

2.6 Grafting of styrene *from* BSA-macroinitiator via SET-LRP: Synthesis of BSA-polystyrene

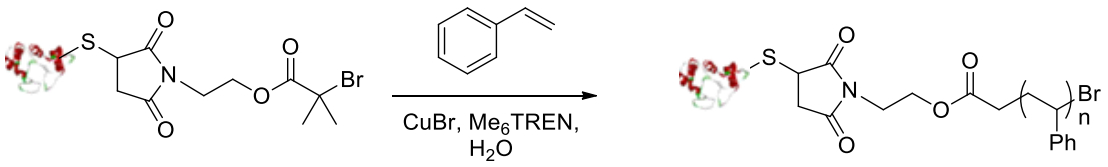


Figure 16: SET-LRP polymerization of styrene from BSA-macroinitiator.

Reagents	Ratios
Styrene	400
BSA-In	1
Me_6TREN	60
CuBr	60
H_2O	0.23 mL

Table 7. Reagent ratios and solvent volume used for the polymerization of styrene from BSA-In.

Since the synthesis of BSA-poly-NIPAAm and BSA-poly-methylacrylate was successful, next step was studying styrene as monomer and optimizing the reaction. As a first step the glass-vial reaction was tested against-the more efficient- plastic syringe reaction. More specifically for the "vial reaction", styrene (400 equiv.) and the BSA-macroinitiator (1.20 mL, 1 equiv.) were placed in an eppendorf tube. CuBr (60 equiv.) were added in the glass vial, dissolved with H_2O and then the ligand Me_6TREN (60 equiv.) was added. Fast stirring was maintained during disproportionation and a syringe exit-needle was used in the sealed vial. After the disproportionation the contents of the eppendorf solutions (monomer and macroinitiator) were sequentially added to the vial. The reaction was left under gentle stirring for approximately one hour. The purification of the bioconjugate was performed via dialysis (using the conditions used for all previous reactions) and the product was characterized with PAGE (Figure 17)

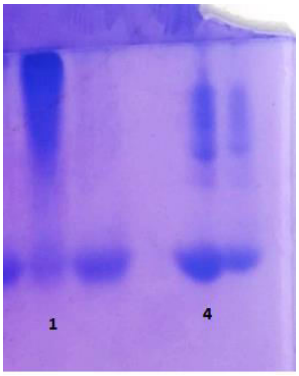
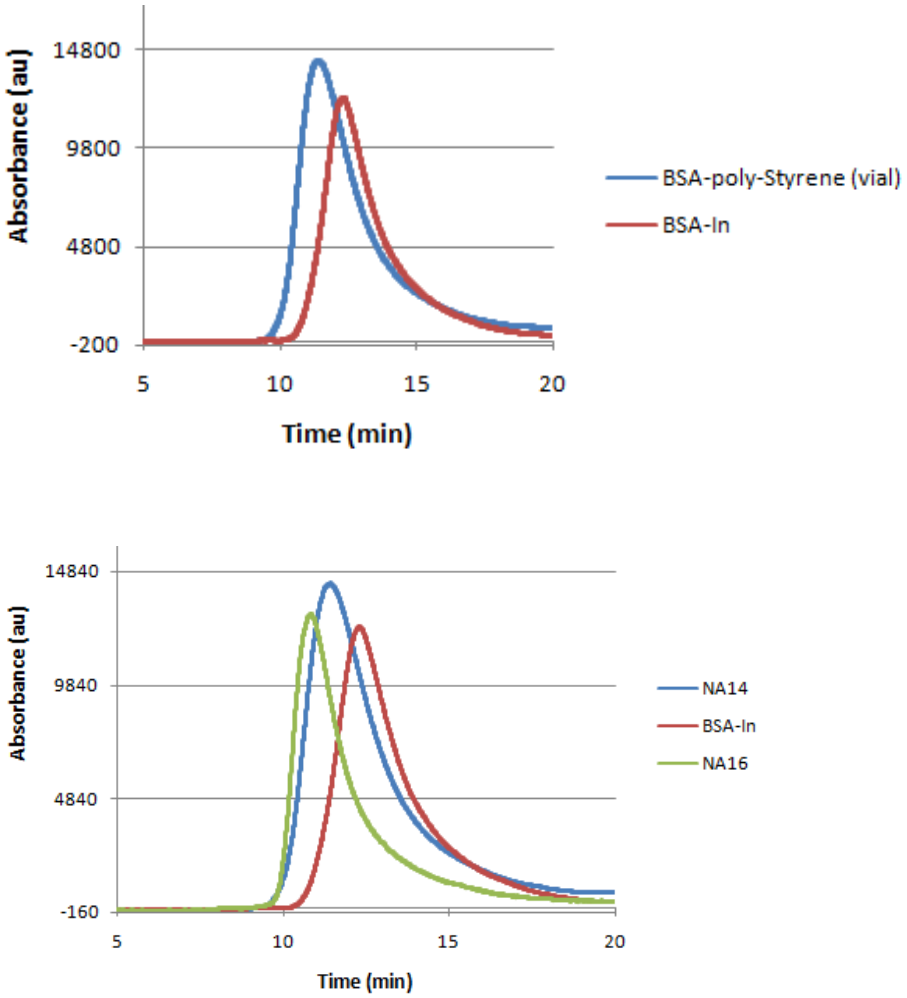


Figure 17: Electrophoretic behavior of BSA-polystyrene, Lane 1: BSA-polystyrene, Lane 4: BSA macroinitiator

The observation of a new dark blue band in the stacking gel well migrating slowly throughout the stacking gel and the frontline of the resolving gel, indicated the formation of a new product. Nevertheless,the presence of a band corresponding to the BSA-macroinitiator, revealed a non-quantitative polymerization. The formation of the bioconjugate was further supported by GPC (GPC Chromatograph, Top). More specifically, the GPC chromatogram of the product was wide, bimodal and partially overlapped with the peak of the macroinititator, which becomes more clear when comparing with a chromatogram of a quantitative reaction yielding BSA-polyMA (GPC Chromatogram 2, Bottom, vide supra).[37]



GPC Chromatogram 2:*Top*) Chromatographic behaviour of BSA-macroinitiator (red trace) and BSA-polystyrene for the "vial reaction" (NA14, blue trace), *Bottom*) Chromatographic behavior of BSA-macroinitiator, BSA-polystyrene "vial reaction" (NA14, blue trace)and BSA-polyMA "vial reaction" (NA16, green trace).

Taking into account this outcome, evaluation of the SET LRP reaction performed in a syringe seemed reasonable and having several benefits. First of all, the use of a plastic syringe was judged to be more compatible to protein/enzymes and also was not need to use larger quantities of reagents (such as the BSA-macroinitiator) in order to eliminate the head space in the reaction vessel. Moreover, this approach would allow monitoring the progress of the polymerization via withdrawing aliquots at selected time intervals.

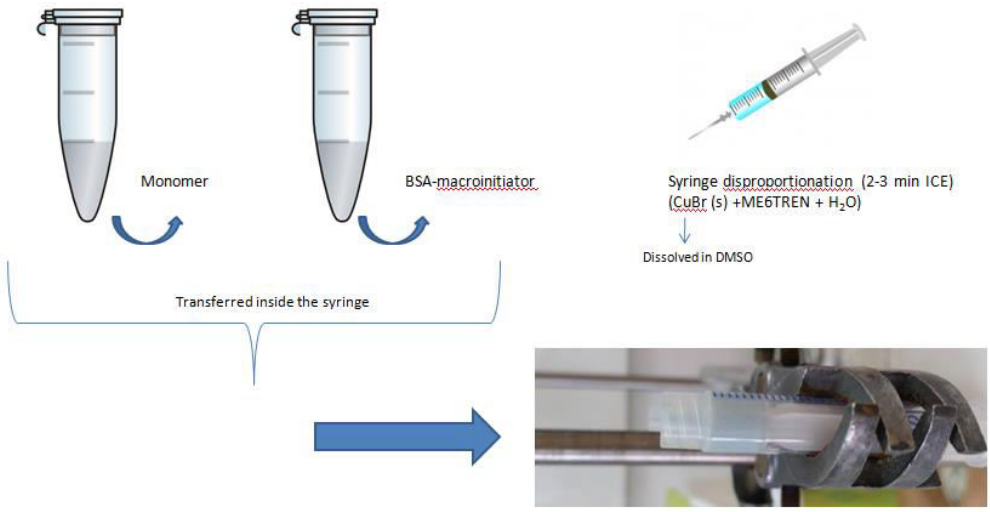


Figure18. Schematic representation of the set-up and synthetic protocol of the SET LRP polymerization performed in a syringe to eliminate head-space.

Reagents	Ratios
Styrene	4000
BSA-In	1
Me_6TREN	40
CuBr	40
H_2O	0.37 mL

Table 8. Reagent ratios and solvent volume used for the SET-LRP polymerization of styrene from BSA-macroinitiator

For the polymerization performed in a syringe, styrene (4000 equiv.) and the BSA-maleimide initiator (0.42 mL, 1 equiv.) were placed into two separate eppendorf tubes. A magnet was placed in the syringe and the ligand Me_6TREN (40equiv.) dissolved in water was added followed by the addition of CuBr (40 equiv.) dissolved with a minute amount of DMSO. The disproportionation reaction was allowed to proceed at 0 °C for approximately 3 min with intense stirring. Finally, the monomer and BSA-macroinitiator solutions were added, air was easily removed to eliminate head space and the reaction was allowed to proceed for approximately 2h under gentle stirring (ca.200rpm).

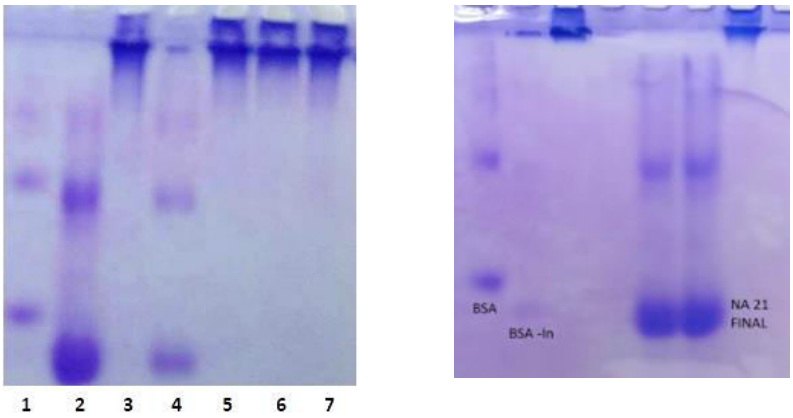
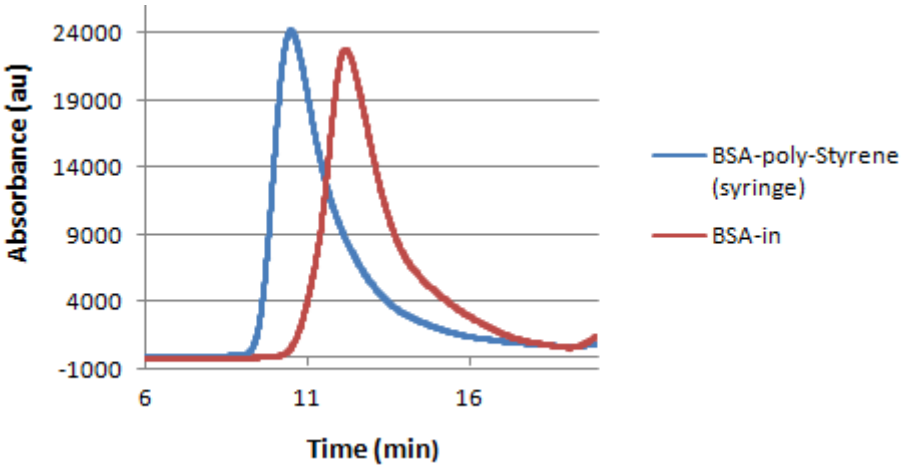


Figure 19: *Left*) Electrophoretic behavior of reaction mixture aliquots collected during the SET-LRP formation of BSA-polystyrene (NA21) performed in a syringe. Lane 1: Native BSA, Lane 3: NA21 20 min, Lane 4:BSA-macroinitiator, Lane 5: NA21 40 min, Lane 6: NA21 60 min, Lane7: NA21 90 min *Right*) Electrophoretic behavior of enriched through dialysis BSA-polystyrene (NA21 final product, 2h). All reaction samples were measured without purification.

Aliquots from the reaction mixture were withdrawn after 20,40, 60 and 90 minutes. Enrichment of the final bioconjugate was performed with extensive dialysis under the conditions mentioned above. After purification, the product was characterized with PAGE (Figure 19). Interestingly, the new dark blue bands in the stacking gel well migrating also at frontline of the resolving gel which were observed at the sample collected after 20 minutes reaction time (Figure 19 Left, lane 3), indicated the formation of a new product. The absence of unreacted BSA-macroinitiator (Figure 19 Left, lane 4), indicated a fast and quantitative reaction.



GPC Chromatogram 3:Chromatographic behaviour of BSA-macroinitiator (red trace) and BSA-polystyrene for the "syringe reaction" (NA21, blue trace).

This was further supported by GPC (GPC Chromatogram 3) which revealed the formation of a new product with elution faster than the elution of the biomacroinitiator. After dialyzing the reaction product against nanopore water, its morphology in water was imaged by Transmission Electron Microscopy(TEM). The TEM micrographs revealed the formation of rather uniform spherical nanostructures with diameters ~30-50 nm which were in agreement with previous studies on BSA-polystyrene self-assembled nanostructures (Figure 20).[37]

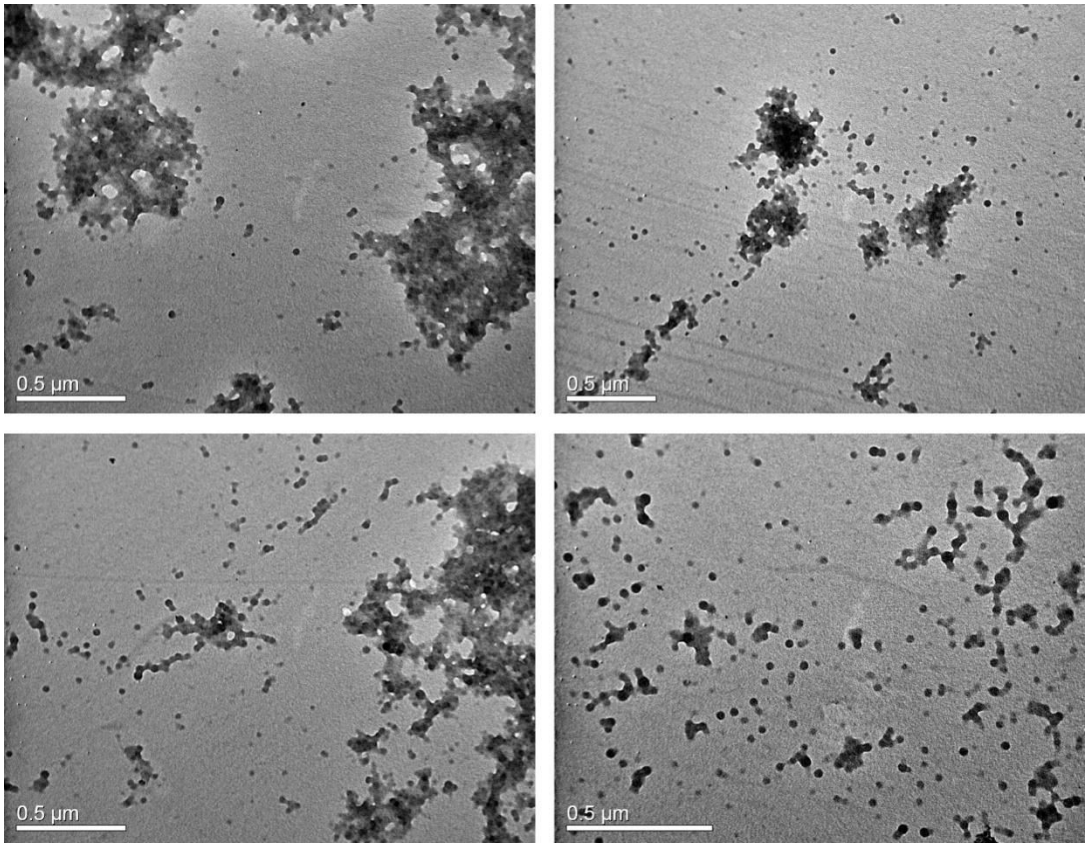


Figure20: TEM micrographs of BSA-polystyrene (NA21).

Since the electrophoresis showed a fast, quantitative formation of the BSA-polystyrene *Giant Amphiphiles* in just 20 minutes, the reaction was repeated and aliquots were collected at shorter time intervals and more specifically after 2,5,10,15,20,40,60,90 min and 2h which was the final aliquot.

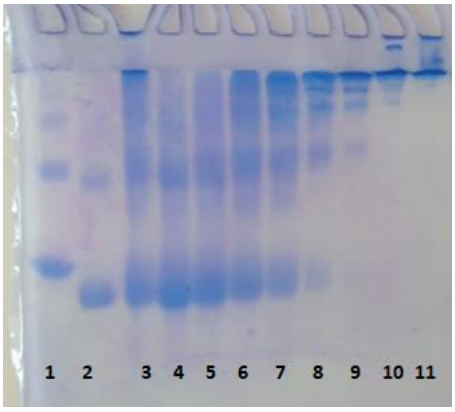


Figure 21:Electrophoretic behavior of reaction mixture aliquots collected during the SET-LRP formation of BSA-polystyrene (NA23, "syringe"-reaction). Lane 1: Native BSA,Lane 2: BSA-macroinitiator,Lane 3: NA23 at 2min, Lane 4: NA23 at 5min,Lane 5: NA23 at 10min, Lane 6: NA23 at 15min, Lane7: NA23 at 20min, Lane 8: NA23 at 40min, Lane 9: NA23 at 1h , Lane 10: NA23 at 90min, Lane 11 final product (NA23, 2h). All reaction samples were measured without purification.

In Figure 21 the course of the SET-LRP polymerization of styrene from BSA-macroinitiator is followed in time. A new band indicating the formation of the amphiphilic bioconjugate is observed during the first few minutes while, the band corresponding to the BSA-macroinitiator disappears after approximately one hour, verifying the quantitative formation of product.[37]

A concerted attempt to optimize the SET-LRP polymerization of styrene from BSA-macroinitiator followed. More specifically, decreasing amounts of Cu(I) and Me_6TREN were used(Tables in Figure 22) and the course of the reactions was followed by withdrawing aliquots of the reaction mixture at fixed amounts of time. However, as observed from PAGE electrophoresis(Figure 22), unreacted BSA-macroinitiator was observed in all fractions proving that ligand and Cu(I) could notbe further decreased than 40 equiv..

Bioconiugates	BSA-Initiator	Ligand	Mon	Cu(I)	EI	V_{H2O}	V_{BSA-In}	V (DMSO) total
NA24 (BSA-poly-Styrene)	1	4	4000	4	×	0.55 ml	0.625 ml	20 µl
NA29 (BSA-poly-Styrene)	1	20	4000	20	×	0.37 ml	0.42 ml	20 µl

Bands	Aliquots
1	NA24 (2')
2	NA24 (5')
3	NA 24 (10')
4	NA 24 (15')
5	NA 24 (20')
6	NA 24 (40')
7	NA 24 (1h)
8	NA 24 (90')
9	NA24 (2h)
10	NA 24 (150')
11	BSA
12	BSA-In
13	NA24 FINAL (3h)
14	-

Bands	Aliquots
1	NA29 (30')
2	NA29 (1h)
3	NA 29 (90')
4	NA 29 (2h)
5	NA 29 (2 ½ h)
6	NA 30
7	BSA

Figure 22: *Left*) Electrophoretic behavior of reaction mixture aliquots collected during the SET-LRP formation of BSA-polystyrene using In:Ligand:Cu(I), 1:4:4 ratios. Lane 1: NA24 2min,Lane 2: NA24 5min, Lane 3: NA24 10min, Lane 4: NA24 15min, Lane 5: NA24 20min, Lane 6: NA24 40min, Lane7: NA24 60min, Lane 8: NA24 90min, Lane 9: NA24 120 min,Lane 10: NA24 150min, Lane 11: Native BSA, Lane 12: BSA-macroinitiator, Lane 13 NA24 final product at 3h.*Right*) Electrophoretic behavior of reaction mixture aliquots collected during the SET-LRP formation of BSA-polystyrene using In:Ligand:Cu(I) 1:20:20). Lane 1:NA29 30min,Lane 2:NA29 60min, Lane 3: NA29 90min, Lane 4: NA29 120min, Lane 5: NA29 150min, NA30 purified product (vide infra),Lane7: Native BSA. All reaction samples were measured without purification, unless otherwise stated.

2.7 Grafting of methyl methacrylate *from* BSA-macroinitiator via SET-LRP: Synthesis of BSA-poly-methylmethacrylate

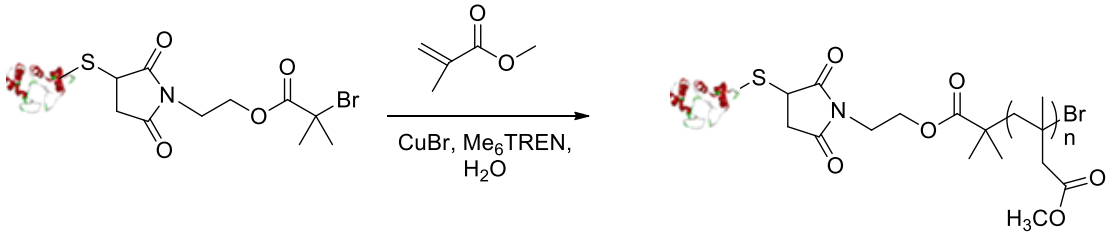


Figure 23: SET-LRP polymerization of methyl methacrylate from BSA-macroinitiator.

Reagents	Ratios
Methylmethacrylate	4000
BSA-In	1
Me_6TREN	40
CuBr	40
H_2O	0.37 mL

Table 9. Reagent ratios and solvent volume used for the polymerization of methyl methacrylate from BSA-In.

Methylmethacrylate (4000 equiv.) and the BSA-maleimide initiator (0.42 mL, 1 equiv.) were initially placed into two separate eppendorf tubes. CuBr (40 equiv.) dissolved with DMSO was added in the syringe followed by the addition the ligand Me_6TREN (40equiv.) in water. The volume of DMSO was minute. The disproportionation reaction was allowed to proceed at 0 °C for approximately 3 min with intense stirring. Finally, the monomer and BSA-macroinitiator solutions were added, air was easily removed to eliminate head space and the reaction was allowed to proceed for approximately 2h under gentle stirring (~200rpm).For the purification of the bioconjugate, the mixture was extensively dialyzed under the conditions previously described for all bioconjugates.

After purification, the product was characterized with by PAGE (Figure 22*Right*, Lane 6).A new band was observed, however, the presence of the band corresponding to the BSA-macroinitiator indicated the partial formation of the bioconjugate. This is an encouraging outcome which dictated optimization studies in the future.

2.8 Grafting of PEGMA *from* BSA-macroinitiator via SET-LRP: Synthesis of BSA-poly-PEGMA.

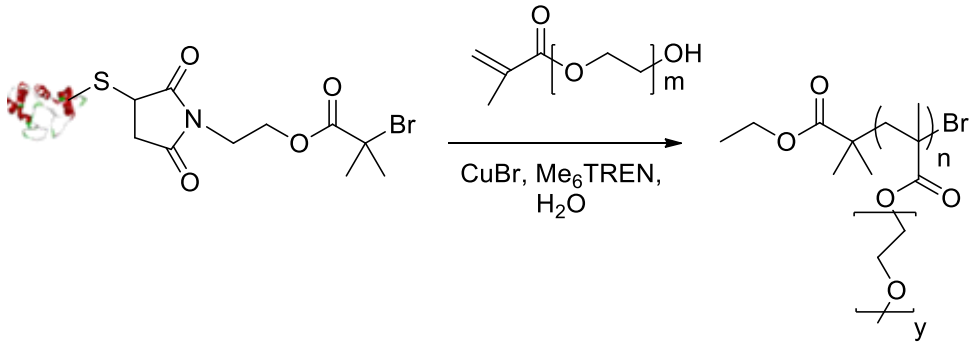


Figure 24:SET-LRP polymerization of PEGMA from BSA-macroinitiator.

Reagents	Ratios
PEGMA	400
BSA-In	1
Me_6TREN	40
CuBr	40
H_2O/Buffer	0.37 mL

Table 10. Reagent ratios and solvent volume used for the polymerization of PEGMA from BSA-In.

With the aim to expand the monomer pool of the SET-LRP approach under non-degassing conditions PEGMA was selected as a hydrophilic monomer. Due to the nature of this monomer (hydrophilic) and its intrinsic characteristics (high molecular weight, polymeric in nature, high viscosity), and based on previous experience in the laboratory the conditions selected were slightly altered to involve lower monomer volumes. More specifically, PEGMA (400 equiv.) and the BSA-maleimide initiator (0.42 mL, 1 equiv.) aqueous solutions were initially placed into two separate eppendorf tubes. A magnet, CuBr (40 equiv.) dissolved with DMSO and the ligand Me_6TREN (40equiv.) in water were added in the syringe. The volume of DMSO was minute.The disproportionation reaction was allowed to proceed at 0 °C for approximately 3 min with intense stirring. Finally, the monomer and BSA-macroinitiator solutions were added, air was easily removed to eliminate head space and the reaction was allowed to proceed for approximately 48 hours under gentle stirring (~200rpm). However, 5 minutes upon the addition of the monomer solution, the formation of a viscous gel was observed. This product was extensively dialyzed under the conditions

Figure 25. BSA-pPEGMA after dialysis.

previously described for all bioconjugates. A gel was recovered after dialysis which due to its nature could not be easily characterized (Figure 25) by either PAGE electrophoresis or GPC. Further studies are necessary to verify the formation of the bioconjugate.

Conclusion

The purpose of this thesis was to take advantage of the improved synthetic protocols offered by very recent research involving copper-mediated polymerization without external deoxygenation or oxygen scavengers,[14] in order to synthesize protein-polymer bioconjugates. *This goal was successful as we achieved the formation of BSA Giant Amphiphiles using a variety of monomers following a SET-LRP approach of grafting from a BSA-macroinitiator without the need of degassing, merely by eliminating the head-space in our reaction vessel.*

From the experimental point of view, we initially tried to adjust the reaction volume to fit the volume of a glass vial with encouraging results. We then modified the setup and used a simple plastic syringe to avoid biomolecule interactions with the glass surface and, more importantly, to completely eliminate head-space by simple means. The latter approach proved to be very efficient. The grafting of a variety of monomers was studied with this setup leading to polystyrene, poly-NIPAAm, poly-methyl acrylate, and poly-methyl-methacrylate Giant Amphiphiles. PEGMA was also studied with an ambiguous outcome. The aqueous copper-mediated SET-LRP grafting of styrene from BSA-macroinitiator in bench top conditions, gave excellent results and was optimized in terms of copper and ligand ratios. The products were characterized with various chromatographic techniques including PAGE electrophoresis and Gel Permeation Chromatography (GPC). The resulting superstructures were imaged with Transmission Electron Microscopy (TEM). Further research should focus on optimization for each monomer and expansion of the monomer pool which can be used following this approach. Mechanistic studies are also judged to be important.

Consequently, we discovered an efficient way to form new amphiphilic bioconjugates via overcoming time-consuming deoxygenation procedures, simply by eliminating the head-space in the reaction vessel.

3.0 Experimental

3.1 Materials used

The materials and reagents used for this thesis were purchased from Sigma-Aldrich, Acros Chemicals, Fisher Scientific, Bio-Rad, Alfa Aesar, Fluka and Armar Isotopes. All reagents were used as received. A 0.35 mM solution of the BSA-biomacroinitiator previously synthesized in the Velonia Lab Group was used for all experiments. Biotech Regenerated Cellulose Dialysis Membranes were purchased fromSpectrum Labs with a MWCO of 10 kDa or 25 kDa. The dialysis bags were used for purification of the bio-conjugates.

3.2 Analytical Techniques

Gel Permeation Chromatography (GPC)

GPC was used for the characterization of the bio-conjugates products, except from BSA-poly-NIPPAm ,BSA-poly-methyl-methacrylate and BSA-poly-PEGMA using as mobile phase a 5mM phosphate buffer pH7.4, 10% MeCN. Samples were diluted in the same solvent prior to analysis. This analytical technique was conducted on aShimadzu VP HPLC system, comprising a DGU-14A solvent degasser, a LC-10AD pump, a CTO-10A column oven, a SIL-10AD auto-injector, a RID-10A refractive index detector and a SPD-10A Shimadzu UV Vis. Spectrometer. All reaction samples were compared to a native BSA solution prepared in the same concentration.

Polyacrylamide Gel Electrophoresis

For the electrophoresis was used a Mini-PROTEAN Tetra Cell was used with a 4% stacking polyacrylamide gel and 10% resolving polyacrylamide gel, under standard non-denaturating conditions. Samples were dissolved in TRIS buffer containing BromophenolBlue and were visualized using Coomassie Brilliant Blue or Silver Staining.

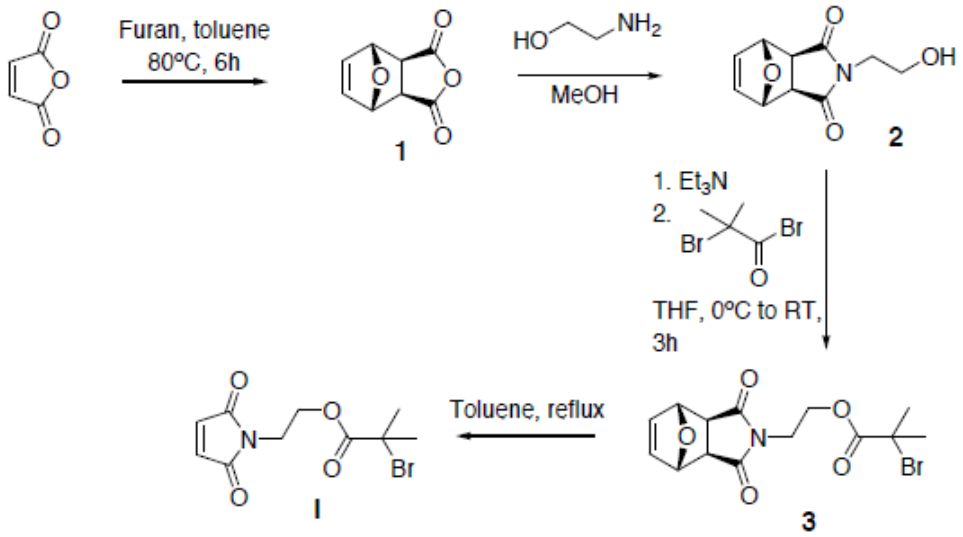
Nuclear magnetic resonance (NMR)

NMR spectra (^{1}H NMR,) were recorded on a Bruker 500 MHz spectrometer system. In order to explain the multiplicities were used the following abbreviations: s=singlet, d=doublet, dd= doublet of doublets, t=triplet, m=multiplet.

Transmission electron microscopy (TEM)

Experiments were performed using a FEI Tecnai G2Electron Microscope. Micrographs were taken using a Tietz CCD camera at a 2048 by2048 pixel resolution.

3.3 Synthesis of the maleimido-initiator I



Maleimido-initiator **I** was synthesized two separate times during the thesis from Velonia Lab Group according to the bibliography.

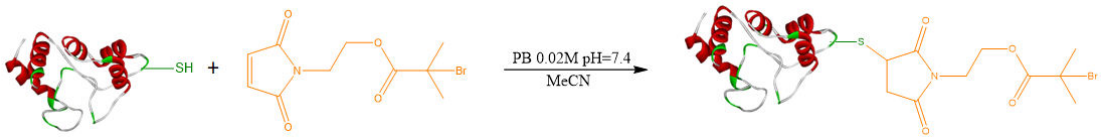
Synthesis of 4-(2-Hydroxyethyl)-10-oxa-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione (2)[39]

The anhydride **1** (2.000 g, 12.04 mmol) was suspended in MeOH (60 mL), in a two-necked round-bottom flask and the mixture cooled to 0 °C. A solution of ethanolamine (0.8 mL, 13.25 mmol) in MeOH (20mL) was added dropwise (over ~ 10 min) through an adding funnel and the resulting solution was stirred for 5 min at 0 °C, then 30 min at ambient temperature. Finally, the solution refluxed for 4 h. After cooling the mixture to ambient temperature, the solvent was removed under reduced pressure, and the white residue was dissolved CH_2Cl_2 (50 mL) and washed two times with 50 mL of water and 50mL of brine. The water layer was washed two times with 20 mL CH_2Cl_2. The organic layer was dried over $MgSO_4$ and filtered. Removal of the solvent under reduced pressure gave the product (0.555 g, 22.01% yield) as a white-yellow (light- yellow) solid. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K): δ = 1.66 (s, 1H, OH), 2.90 (s, 2H, CH), 3.70-3.72 (m, 2H, NCH_2), 3.76-3.78 (m, 2H, OCH_2), 5.30 (d, 2H, CH), 6.53 (d, 2H, CHvinyl).

Synthesis of 2-Bromo-2-methyl-propionic acid 2-(3,5-dioxo-10-oxa-4-aza-tricyclo[5.2.1.0^{2,6}]dec-8-en-4-yl)-ethyl ester (3) [39]

The alcohol **2** (0.555 g, 2.65 mmol) and Et_3N (0.37 mL, 2.65 mmol) were suspended in THF (32mL), in a two-necked round-bottom flask and the mixture (the solution remained slightly turbid) was cooled to 0 °C. Then a solution of 2-bromo isobutyryl bromide (0.330 mL, 2.67 mmol) in THF (10 mL) was added dropwise (over a period of 30 min) with an added funnel.The white suspension was stirred for 3 h at 0 °C and subsequently overnight at ambient temperature. TLC revealed the appearance of the desired product. The ammonium salt was filtered off and the solvent removed under reduced pressure to give a pale-yellow residue. The pale-yellow residue was purified by chromatography column (CC, SiO_2, diethyl ether/ethyl acetate 1:5) to give the final product **3** as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$, 298 K): δ = 1.90 (s, 6H, CH_3), 2.87 (s, 2H, CH), 3.81-3.83 (t, 2H, NCH_2), 4.32-4.34 (t, 2H, OCH_2), 5.26-5.27 (t, 2H, CHO), 6.51-6.52 (t, 2H, CH_{vinyl}).

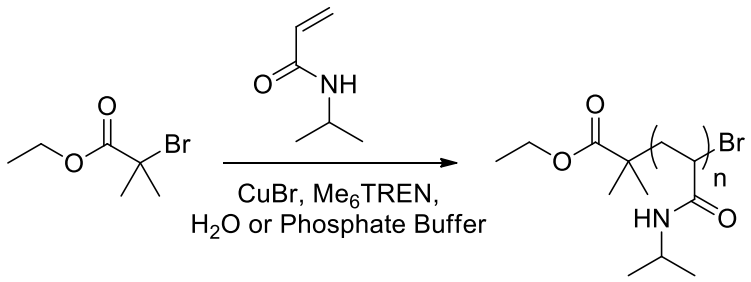
3.4 Synthesis of BSA-macroinitiatorI [39]



This synthesis was performed twice during the thesis, but not actually synthesized from me.

In a solution of BSA (0.348 mM) in phosphate buffer (20 mM, 13 mL, pH 7.4), in a 15 mL falcon tube, a solution of the malemido-initiator **I** (1260.3 mM, 41 equiv.) in acetonitrile (0.1529 mL) was slowly added. The reaction mixture was gently shaken for 65 hours at room temperature. To eliminate the excess of the ATRP initiator **I**, the mixture was extensively dialyzed initially against phosphate buffer (5mM, pH 7.4) and then against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. Electrophoresis showed 100% yield. The final product solution was divided in 15 falcon tubes for freeze-drying. Samples of freeze-dried bioconjugate**II** were stored at -20 °C.

3.5 Experimental synthetic process of poly-NIPAAm with initiator EBiB



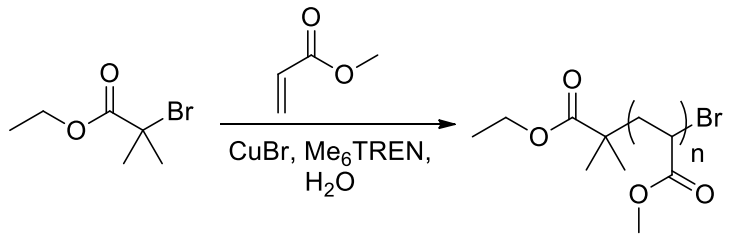
The synthesis was performed twice for each reaction, one with each solvent.

NIPAAm(0.386 g, 3.410mmole, 40 equiv.) and nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (1.02 ml) were placed on an eppendorf (1) and afterwards rotated on a vortex until dissolution. In another eppendorf (2) BSA (0.026 g, 0.39x 10^{-3}mmole ,4.64x 10^{-3}equiv.) , EBiB(16.45 µL, 0.0843mmole, 1 equiv.) and nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (0.51 ml) was added and then rotated on a rotator at low rpm until a homogeneous solution resulted. Then on a vial were added with the specific sequence CuBr (0.004 g, 0.028mmole, 0.3 equiv.), nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (0.257 ml) and Me_6TREN (28.3 µL, 0.106mmole, 1.3equiv.). The moment Me_6TREN was added the vial was tapped and the mixture rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) and (2) solutions were added consecutively via syringe into the vial and the reaction mixture was left stirring for 1h.The reaction with acetone and diethylether and then purified. After the purification, the product was analyzed with ^{1}H NMR,(500 MHz, $CDCl_3$): δ = 1.15 (m, CH_3), 1.64(s, CH_2), 2.37 (m, CH) , 3.99 (S, CH), 4.17 (m, CH), 5.61 6.01 6.28 (m, vinyl CH), that verified the partial formation of the desired product.

At a second try, NIPAAm(0.355 g, 3.130mmole, 40 equiv.) and nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (0.935 ml) were placed on an eppendorf (1) and afterwards rotated on a vortex until dissolution On an another eppendorf (2) were placed BSA (0.024 g, 0.361x 10^{-3}mmole ,4.67x 10^{-3} equiv.) , Ebib (15.08µL, 0.10275mmole, 1 equiv.) and nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (0.4675 ml) and then rotated on a rotator at low rpm until to have an homogeneous solution. Then on a vial were added with the specific sequence CuBr(3.6 mg , 2.5096x 10^{-2}mmole, 0.3 equiv.) , nanopure H_2O or 0.2 mMPhosphate Buffer pH= 7.4 (0.2356 ml) and Me6TREN (25.94µL, 0.113mmole, 1.46 equiv.) . The moment Me6TREN was added the vial has to be tapped the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) solution was first added

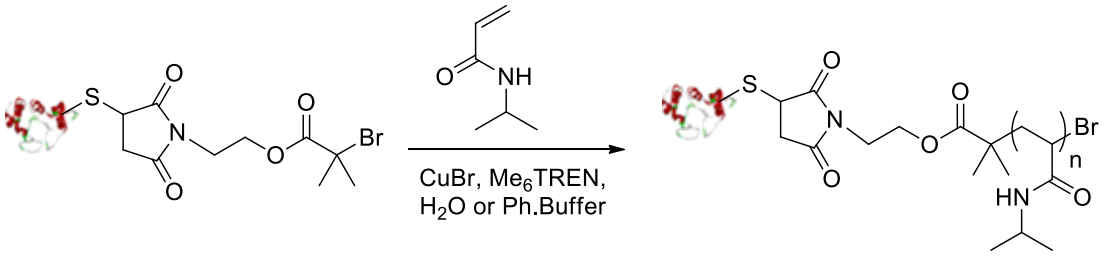
via syringe into the vial and then eppendorf's (2) solution with the same way. The reaction mixture was stirred for 1h and dissolved with acetone and diethylether and ethylacetate and then purified. After the purification, the product was analyzed with ^{1}H NMR(500 MHz, $CDCl_3$): δ = 1.16 (m, CH_3), 1.64(s, CH_2), 2.21 (m, CH) , 4.00 (s, CH), 4.12 (m, CH), 5.62 6.02 6.26 (m, vinyl CH), that verified the successful formation of the desired product.

3.6Experimental synthetic process of poly-MA with initiator Ebib



MA (0.2817 ml, 3.128 mmole, 40 equiv.) and nanopure H_2O (0.7534 ml) were placed on an eppendorf (1) and afterwards rotated on a vortex until dissolution.On another eppendorf (2) were placed BSA (24 mg, 0.3609x 10^{-3}mmole, 4.627x 10^{-3}equiv.), Ebib (11.5 µL, 78.36 x 10^{-3}mmole, 1 equiv.) and nanopure H_2O (0.3767 ml) and then rotated on a rotator at low rpm until to have an homogeneous solution. Then on a vial were added with the specific sequence CuBr(4.5 mg, 0.03137 mmole, 0.4 equiv.), nanopure H_2O (0.19 ml) and Me_6TREN (8.3 µL, 0.031mmole, 0.4 equiv.). The moment Me_6TREN was added the vial has to be tapped the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place Moreover, the use of a syringe- exit needle on the top of the vial during the disproportionation process was tried in order for the trapped O_2 inside the vial to get away. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) solution was first added via syringe into the vial and then eppendorf's (2) solution with the same way. The reaction mixture was stirred for 1h and dissolved with dicloromethane and then purified. After the purification, the product was analyzed with ^{1}H NMR(500 MHz, $CDCl_3$): δ =1.24 (s, CH_3), 1.68 (s, CH_2), 2.30 (s, CH), 3.66 (s, H_3CO), 4.13(m,CH_2), 4.15 (m,CH-Br), that verified the successful formation of the desired product.

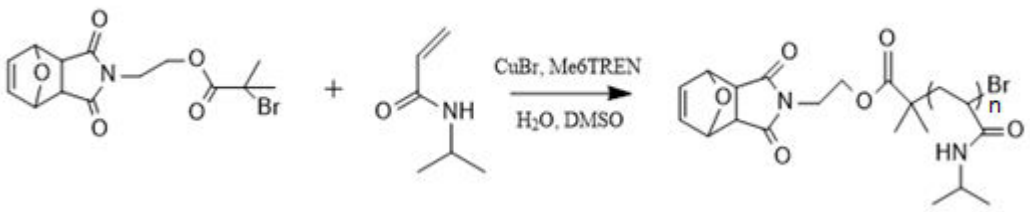
3.7 Experimental synthetic process of BSA-poly-NIPAAm with BSA-maleimide initiator



The synthesis was perfomed twice for each reaction, one with each solvent.

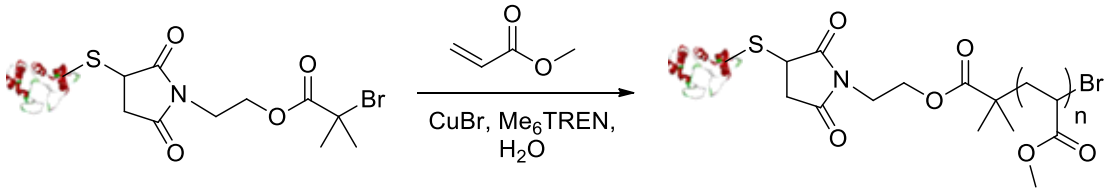
NIPAAm (0.0222 g, 0.196 mmole, 400 equiv.) and the already synthesized BSA-maleimide initiator 0.35mM (1.40 ml, $0.49x10^{-3}$mmole, 1 equiv.) were placed on an eppendorf (1) and afterwards rotated on a rotator at low rpm until to have an homogeneous solution . Then on a vial were added with the specific sequence, nanopure H_2O or 0.2 mM Phosphate Buffer pH= 7.4 (0.23 ml), CuBr (4.5 mg , 0.0314mmole, 60 equiv.) and Me6TREN (8.3µL, 0.31mmole, 60 equiv.) . The moment Me6TREN was added the vial has to be tapped the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. Moreover, the use of a syringe- exit needle on the top of the vial during the disproportionation process was tried in order for the trapped O_2 inside the vial to get away. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) solution was added via syringe into the vial. The reaction mixture was stirred for 1h. For the purification of the bioconjugate, the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA, then against phosphate buffer (20 mM, pH 7.4), ~2% EDTA, twice, and finally against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was analyzed with ^{1}H NMR (500 MHz, D_2O): δ =1.02 (s, CH3),1.15 (s,CH3) , 1.46 (s, CH2), 1.89 (s, CH),3.78 (s, CH), PAGE-electrophoresis which verified the success of the reaction for both reactions.

3.8 Experimental synthetic process of BSA-poly-NIPAAm with protected maleimide initiator



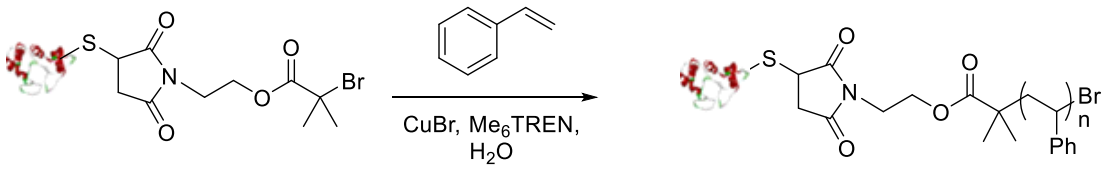
NIPAAm (0.124g, 1.12 mmole, 50 equiv.)and nanopure H_2O (0.24 ml) were placed on an eppendorf (1) and afterwards rotated on a vortex until dissolution. On another eppendorf (2) were placed the protected maleimide initiator (8 mg, 22.4x 10^{-3}mmole, 1 equiv.) and DMSO (20µL) and then rotated on a vortex at high rpm until to have an homogeneous solution. If it's not dissolved it's better for the mixture to be sonicated. Then inside of a syringe were added with the specific sequence CuBr(1.28 mg, 8.96x 10^{-3}mmole, 0.4 equiv.) , nanopure H_2O (0.23 ml) and Me6TREN (2.4µL, 8.96x 10^{-3}mmole, 0.4 equiv.). The moment Me6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) solution was first added inside of the syringe via the disproportionation syringe and then eppendorf's (2) solution with the same way. The reaction has been left overnight and the resulting polymer was purified from unreacted monomer by repeated dissolution in THF and precipitation from hexane. After the purification, the product was analyzed with ^{1}H NMR (500 MHz, $CDCl_3$): δ =1.12 (s, CH_3), 1.62 (s, CH_2), 2.61 (s, CH), 3.98 (s, CH), 3.73-3.75 (m, 2H, OCH2, 2H, NCH2), 5.24 (2H, CH), 6.51 (2H, CHvinyl).

3.9 Experimental synthetic process of BSA-poly-Methyl Acrylate with BSA-maleimide initiator



MA (161.3 µL, 1.96 mmole, 4000 equiv.) and the already synthesized BSA-maleimide initiator 0.35mM (1.40 ml, 0.49x 10^{-3}mmole, 1 equiv.) were placed on an eppendorf (1) and afterwards rotated on a rotator at low rpm until to have an homogeneous solution . Then on a vial were added with the specific sequence, nanopure H_2O (0.23 ml), CuBr(2.8mg ,19.6x 10^{-3}mmole, 40 equiv.) and Me6TREN (5.24µL, 19.6x 10^{-3}mmole, 40 equiv.). The moment Me_6TREN was added the vial has to be tapped the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. Moreover, the use of a syringe- exit needle on the top of the vial during the disproportionation process was tried in order for the trapped O_2 inside the vial to get away. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's(1) solution was added via syringe into the vial. The reaction mixture was stirred for 1h. For the purification of the bioconjugate, the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA , then against phosphate buffer (20 mM, pH 7.4), , twice, and finally against phosphate buffer (20 mM, pH 7.4),~2% EDTA twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was analyzed withPAGE-electrophoresis and GPC which verified the success of the reaction for both reactions.

3.10 Experimental synthetic process of BSA-poly-Styrene with BSA-maleimide initiator



The specific synthetic approach was tried several times, the first reaction took place on the vial and the subsequent ones inside of a syringe.

Styrene (19.3 µL, 0.168mmole, 400 equiv.) and the already synthesized BSA-maleimide initiator 0.35mM (1.20 ml, 0.42x 10^{-3}mmole, 1 equiv.) were placed on an eppendorf (1) and afterwards rotated on a rotator at low rpm until to have an homogeneous solution. Then on a vial were added with the specific sequence, nanopure H_2O (0.23 ml), CuBr (3.9mg,26.88x 10^{-3}mmole, 60 equiv.) and Me_6TREN (7.18µL, 26.88x 10^{-3}mmole, 60 equiv.). The moment Me6TREN was added the vial has to be tapped the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. Moreover, the use of a syringe- exit needle on the top of the vial during the disproportionation process was tried in order for the trapped O_2 inside the vial to get away. The mixture's colourshould rapidly change into a light blue. Afterwards eppendorf's (1) solution was added via syringe into the vial. The reaction mixture was stirred for 1h. For the purification of the bioconjugate, the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA, ~10% DMSO , then against phosphate buffer (20 mM, pH 7.4),~2% EDTA~10% DMSO, twice, and finally against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was analyzed withPAGE-electrophoresis and GPC which verified the partial success of the reaction for the reaction.

Styrene (67.6 µL, 0.588 mmole, 4000 equiv.) and the already synthesized BSA-maleimide initiator 0.35mM (0.42 ml, 0.147x 10^{-3}mmole, 1 equiv.) were placed on an eppendorf and afterwards rotated on a rotator at low rpm until to have an homogeneous solution. Then inside of a syringe were added with the specific sequence CuBr(0.8 mg , 5.88x 10^{-3}mmole, 40 equiv.) dissolved on 10 µL DMSO , nanopure H_2O (0.37 ml) and Me_6TREN (1,57µL, 5.88x 10^{-3}mmole, 40 equiv.)dissolved on 10 µL DMSO. The moment Me_6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colourshould rapidly change into a light blue. Afterwards eppendorf's

solution was added inside of the syringe via the disproportionation syringe. The reaction has been left for 2h and there were collected aliquots at 20’,40’,60’,90’.For the purification of the bioconjugate, the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA, ~10% DMSO , then against phosphate buffer (20 mM, pH 7.4),~2% EDTA~10% DMSO, twice, and finally against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was analyzed withPAGE-electrophoresis and GPC which verified the success of the reaction, the formation of the product from 20 minutes.

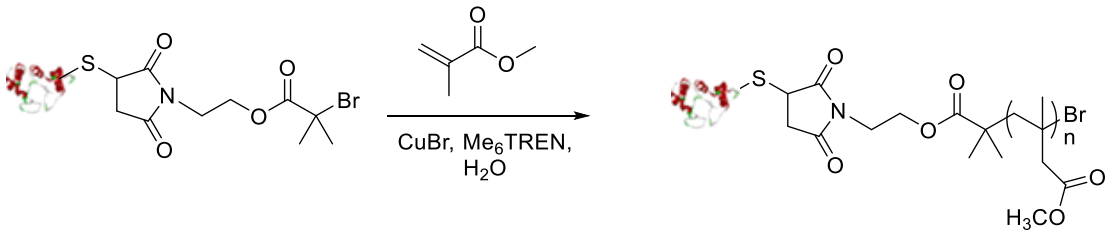
The next reaction had the same ratios with the previous one, with the only difference that there were collected aliquots instantly at 2’,5’,10’,15’,20’,40’,60’,90’. The final product was purified at the same way and it was analyzed with PAGE-electrophoresis and which verified the success of the reaction,

Styrene (98.86μL, 0.86 mmole, 4000 equiv.) was placed on an eppendorf (1). On an another eppendorf (2) was added the BSA-maleimide initiator 0.35mM (0.625 ml, 0.000215mmole, 1 equiv.), Then inside of a syringe were added with the specific sequence CuBr (0.128 mg , 0.86x 10^{-3}mmole, 4 equiv.) dissolved first on 10μL DMSO, nanopure H_2O (0.55 ml) and Me_6TREN (0.23μL, 0.86x 10^{-3}mmole, 4 equiv.) dissolved first on 10μL DMSO. The moment Me_6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture’s colour should rapidly change into a light blue. Afterwards eppendorf’s (1) solution was first added inside of the syringe via the disproportionation syringe and then eppendorf’s (2) solution with the same way. The reaction has been left for 3h there have been collected aliquots at 2’,5’,10’,15’,20’,40’,60’,90’,120’,150’. The final product had not been purified, but instantly analyzed withPAGE-electrophoresis, which revealed the failure of the reaction with these ratios.

Styrene (67.6μL, 0.388 mmole, 4000 equiv.) was placed on an eppendorf (1). On an another eppendorf (2) was added the BSA-maleimide initiator 0.35mM (0.42 mL, 0.000147mmole, 1 equiv.), Then inside of a syringe were added with the specific sequence CuBr (0.42mg, 2.94x 10^{-3}mmole, 20 equiv.) dissolved first on 10μL DMSO, nanopure H_2O (0.37 ml) and Me_6TREN (0.78μL, 2.94x 10^{-3}mmole, 20 equiv.) dissolved first on 10μL DMSO. The moment Me6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture’s colourshould rapidly change into a light blue. Afterwards eppendorf’s (1) solution was first added inside of the syringe via the disproportionation syringe and then eppendorf’s (2) solution with the same way. The reaction has been left for 2hand there have been collected aliquots at 30’,60’,90’. The final product had not

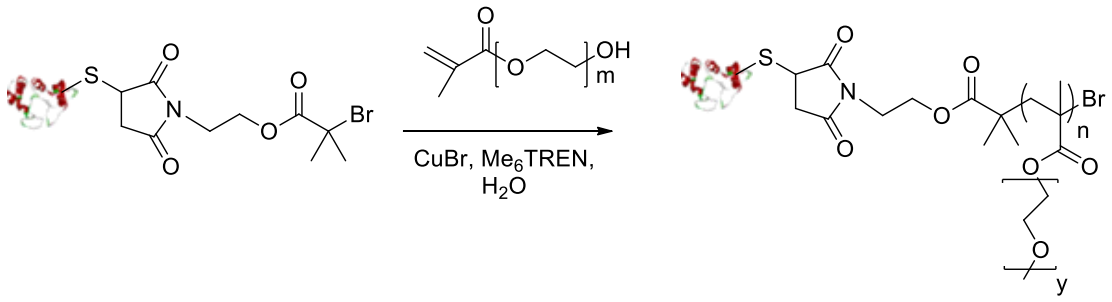
been purified, but instantly analyzed with PAGE-electrophoresis, which revealed the failure of the reaction with these ratios.

3.11 Experimental synthetic process of BSA-poly-Methyl Methacrylate with BSA-maleimide initiator



MMA (62.9 µL, 0.588 mmole, 4000 equiv.) and the already synthesized BSA-maleimide initiator 0.35mM (0.42 ml, 0.147x 10^{-3}mmole, 1 equiv.) were placed on an eppendorf and afterwards rotated on a rotator at low rpm until to have an homogeneous solution. Then inside of a syringe were added with the specific sequence CuBr (0.8 mg, 5.88 10^{-3}mmole, 40 equiv.) dissolved on 10 µL DMSO, nanopure H_2O (0.37 ml) and Me_6TREN (1,57µL, 5.88x 10^{-3}mmole, 40 equiv.)dissolved on 10 µL DMSO . The moment Me_6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colourshould rapidly change into a light blue. Afterwards eppendorf's solution was added inside of the syringe via the disproportionation syringe. The reaction has been left overnight.For the purification of the bioconjugate, the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA, then against phosphate buffer (20 mM, pH 7.4),~2% EDTA twice, and finally against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was analyzed with PAGE-electrophoresis which verified the partial formation of the product with remained unreacted bioinitiator.

3.12 Experimental synthetic process of BSA-poly-PEGMA with BSA-maleimide initiator



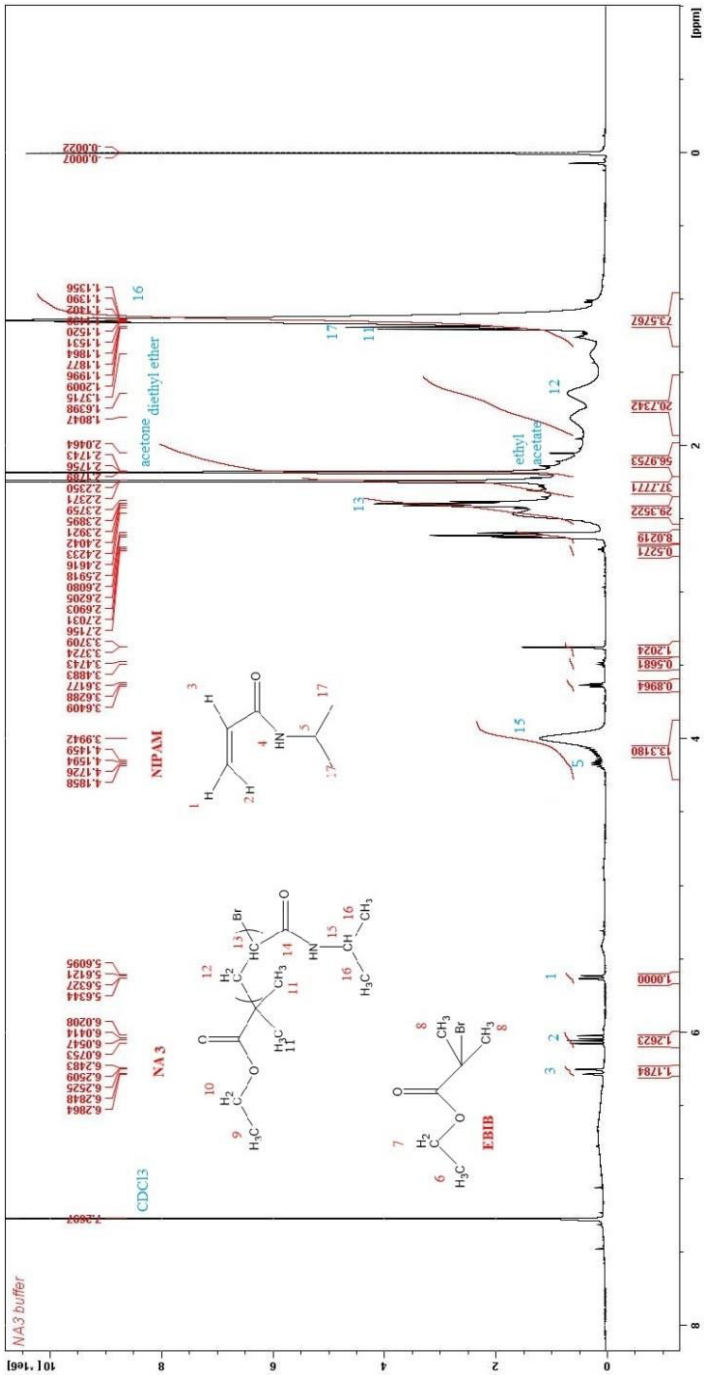
PEGMA (256.58µL, 0.588 mmole, 4000 equiv.) was placed on an eppendorf (1). On an another eppendorf (2) was added the BSA-maleimide initiator 0.35mM (0.42 ml, 0.147x10^{-3}mmole, 1 equiv.), Then inside of a syringe were added with the specific sequence CuBr (0.8 mg , 5.88 x10^{-3}mmole, 40 equiv.) dissolved first on 10µL DMSO, nanopure H_2O (0.37 ml) and Me6TREN (1.57µL, 5.88 x10^{-3}mmole, 40 equiv.) dissolved first on 10µL DMSO . The moment Me_6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colour should rapidly change into a light blue. Afterwards eppendorf's (1) solution was first added inside of the syringe via the disproportionation syringe and then eppendorf's (2) solution with the same way. The reaction has been left for 2days, however after 5 minutes of the bioinitiator's addition, the mixture has been thickened and formed a gel with the non-stirring effect as a consequence. The final product had been purified, as the mixture was extensively dialyzed initially against 10% MeCN in phosphate buffer (5mM, pH 7.4),~2% EDTA, then against phosphate buffer (20 mM, pH 7.4),~2% EDTA twice, and finally against phosphate buffer (20 mM, pH 7.4), twice, using regenerated cellulose dialysis membranes with a MWCO of 25 kDa. After the purification, the product was not be able to be analyzed with PAGE-electrophoresis.

Concerning the blank test reaction for PEGMA, (256.6µL, 0.588 mmole, 4000 equiv.) was placed on an eppendorf (1). On an an othereppendorf (2) was added BSA (10 mg, 0.417 x10^{-3}mmole, 1 equiv.) dissolved on 0.1 ml H_2O. Then inside of a syringe were added with the specific sequence CuBr (0.8 mg , 5.88 x10^{-3}mmole, 40 equiv.) dissolved first on 10µL DMSO, nanopure H_2O (0.37 ml) and Me6TREN (1.57µL, 0.588 x10^{-3}mmole, 40 equiv.) dissolved first on 10µL DMSO . The moment Me6TREN was added to the syringe the trapped oxygen should be pushed outside of the syringe and the mixture should be rotated at high rpm for 2-3 min on ice in order for disproportionation to take place. The mixture's colourshould rapidly change into a light blue.

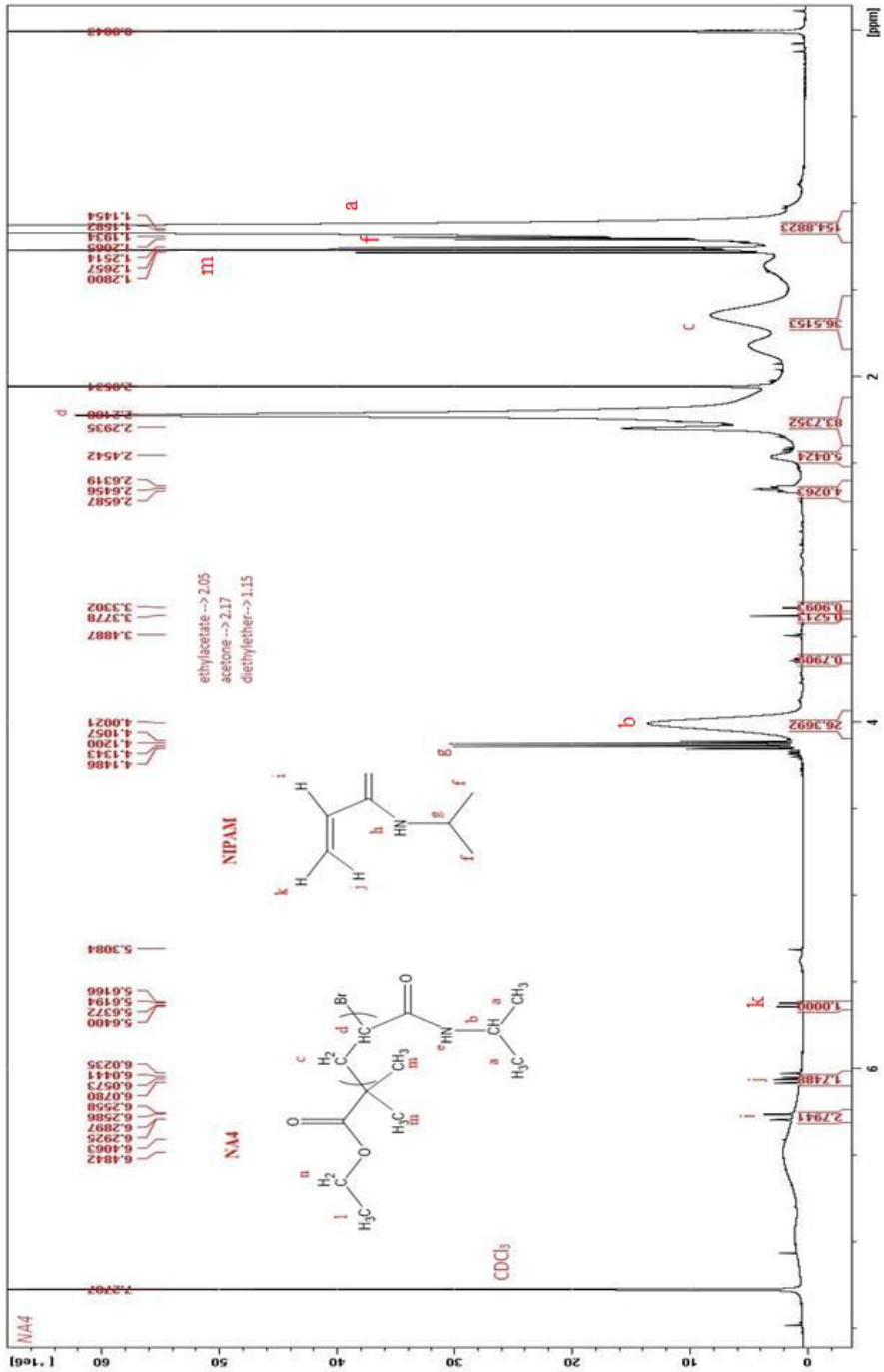
Afterwards eppendorf's (1) solution was first added inside of the syringe via the disproportionation syringe and then eppendorf's (2) solution with the same way. The reaction has been left for several hours, without the thickening taking place indicating that the previous reaction may was successful.

NMR Spectra

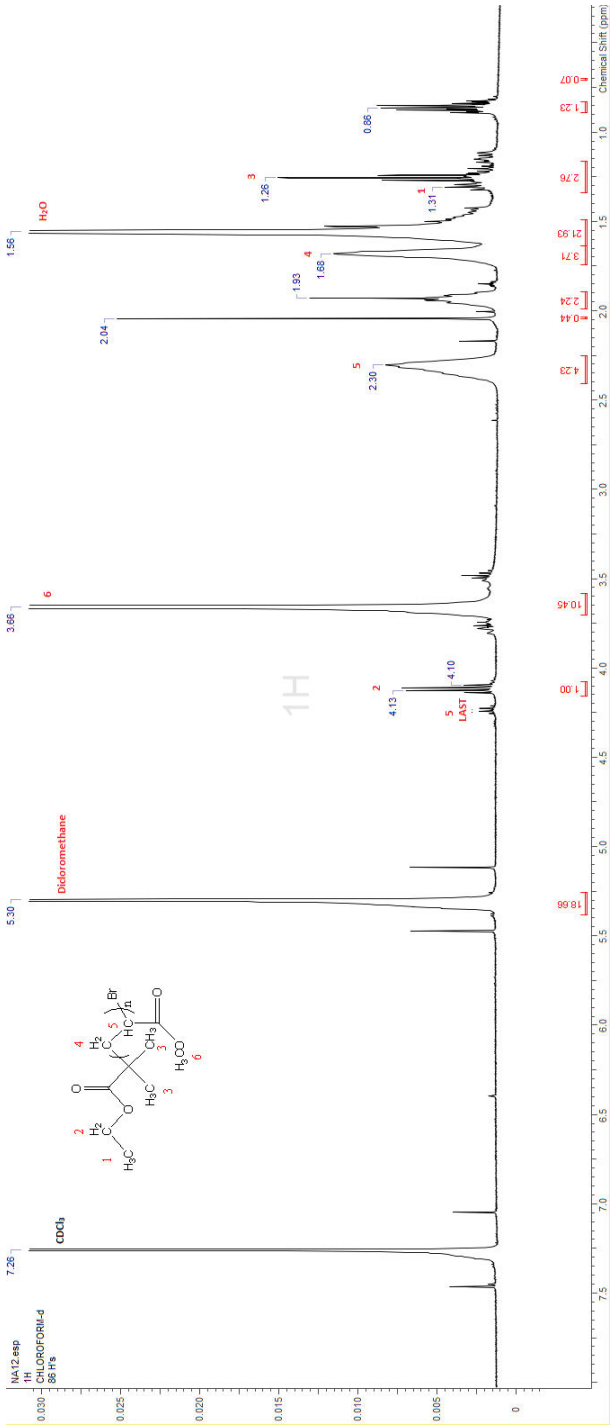
NMR Spectrum 1:EBiB-pNIPAAM ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.15 (m, CH_3), 1.64(s, CH_2), 2.37 (m, CH) , 3.99 (S, CH), 4.17 (m, CH), 5.61 6.01 6.28 (m, vinyl CH). [38][41]



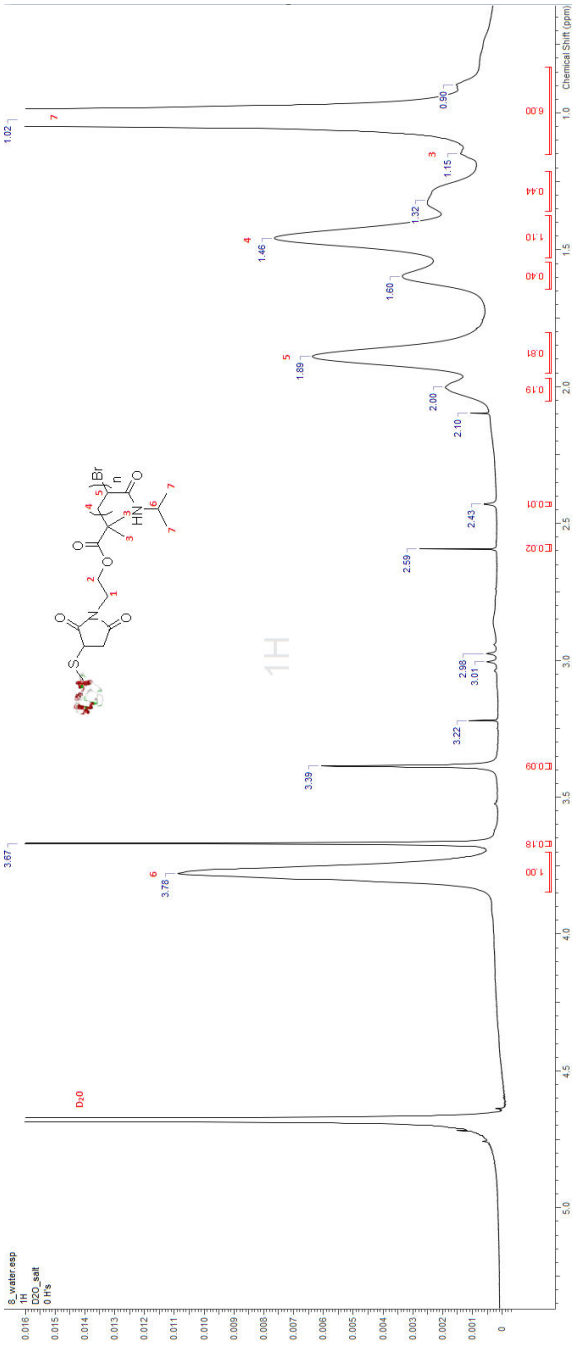
NMR Spectrum 2: EBiB-pNIPAAM. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.16 (m, CH_3), 1.64(s, CH_2), 2.21 (m, CH) , 4.00 (s, CH), 4.12 (m, CH), 5.62 6.02 6.26 (m, vinyl CH).[38][41]



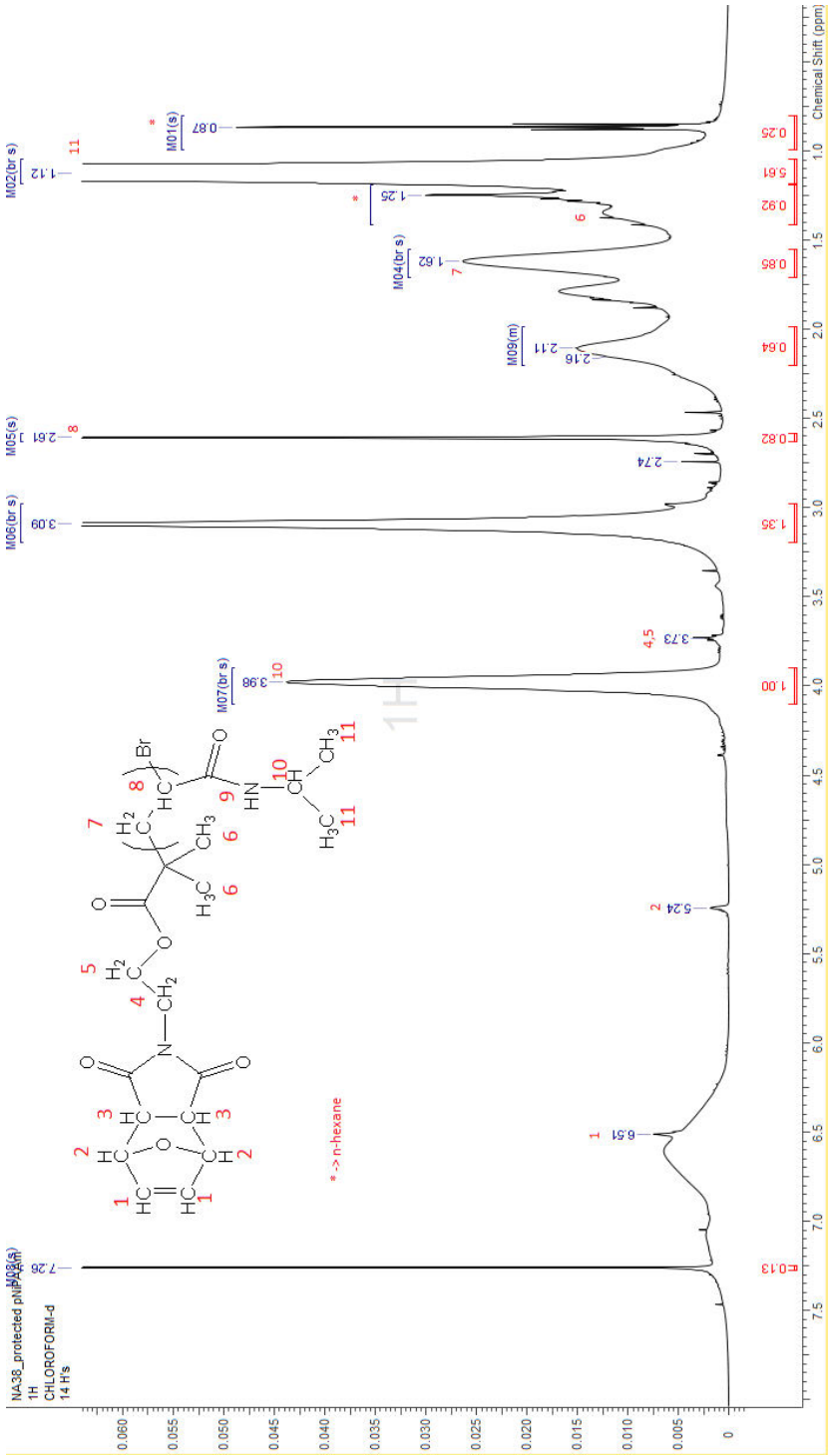
NMRSpectrum 3: EBiB-pMA. ^{1}HNMR (500 MHz, $CDCl_3$): δ =1.24 (s, CH_3), 1.68 (s, CH_2), 2.30 (s, CH) , 3.66 (s, H_3CO), 4.13(m,CH_2), 4.15 (m,CH-Br)[39].



NMR Spectrum 4 : BSA-pNIPAAm.^{1}H NMR (500 MHz, D_2O): δ =1.02 (s, CH3),1.15 (s,CH3) , 1.46 (s, CH2), 1.89 (s, CH) , 3.78 (s, CH).[35]



NMRSpectrum 5: protected maleimide-pNIPAAm . ^{1}HNMR (500 MHz, $CDCl_3$): δ =1.12 (s, CH_3), 1.62 (s, CH_2), 2.61 (s, CH), 3.98 (s, CH), 3.73-3.75 (m, 2H, OCH_2, 2H, NCH_2), 5.24 (2H, CH), 6.51 (2H, CH_{vinyl}).[38]



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