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Polyphosphate-Peptide-Conjugates

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vorgelegt von

Laura Müller

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Hiermit versichere ich, die vorliegende Arbeit selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt zu haben. Alle Stellen, die wörtlich oder sinngemäß aus Veröffentlichungen oder anderen Quellen entnommen sind, wurden als solche eindeutig kenntlich gemacht. Diese Arbeit ist in gleicher oder ähnlicher Form noch nicht veröffentlicht und auch keiner anderen Prüfungsbehörde vorgelegt worden.

Mainz, den 27. November 2013 _____

Laura Müller

Für meine Familie

„Wichtig ist, dass man nicht aufhört zu fragen."

Albert Einstein

Danksagung

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Inhalt

Abstract

According to the fact that in our days nearly a third of all drug candidates in clinical trials are polypeptides, protein and peptide drugs hold great promise as therapeutic agents. But there are difficulties with their stability and lifetime in blood circulation due to proteolytic degradation, low solubility and rapid kidney clearance.

To overcome these problems, PEGylation of drugs, i.e. covalent attachment of poly(ethylene glycol) (PEG), is a widely used method for preparation of pharmaceuticals; however this method faces several problems: even if PEG is biocompatible, it is not degradable and it is not completely clear yet, how this will affect the human body in a long term treatment and some initial studies reveal that the prolongued use of PEG can have consequences e.g. for the kidneys.

Polyphosphoesters (PPEs) are, in contrast, biodegradable polymers having several advantages compared to PEG making them attractive for biomedical application. They are very similar in structure to biomacromolecules such as nucleic acids and compared to commonly degradable aliphatic polyesters can be functionalised easily due to the pentavalency of the phosphorous atom.

This work was devoted to investigate a new class of drug-delivery-systems by combining the properties of polyphosphates with (cell-penetrating) peptides. For this purpose, different hydrophilic polyphosphates with different in-chain and chain-end functionalities were synthesized by ring-opening polymerisation with different organo catalysts. For this reason a novel protected monomer was synthesized and copolymerized with other phosphates. Peptides with differences in size and properties were conjugated to the polyphosphates via different functionalities which were introduced via the initiator. Functionalization of polymers was determined by ^{1}H DOSY NMR spectroscopy and the modification with peptides was confirmed by high pressure liquid chromatography as well as NMR spectroscopy.

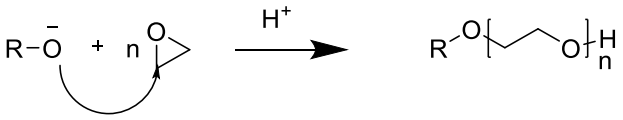
1. Introduction

The introduction gives a short insight into polyphosphates including synthetic strategies and advantages towards common poly(ethylene glycol). Cell-penetrating peptides are discussed followed by possible ways, to covalently connect both.

1.1 Polyetheylene glycol (PEG) and PEGylation

Poly (ethylene glycol)s are polymers of ethylene oxide and are therefore also known as poly (ethylene oxide) (PEO).They play an important role in daily life as ingredients of for example food, cosmetics and pharmaceuticals.[1]

In general, PEG can be synthesized by ring-opening polymerisations with anionic, cationic or coordinative mechanism. The anionic ROP offers the advantages of molecular weight and structure control and is therefore a widely used method in laboratories and technical industry. What makes PEG interesting for applications are its water solubility and low toxicity.[1]



Scheme 1: anionic ROP of PEG

In Scheme 1 the structure of poly (ethylene glycol) is shown. PEG is an aliphatic ether. This suggests the speculation that the polymer is water insoluble like its homologues poly (methylene oxide) or poly (propylene oxide). But the very reverse is found, PEG is highly water soluble. This phenomenon is based on the distance between the oxygen atoms within the polymer chain that is similar to the distance between the oxygen atoms in liquid water. This enables the formation of a hydrogen bonded network and guarantees solubility.[2]

The other goal of PEG is its low toxicity for both, oral and intravenous incorporation. Studies showed, that the middle lethal dose for mice when incorporated oral is more than 50 kg per kilogram of body weight.[1]

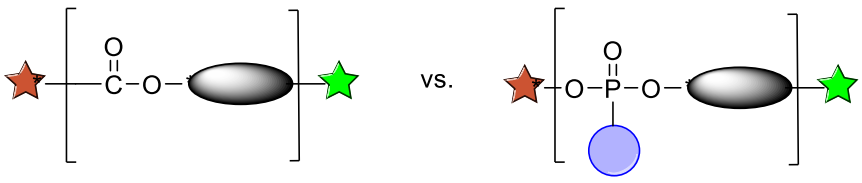
Both features make poly (ethylene glycol) very attractive for biomedical application. As already foreshadowed in the abstract, the covalent attachment of PEG to drugs is a widely used method for the preparation of pharmaceuticals and called "PEGylation".[3] This way, longer lifetimes in blood-circulation of conjugated proteins or polypeptides as well as slower kidney clearance are achieved. Examples for PEGylated proteins that are in clinical practice are Pegasys® (PEGylated Interferon α-2a) for the treatment of hepatitis C and Cimzia® (PEGylated anti-TNFα Fab) for the treatment of morbus Crohn.[1]

Despite the fact, that PEGylation has many advantages and is a commonly used method for the preparation of pharmaceuticals, this method imports the problem of life-time storage in human body due to PEGs non-biodegradability.

1.2 Polyphosphoesters

The most commonly degradable polymers in our days that are used in clinical trials and therapeutic agents are aliphatic polyesters like polylactide (PLA) or poly (*ε*-caprolactone). Advantages of those materials are their biocompatibility and biodegradability, but there are also some drawbacks. When degraded by hydrolytic cleavage, acidic by-products lead to a decrease in pH causing inflammations in tested mice.[4]

Because of the tetravalency of carbon, the introduction of functional groups within the polyester backbone is only possible along the main chain. Polyphosphoesters (PPEs) on the other hand can be functionalised easily due to the pentavalency of the phosphorous atom via an additional ester bond.

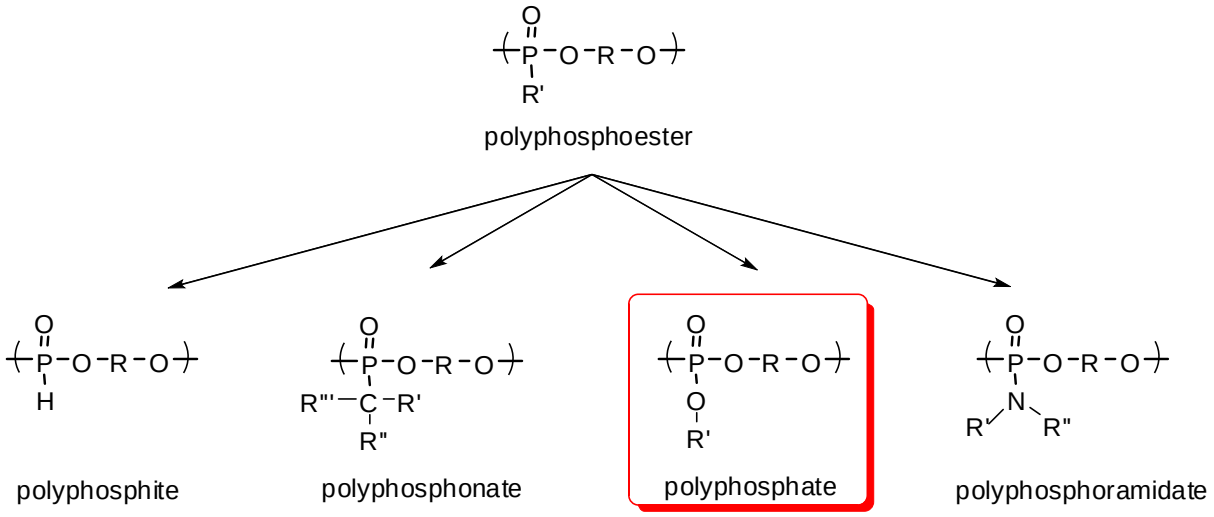


Scheme 2: functionlization-possibilities of aliphatic polyesters compared to PPEs

Biodegradable PPEs have been investigated as biomimetic materials for nearly three decades now, but have been originated much earlier as flame retardants[5] and are widely used in that field today. In

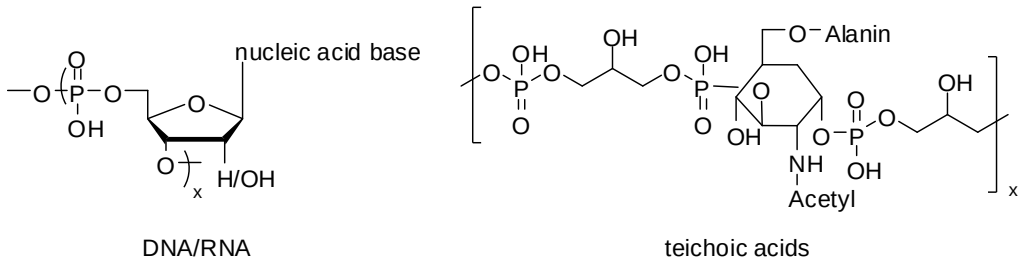
the 1970s, Penczek and coworkers illustrated different synthetic approaches, that will be discussed later-on in this introduction.[6]

In general, PPEs are polyesters of the corresponding phosphorous acid that builds up the main chain of the polymer and can be classified in four types depending on the side chain.



Scheme 3: classification of PPEs[7]

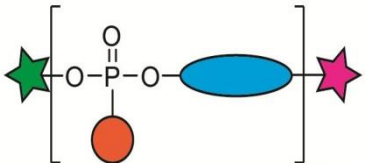
This thesis concentrates on the subclass of polyphosphates. As shown in Scheme 3, polyphosphates are polyesters of phosphoric acid and contain P-O-C bonds in the polymer backbone as well as in the side chains. For pharmaceutical application, especially poly(alkylene phosphate)s are of interest according to their biocompatibility, biodegradability and structural analogy to natural biomacromolecules like DNA or teichoic acids.[7,8]



Scheme 4: natural biomacromolecules containing phosphorous

According to the chemical versatility of the monomeric phosphate, it is possible to design functional materials with tuneable architectures and a multitude of properties,[8] which makes polyphosphates interesting for pH or thermoresponsive materials,[9] tissue engineering, drug and gene delivery.[6,8]

The general structure of polyphosphates in Scheme 3 already indicates that there are many possibilities for their functionalization. Functional groups can be introduced depending on the method of synthesis in the polymer backbone, via side-chains and by endgroup-modification.



Scheme 5: functionalization opportunities of PPEs

The polymer backbone can be varied in length of the methylene linker or by introducing aromatic or unsaturated groups. This way, water-soluble polymers like poly(ethyl ethylene phosphate) as well as completely water-insoluble polymers[8] are accessible similar to conventional polyesters.

By side-chain modification, bioactive groups as well as polymerizable groups can be introduced. The latter are of special interest as crosslinkers for polymeric hydrogels.[7]

The polymer endgroups can be functionalized depending on their synthetic route either by α- or ω-functionalization and will be discussed within the next chapters.

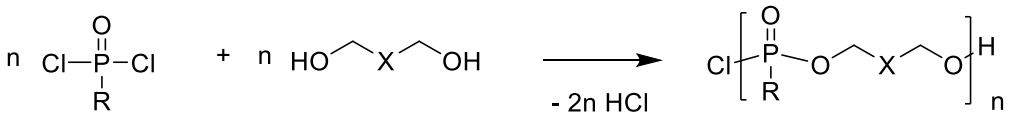
1.2.1 Synthesis of polyphosphates

As already mentioned, polyphosphates can in analogy to aliphatic polyesters be synthesised by five different synthetic routes, namely polycondensation,[10] polyaddition,[11] transesterification,[12] ring-opening polymerisation (ROP) of cyclic monomers[11] and olefin metathesis.[8,13]

This chapter gives a short overview of the different synthetic strategies mentioned above.

Polycondensation

Polycondensation has been an often used method for the preparation of polyphosphates and is usually carried out between aliphatic or aromatic dihydroxy compounds and aryl or alkyl phosphoric dihalides.[7,11]



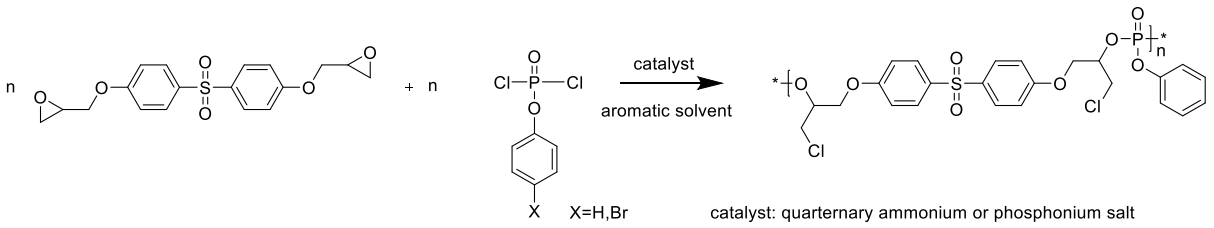
Scheme 6: polycondensation for preparation of polyphosphates

Various polyphosphates were synthesized by solution polycondensation and has been used as flame retardants.[7] For example, Kishore and coworkers synthesized polymers by polycondensation of bisphenol A with different alkyl phosphorodichloridates, in order to investigate their thermal stability. They found out, that the thermal stability of the polymers was not affected in a significant way by changing the alkyl pendant group.[14]

Drawbacks of the polycondensation method for the synthesis of polyphosphates, as well as for other aliphatic polyesters, is the occurrence of transesterification as side reaction that leads to broad molecular weight distributions and typically low molecular weights; also functional group tolerance is limited due to the reaction conditions.

Polyaddition

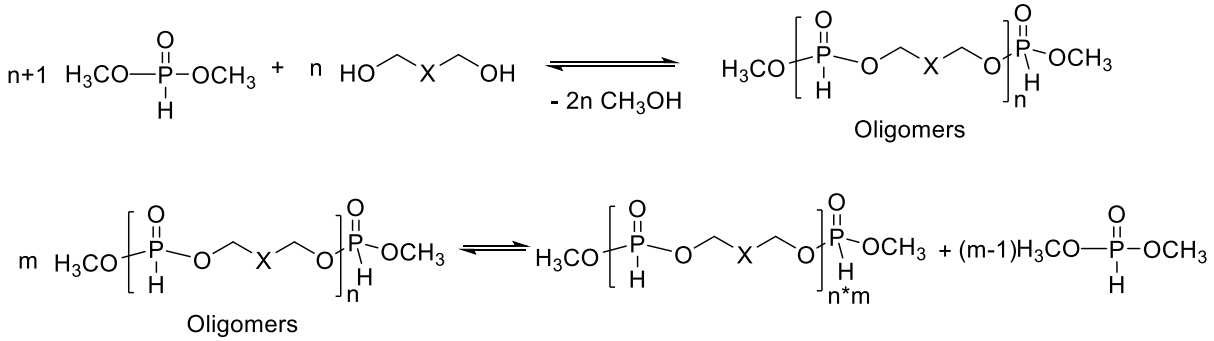
Liaw and coworkers synthesized polyphosphates by addition of diglycidyl ether of bisphenol S with aryl phosphorodichloridates that contain reactive chloromethyl groups.[15]



Scheme 7: polyaddition for preparation of polyphosphates

Transesterification

Penczek *et al.* synthesized low molecular weight polymers by condensation polymerisation of diols with *H*-phosphonates. Due to a molecular weight limitation of this process, they changed to transesterification polymerisation afterwards. This allowed the preparation of polymers with molecular masses up to $30x10^3$ g/mol.[5]

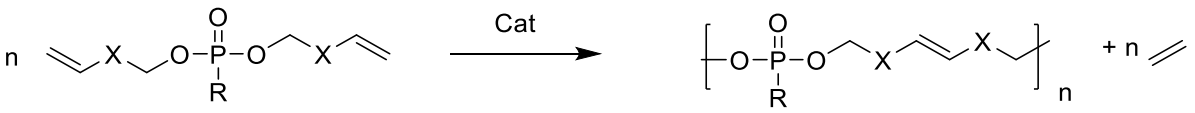


Scheme 8: transesterification for preparation of polyphosphates

Olefin Metathesis

Recently, there were two new synthetic approaches developed in our research group. Both are based on metathesis, but differ in monomers.

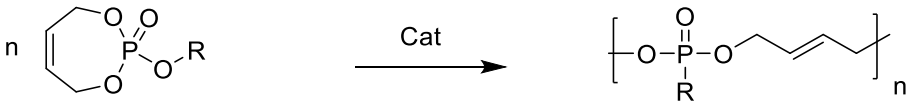
Marsico *et al.* developed a method for the synthesis of unsaturated PPEs via the acyclic diene metathesis (ADMET) polymerisation allowing to control the microstructure of the polymers backbone as well as side chains. Polymers with molecular weights between 7,000 and 50,000 g/mol and reasonable molecular weight distribution were obtained.[8]



Scheme 9: ADMET approach for preparation of polyphosphoesters

Metathesis polymerisation tolerates many functional groups and therefore allows the formation of various high functional polyphosphates.

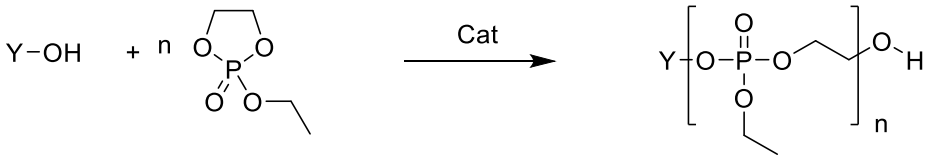
Steinbach *et al.* expanded the idea of metathesis polymerisation to obtain PPEs from the step-growing to a chain growing mechanism. Seven-membered cyclic monomers were polymerised via ring-opening metathesis polymerisation (ROMP) using a Grubbs catalyst 3rd generation to obtain polyphosphates with molecular weights up to 5,000 g/mol and a molecular weight distribution of ~2.[13]



Scheme 10: ROMP approach for preparation of PPEs

Ring-opening polymerisation (ROP)

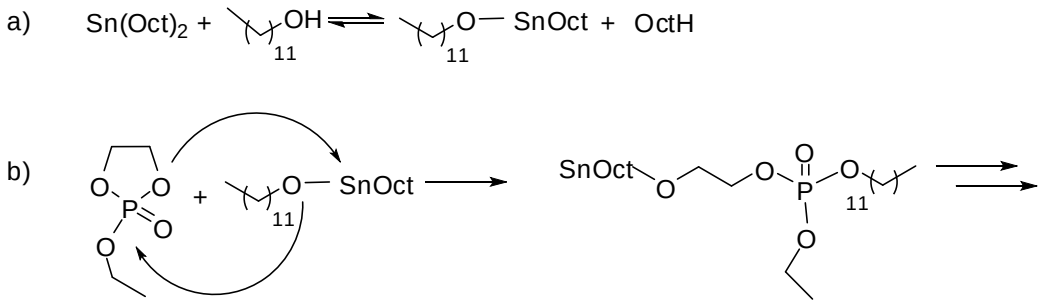
The ring-opening polymerisation of cyclic phosphoester monomers with a hydroxyl-bearing initiator is the most common way to synthesize polyphophoesters.[16] Advantages of this method towards the above described ones are the possibility of controlling the resulting polymers structure and the control of molecular weight including its distribution.



Scheme 11: ROP for preparation of polyphosphates

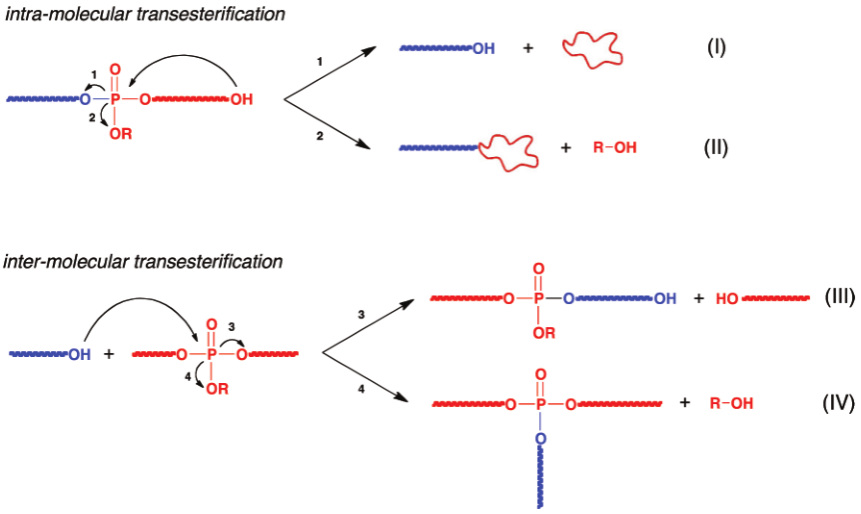
The polymerisation can be catalysed either by enzymes,[17] metallic compounds or organo-catalysts. Cationic polymerisation of cyclic monomers with $CF_3SO_3CH_3$, $CF_3SO_3C_2H_5$, $(CF_3SO)_2O_2$, or $Ph_3C^+AsF_6^-$ as initiators resulted in polymers with low molecular weights.[11] When using *n*-C_4H_9Li, (*i*-$C_4H_9)_3Al$, $(C_5H_5)_2Mg$ or *t*-C_4H_9OK as initiators for anionic ROP, polymers with molecular weights up to 10^5 g/mol were obtained.[18]

As organo-metallic catalysts, triisobutylaluminium was used by Iwasaki *et al*. to copolymerise ethyl ethylene phosphate and 2-isopropoxy-2-oxo-1,3,2-dioxaphospholane (IPP).[16] Wang and co-workers used stannous octoate to polymerise ethyl ethylene phosphate (EEP) and were able to prove that polymerisation is co-initiated by catalyst and dodecanol, which was used as hydroxyl initiator.[19] The reaction is first-order and underlies a coordination-insertion polymerisation mechanism.



Scheme 12: Co-initiation process of stannous octoate catalysed polymerisation

With extended reaction times, side chain transfer leads to branched polymeric structures due to the presence of the ester side groups along the backbone. By controlling the conditions of the polymerisation, these side reactions can be suppressed and perfectly linear polyphosphates with well-defined structures can be obtained.[19]

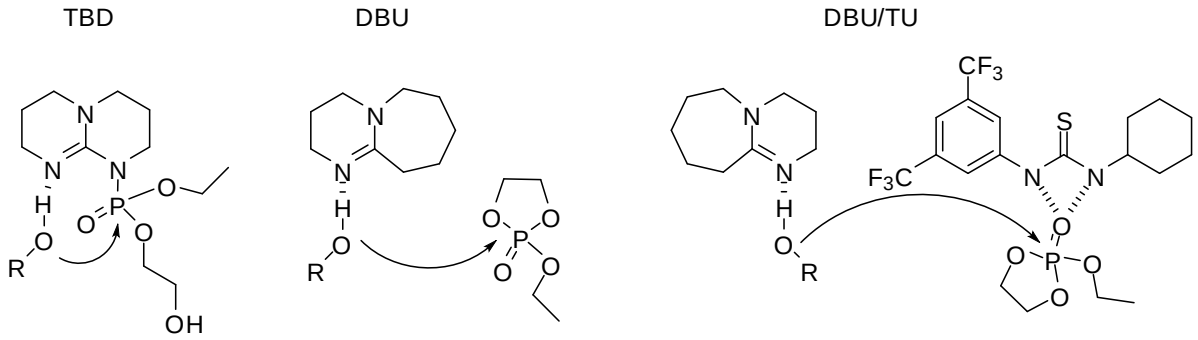


Scheme 13: transesterification as side-reaction during ROP[4]

Even though it is possible to synthesize PPEs with metallic catalysts, side reactions cannot be prevented completely and therefore the molar masses of obtained polymers are limited. In addition, PPEs are often used for biomedical application and the metallic catalysts described previously are cytotoxic and have to be removed completely after synthesis.

According to that, metal-free synthetic approaches have been developed during the past decade. Pioneering with his work, Hedrick *et al.* used organocatalysts to carry out ROP of lactones and lactide copolymers in a controlled and living nature.[20] The organo-catalysts act as bases or nucleophiles and activate initiator and/or monomer. It turned out, that 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,5,7-triazabicyclo[4.4.0]undec-5-ene (TBD) are highly active catalysts for ROP of aforementioned monomers and allow good control over molecular weight and polydispersity.[21,22] TBD is in contrast to DBU and the metal-catalysts non-toxic and in addition the organo catalysts can be removed easily by precipitation or extraction. DBU acts as monofunctional catalysts, which means, that only the alcohol of the initiator is activated. TBD on the other hand is a so called bifunctional catalyst and activates initiator as well as monomer. This allows shorter reaction times with consistent molecular weight control. Yamaguchi and Iwasaki first reported the usage of these organocatalysts for the ring-opening polymerisation of cyclic phosphoesters.[16] The polymerisation of 2-isopropoxy-2-oxo-1,3,2-dioxaphospholane with DBU was carried out in bulk at 0°C, whereas the polymerisation with TBD was carried out in toluene. According to the fact, that the ring-opening polymerisation of cyclic phosphates needs highly anhydrous conditions, argon was used as inert gas. Polyphosphates with molecular weights up to 30,000 g/mol and a narrow distribution were obtained, but full conversion of monomers was not achieved.[16] Recently, Wooley and co-workers reported on the ring-opening polymerisation of a cyclic phosphate bearing a pendant alkyne chain by using DBU as catalyst.[23]

Lecomte and co-workers investigated a new organo-catalyst system for the polyphosphate preparation via ROP that consists out of DBU and thio-urea as co-catalyst.[4] This way, not only the hydroxyl group of the initiator but also the monomers are activated.



Scheme 14: Activation of monomers/initiator by different organo-catalysts[23]

2-isobutoxy-2-oxo-1,3,2-dioxaphospholane (*i*BP) was polymerised in toluene at 0°C with the DBU/TU co-catalyst system. Full conversion was achieved after 80 minutes and polymers with molecular weights up to 67,000 g/mol and molar mass distributions around 1.06 were obtained.[23] Transesterification as side reaction was avoided so far.

1.2.2 Applications for polyphosphoesters

As already mentioned, investigation on PPEs originated for flame-retardation applications by Penczesk *et al.* around 1950.[6] But according to their properties such as biodegradability, biocompatibility and structural similarity to naturally occurring nucleic acids, PPEs have been studied as biomaterials for tissue engineering, drug and gene delivery more recently. One example is the poly(lactide-*co*-ethylphosphate) copolymer, that has been used as drug carrier for paclitaxel in preclinical and clinical studies.[6]

Paclitaxel is widely used as a chemotherapeutic in the treatment of breast and ovarian cancer, but also for lung, head and neck cancer. Paclitaxel binds along microtubules and supresses their dynamics, which leads to mitotic arrest and apoptosis in dividing cells.[24]

One example for paclitaxel loaded copolymer is PACLIMER® that contains 10% (w/w) of the drug. When the reclusive chemotherapeutic is injected intravenously, a high plasma concentration is the consequence, followed by rapid exponential decay. When paclitaxel is injected in form of PACLIMER®, the chemotherapeutic is released continuously and the concentration maintained at a therapeutical level over weeks.[6]

1.3 Cell-penetrating peptides

To act as therapeutic agents, drugs have to be delivered into the inside of a cell. For that, there are many peptides known, which have cell-penetrating abilities because of their specific amino acid sequence.

Biological membranes are mainly composed of proteins and amphiphatic lipids, which assemble spontaneously into a bilayer. This bilayer acts as a solvent for the membrane proteins[25] and forms a barrier between the cytosolic and extracellular part of a cell. This separation is essential for life, but also makes high demands on the delivery of therapeutic agents into cells. Therefore, the discovery of peptides that translocate across the plasma membrane of cells and permit intracellular transport of conjugated therapeutics has opened new possibilities in biomedical research.[26]

1.3.1 Endocytosis versus penetration

Endocytosis means the uptake of extra-cellular nutrients into the inside of the cell and is also utilized by viruses, microorganisms and toxins to achieve entry into a cell.[27] The most popular endocytotic pathway is the receptor-mediated one via clathrin-coated pits. The mechanism of endocytosis includes the absorption to the plasma membrane (or a membrane-bound receptor) and is followed by a vesicle formation that is energy-dependent. Usually, hydrophilic macromolecules enter a cell this way.[26] After vesicle-formation, the uptaken molecules are sorted into different compartments that are tagged for destruction or recycling. As shown in Figure 1, also the clathrin molecules can be recycled for further endocytosis.

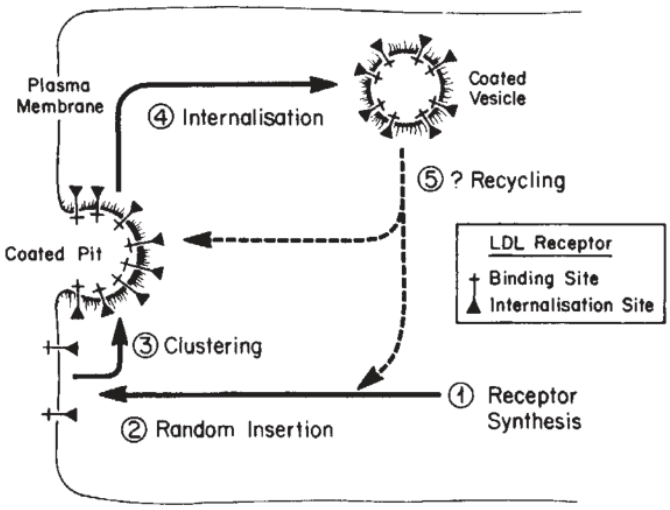


Figure 1: mechanism of clathrin-mediated endocytosis[28] (LDL=low density lipoprotein)

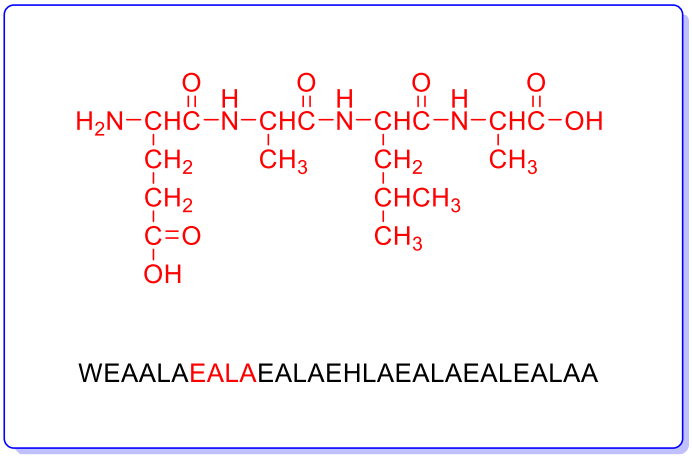
According to the fact, that release of the up-taken molecules into the cytoplasm is rare, endocytosis is not qualified for the delivery of biomacromolecules into the cytoplasmic or nuclear compartments.[26]

Cell-penetration on the other hand is an energy independent mechanism, in which a peptide is translocated across the membrane. This opens the possibility of a release of conjugated loads into the cytoplasm and nucleus of the cell. Due to the fact, that no receptor is needed for that process, cell-penetration is not cell-specific and allows the delivery of hydrophilic macromolecules into any cell-type.[26]

1.3.2 GALA and KALA

Examples for cell-penetrating peptides are synthetic GALA and KALA. Both consist out of thirty amino acids.

The name GALA derives from the peptides repeating unit glutamic acid-alanine-glutamic acid-leucine.



Scheme 15: repeating unit of GALA and one-letter code

Thanks to particular amino acids within the repeating unit, there are several features combined in the peptide GALA. Glutamic acid imparts pH-dependency, alanine imparts high potential to form an α-helix and acts as a spacer between the polar and apolar parts and leucine adds high hydrophobicity. Alltogether, this results in different secondary structures for different pH-values. When the pH of a system is decreased from 7.5 to 5, one can observe a change in conformation from random coil to an α-helix.

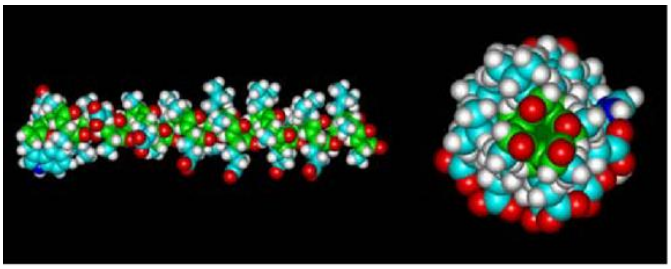
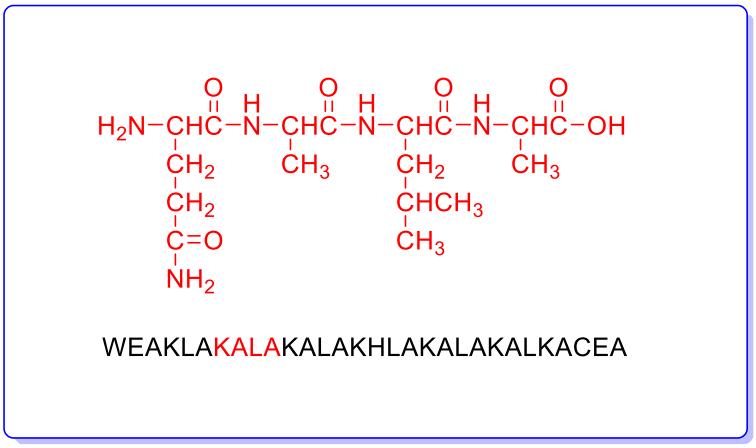


Figure 2: GALA in helical conformation[25]

One explanation for this behaviour could be the degree of protonation of glutamic acid.[25] At neutral pH, electrostatic repulsion between deprotonated carboxylic acid moieties is expected to destabilize the helix, but when these moieties are protonated at pH 5 the formation of an α-helix is promoted.

Ten GALA helices are able to form a transmembrane peptide pore with hydrophobic parts on the outside in contact with the lipid bilayer and hydrophilic parts inside the pore.

KALA is like GALA a synthetic cell-penetrating peptide that also consists of thirty amino acids.[25] The only difference is the change of glutamic acid with lysine within its repeating unit.



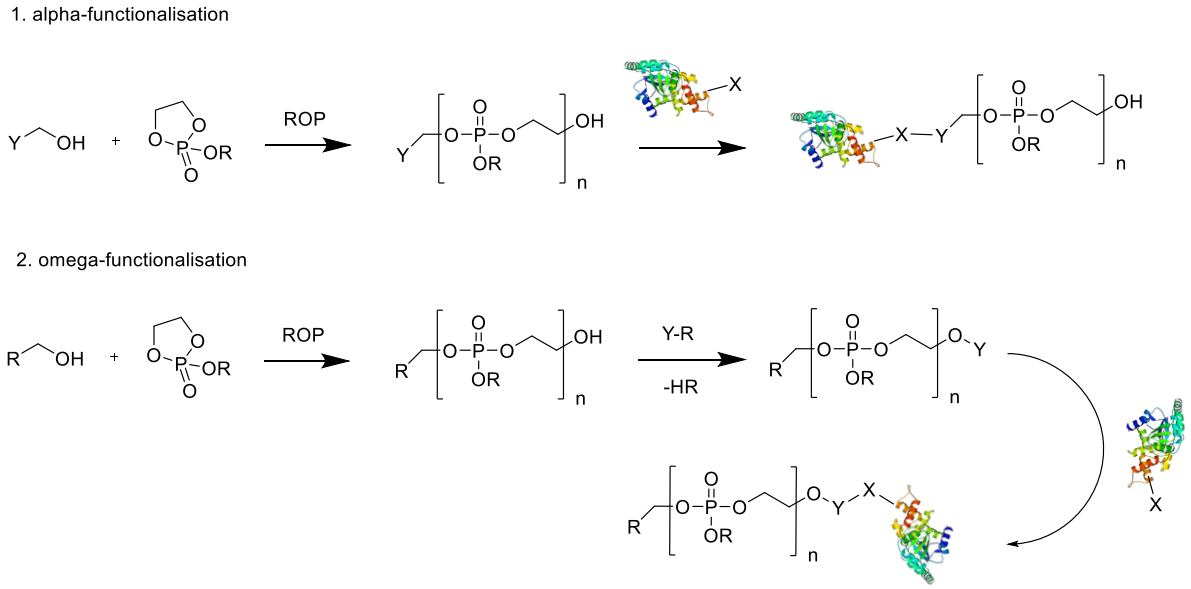
Scheme 16: repeating unit of KALA and one-letter code

The peptide is also able to change its conformation, but vice versa.[29] When the pH of a system is increased from pH 5 to 7.5 there is also a change in secondary structure from random coil to α-helix. The great benefit of KALA in contrast to GALA is its ability to bind DNA or *si*RNA. Under physiologically conditions, lysine is protonated and according to this positively charged. This offers the ability to bind to the negatively charged parts of nucleic acid and also of intracellular trafficking.[25,29]

1.4 Bioconjugation of peptides or proteins

Peptides and proteins offer a wide range of possibilities for covalent linking due to their diversity of functional groups within the specific side chains of the single amino acids and the functionality of the peptides *C*- and *N*-termini.

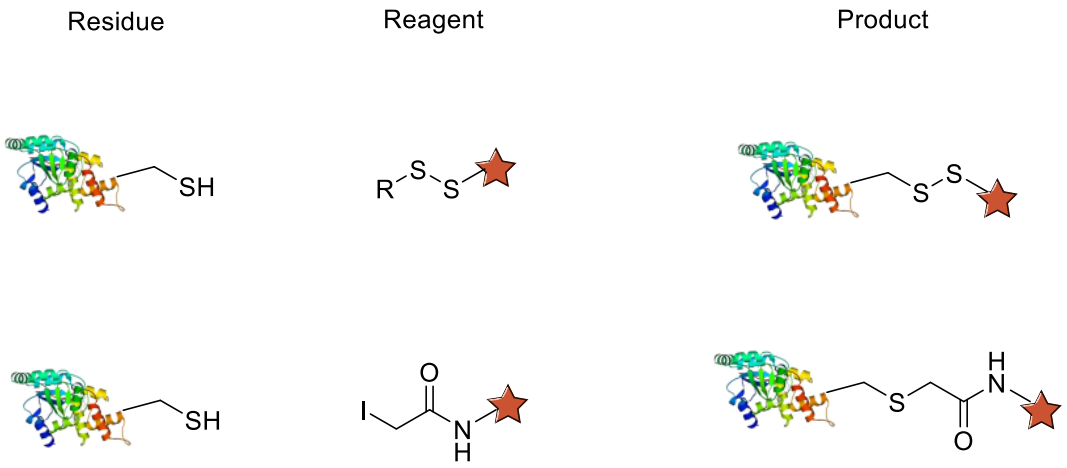
For covalent bioconjugation of polyphosphates with peptides, the polymer has to be activated by functionalization. In general, there are two different ways of introducing functional groups into a polymer chain, namely α- and ω-functionalization (Scheme 17).



Scheme 17: pre- and post-functionalization of polyphosphates

In Scheme 17 the two possible ways of activating of polyphosphates are shown. On the one hand it is possible to introduce a functional group within the initiator and start polymerisation. This method is called alpha-functionalization. On the other hand it is possible to post-modify the polymer after synthesis via modification of the terminal hydroxyl group. This method is therefore called omega-functionalization.

Cysteine and lysine are the most often modified residues within a protein. Examples for addressing the thiol group of a cysteine side chain within a peptide are shown below.

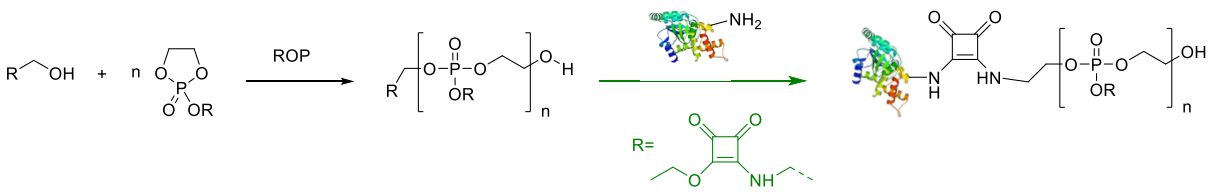


Scheme 18: bioconjugation with peptides adressing cysteine[30]

As shown in Scheme 18, the thiol group of cysteine can form a disulfide with disulfide modified polymers by undergoing a disulfide exchange. In this thesis, the residue will be a pyridine-residue in order to obtain 2-mercaptopyridine as by-product. This substance is UV-active and allows the observation of the educts´ conversion.

In addition, Michael-addition with a,b-unsaturated carbonyl compounds and the thiol group is possible, which leads to thioethers.[30]

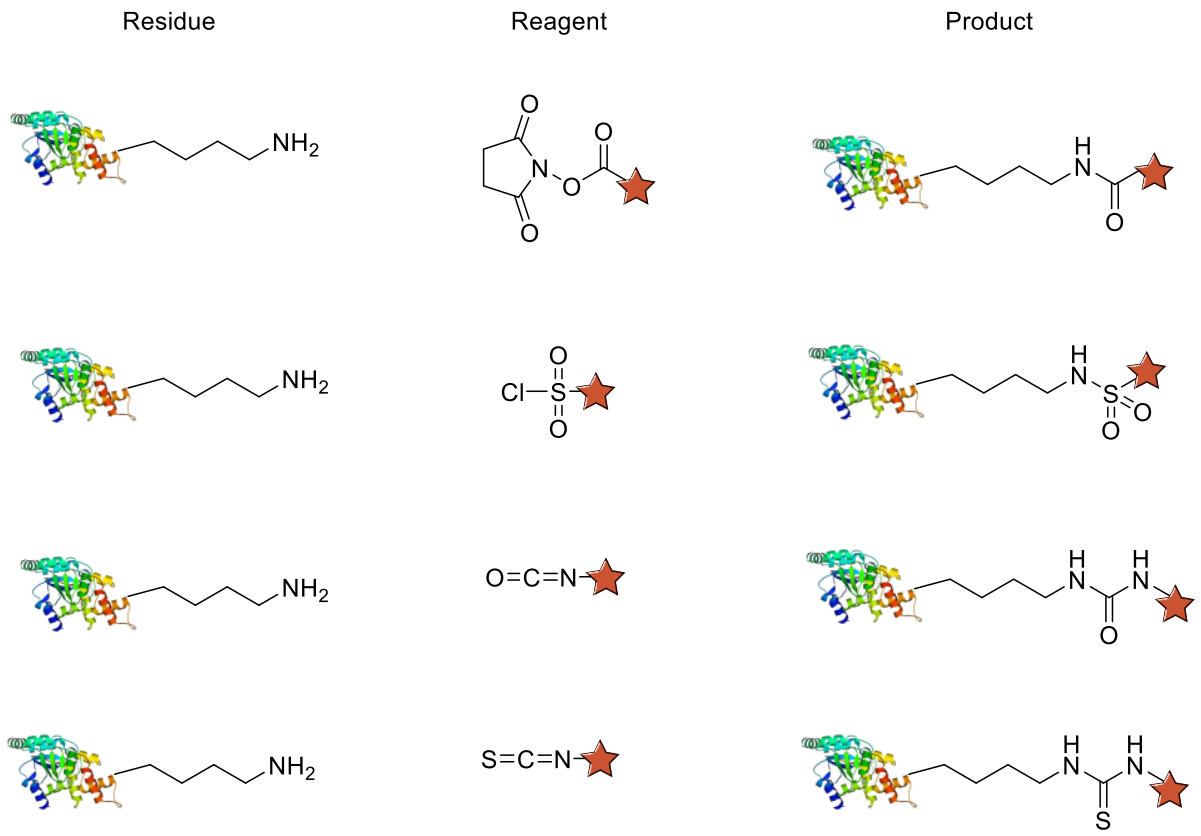
Lysine side chains can be addressed by many functional groups. Wurm *et al.* used initiators based on squaric acid for the ring-opening polymerisation of cyclic phosphates in order to introduce amine-sensitive groups into the polymer chain.[31,32] Benefits of squaric acid derivatives are according to their work a high chemoselectivity towards amino groups and a long stability against hydrolysis.



Scheme 19: Bioconjugation with squaric acid containing initiator

Other classical bioconjugation reactions addressing the lysine side chain underlie the omega-functionalisation of polymers. This way, amides, urea, thiourea and sulfonamides can be formed by modifying lysine residues. As shown in Scheme 20, the terminal hydroxyl groups of the polymer have

to be modified as *N*-hydroxysuccinimide-activated esters, sulfonyl chlorides, isocyanates or isothiocyanates.



Scheme 20: classical bioconjugation reactions with lysine residues[30]

So far, alpha- and omega-functionalization have been discussed separately, but it is also possible to combine these two methods. For example, when propargyl alcohol is used as initiator for the ring-opening polymerisation of phosphates, the triple bond can be used for further modification via click chemistry with azide-functionalized peptides. In addition, it is also possible to functionalize the polymers terminal hydroxyl group in order to address lysine residues within a peptide. This way, polymer-peptide conjugates with more than one attached peptide are formed.[23]

2. Perspective

The conjugation of polymers with drugs is a widely used method to increase solubility and life-time in blood-circulation of several drugs. The most famous example is PEGylation of drugs. Three decades ago, PEG was accepted as drug delivery system for protein therapeutics, but in our days, PEGylation of proteins and peptides is used to transform those drugs into even more potent ones.[33]

By covalent linking of poly(ethylene glycol) with drugs, their solubility as well as stability are increased. In addition, passive targeting due to the EPR-effect (enhanced permeability and retention effect) is possible.[34] The EPR-effect means the passive accumulation of the conjugate in tumor-tissue due to a different physiology and bio-chemistry of this tissue compared to healthy one. One can compare this phenomenon with a filter.

A huge disadvantage of this method is the already mentioned fact, that PEG is not degradable. The daily up-taken amount of this polymer in form of food, drugs and so on is stored in vacuoles in human body. There are already some studies on mice that address this problem in order to study, how this storage will affect the human body. It is already shown, that the kidney is attacked by PEG, but it is not completely clear yet, what PEG-storage means to the human body in a long term view.[3]

Polyphosphates on the other hand are bio-degradable and show great potential for biomedical applications. The main motivation of this thesis is to create a new class of drug-delivery systems by combining the properties of aforementioned polymers with cell-penetrating peptides. These drug delivery systems offer many options of fine tuning. Especially *si*RNA or plasmid-DNA are supposed to bind non-covalently to the positively charged parts of the peptide. This method would avoid covalent modification of drugs, which often comes along with a change in activity.[33]

The water-soluble polyphosphates cause enhanced solubility of the drug delivery system and longer lifetime in blood-circulation. Due to the fact that these polymers can be hydrolysed easily by enzymes, accumulation in human body is avoided and therefore those polymers outplay PEG on that score.

Cell-penetrating peptides on the other hand are necessary for enhanced cellular uptake by percolating biological membranes.

Polyphosphates as well as peptides can be varied in structural features to optimize transport and cellular uptake of drugs. Another possible modification would be the coupling of polyphosphate-peptide-conjugates with antibodies. This way, active targeting with selective release of the bioactive compound can be achieved.

For this purpose, the following tasks need to be fulfilled:

- Two different phosphate monomers, namely ethyl ethylene phosphate (EEP) and 2-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)-2-oxo-1,3,2-dioxaphospholane (GEP), were synthesized
- The new monomer 2-(2-(benzyloxy)ethoxy)-1,3,2-dioxaphospholane-2-oxide was synthesized by Tobias Steinbach and polymerisation behaviour was under investigation as part of this work
- Different polymers and copolymers were synthesized by ring-opening polymerisation with stannous octoate as catalyst
- Protected polymers were deprotected either by hydrogenation or under acidic conditions
- The polyphosphates were conjugated to different peptides

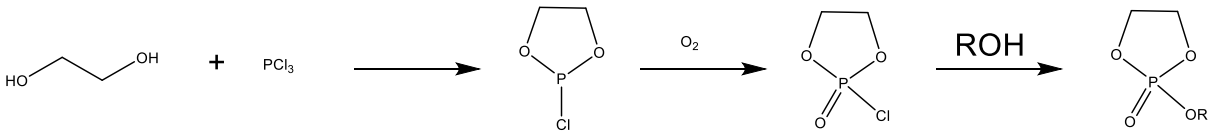
3. Results and Discussion

This chapter focusses on the synthesis and characterization of different monomers and their corresponding polymers, which were used for further modification, namely bio-conjugation with peptides and different deprotection methods. Benefits and draw-backs of the different synthetic strategies will be discussed.

3.1 Monomers

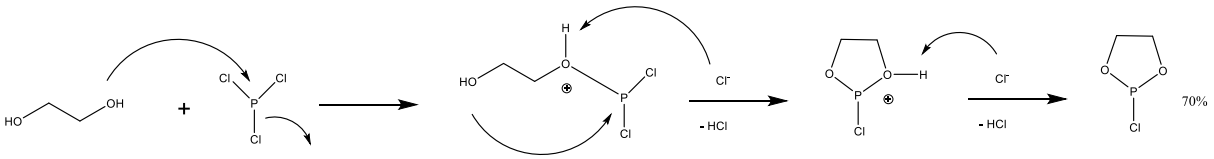
Three different cyclic ethylene phosphate monomers were synthesized to obtain water-soluble polyphosphates via ROP.

In general, the cyclic monomers can be synthesized within three steps:



Scheme 21: General scheme for the cyclic phosphate monomers used in this thesis

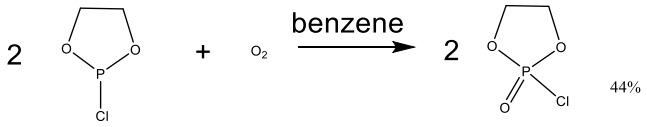
In a first step, a ring is formed via nucleophilic substitution. Phosphorous trichloride can be attacked twice by the two hydroxyl groups of ethylene glycol. Hydrogen chloride is released during the reaction, which was removed by flushing the reaction mixture with inert gas and afterwards neutralized with sodium hydroxide. Because of the fact that all three reaction steps are sensitive to air moisture, dry chemicals and inert gas atmosphere are required.



Scheme 22: mechanism of ring formation to obtain 2-chloro-1,3,2-dioxaphospholane

By fractional distillation under reduced pressure 70% yield were obtained, which is in good agreement with literature.

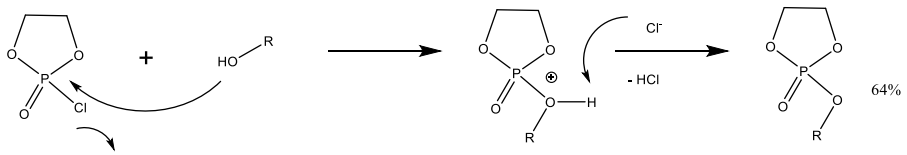
After the formation of 2-chloro-1,3,2-dioxaphospholane, the phosphorous atom is oxidized with oxygen to form 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP).



Scheme 23: Oxidation of 2-chloro-1,3,2-dioxaphospholane to 2-chloro-2-oxo-1,3,2-dioxaphospholane (COP)

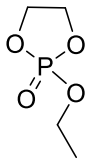
It is noteworthy here that the workup of this compound needs to be done highly carefully with any exclusion of moisture to achieve acceptable yields which are in most cases still lower than reported in literature.[35]

In a last step, the phosphorous can again be attacked by an alcohol acting as nucleophile to form an ester. Again, hydrogen chloride is formed during the reaction, which has to be removed from the reaction mixture. This time, pyridine is used to form an insoluble precipitate, which can be removed by filtration under inert conditions (Scheme 24). The product can be purified by fractional distillation under reduced pressure with reasonable yields between 37 and 64%.



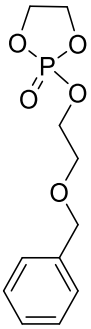
Scheme 24: Esterification of COP to EEP

In case of ethanol as nucleophile, 2-ethoxy-2-oxo-1,3,2-dioxaphospholane (EEP) is received (Scheme 25):



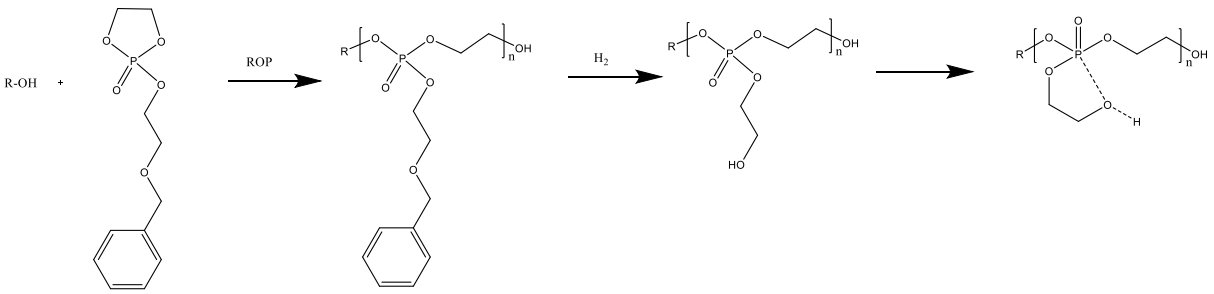
Scheme 25: ethyl ethylene phosphate (EEP)

In case of 2-(benzyloxy)-ethanol, 2-(2-(benzyloxy)ethoxy)-1,3,2-dioxaphospholane-2-oxide is received, that will furthermore also be called benzyl protected hydroxyl-ethyl ethylene phosphate monomer (HEEP) to simplify matters (Scheme 26):



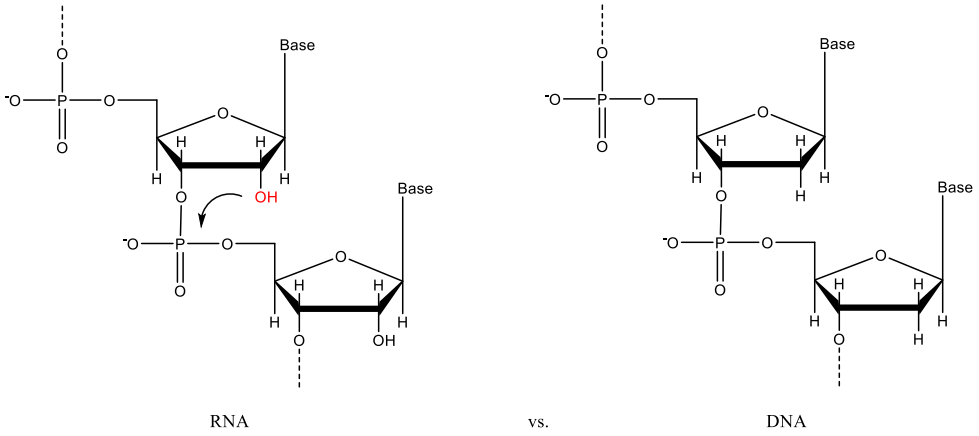
Scheme 26: 2-(2-(benzyloxy)ethoxy)-1,3,2-dioxaphospholane-2-oxide (HEEP)

The idea of HEEP is to increase the time of degradation of the polyphosphate. By removing the benzyl groups, there are more available hydroxyl groups, which increase the probability of hydrolysis. This is shown in Scheme 27.



Scheme 27: polymerisation of HEEP to P(HEEP) with following hydrogenation

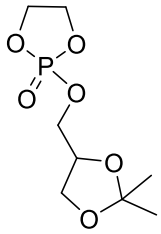
This phenomenon of an intramolecular nucleophilic attack of hydroxyl groups leading to degradation is inspired and comparable to the hydrolysis of human RNA.



Scheme 28: RNA hydrolysis

In Scheme 28 a part of a RNA- and accordingly a DNA-profile are shown. RNA has free available hydroxyl groups within the saccharides, which can attack the polyphosphate backbone leading to hydrolysis of the RNA-profile. DNA on the other hand misses those freely available hydroxyl groups and therefore does not undergo hydrolysis easily.

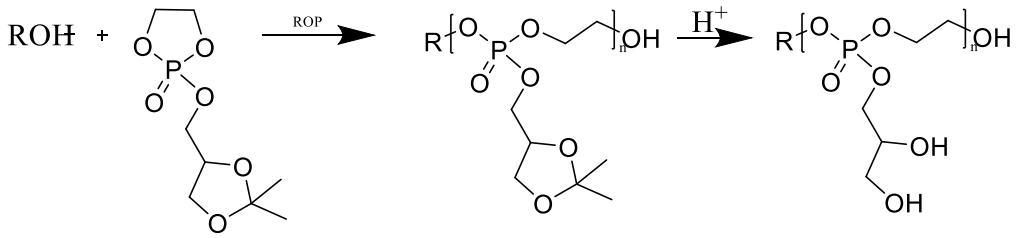
When using solketal ((2,2-dimethyl-1,3-dioxolan-4-yl)methanol) as the respective alcohol in the third step of the monomer synthesis, 2-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)-2-oxo-1,3,2-dioxaphospholane (GEP) is received as a cyclic monomer:



Scheme 29: 2-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)-2-oxo-1,3,2-dioxaphospholane (GEP)

In analogy to HEEP, GEP should be able to alter the rate of degradation of the corresponding polymer. Here, every monomer unit carries two hydroxyl groups and the deprotection is possible via slightly acidic conditions that are not too harsh to attack the polymer backbone. By combining the

HEEP and GEP monomers to copolymers, orthogonal deprotection should be possible to fine tune degradation times or to generate highly functional PPEs and will be discussed later on in this thesis.



Scheme 30: acidic hydrolysis of the acetal protective groups in P(GEP)

3.1.1 Monomer Characterization

The intermediate COP was characterised by NMR spectroscopy. The ^{1}H NMR spectrum, 300 MHz in $CDCl_3$ is shown below:

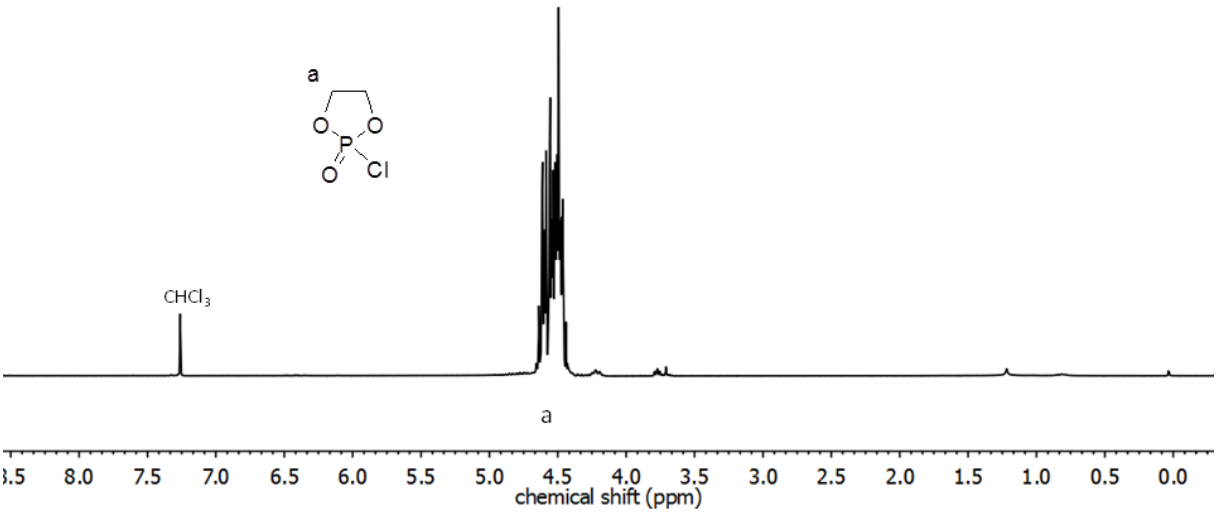


Figure 4: ^{1}H NMR of COP

All three monomers were fully characterised by NMR-spectroscopy. The corresponding ^{1}H NMR-spectra are described and shown below.

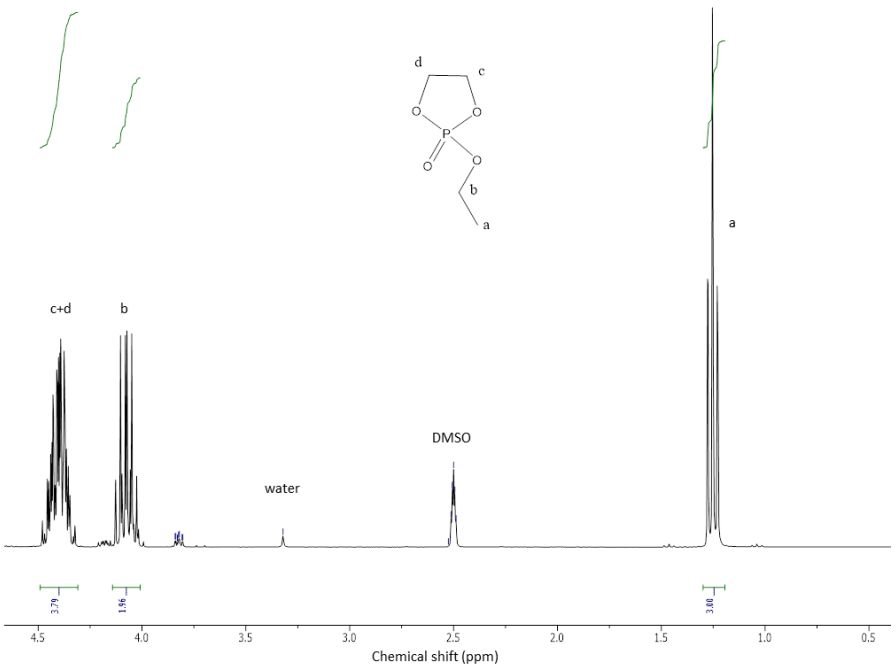


Figure 5: ^{1}H NMR EEP in DMSO-d_6

In Figure 5 the ^{1}H NMR spectrum of EEP is shown (300MHz in DMSO-d_6). The corresponding signals of the ring protons can be detected as multiplet between 4.32-4.48 ppm. Signal **b** belongs to the methylene group of the specific side chain of the cyclic monomer and splits up also in a multiplet due to ^{3}J coupling with P. The signal of the methyl group has a chemical shift of 1.25 ppm and is detected as a triplet. The ^{13}C NMR spectrum shows signals at 66.35 ppm corresponding to the ring protons, 64.18 ppm belonging to the methylene group and at a chemical shift of 15.96 ppm belonging to the methyl group. The ^{31}P NMR spectrum shows a specific single peak with a shift of 16.83 ppm.

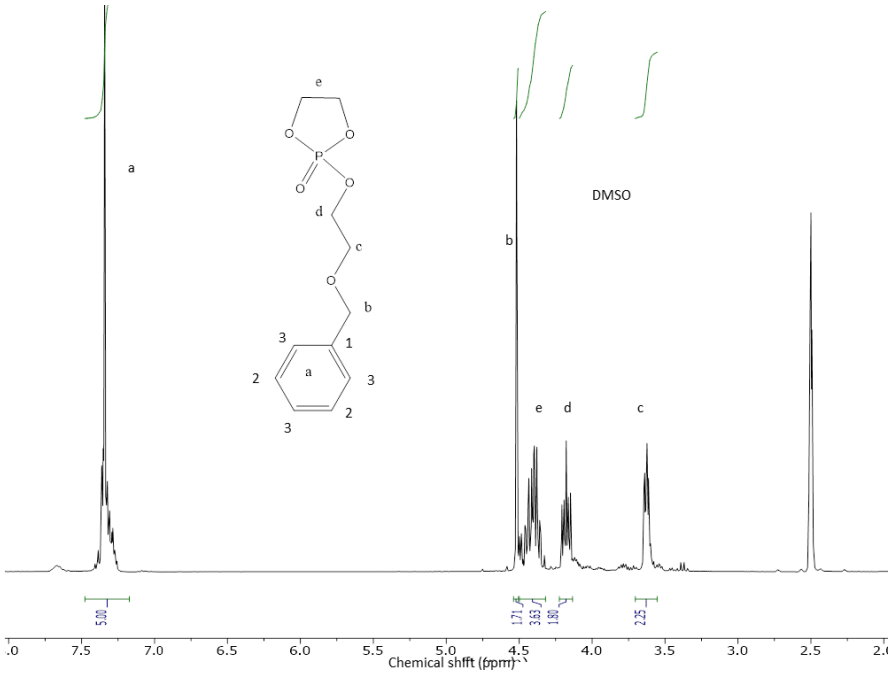


Figure 6: ^{1}H NMR of HEEP

In Figure 6 the ^{1}H NMR spectrum of HEEP is shown (300 MHz in DMSO-d_6). Again, the protons of the ethylene group **e** show the typical resonances between 4.28 and 4.50 ppm. Singlet **a** with a chemical shift of 7.34 ppm belongs to the aromatic protons of the monomer´s side chain as well as singlet **b** with a chemical shift of 4.52 ppm belonging to the methylene protons of the protecting group. The methylene groups **c** and **d** show resonances as mulitplets with shifts of 4.13-4.23 ppm and 3.56-3.69 ppm.

With ^{13}C NMR spectroscopy, the differentiation between the different aromatic carbons is possible. Carbon **a1** shows resonances at 138.13 ppm with lower intensity due to the Kern-Overhauser-effect. Carbon **a3** was detected at 128.21 ppm and carbon **a2** at a chemical shift of 127.47 ppm. According to their non-aromaticity, all other carbons were detected more upfield. The methylene carbon **b** of the protecting group shows resonances at 71.91 ppm and the ethylene carbons **e** at 68.57 ppm. The two methylene carbons **c** and **d** were detected at 67.18 and 66.38 ppm (Figure 7).

The ^{31}P NMR spectrum of HEEP shows again a single typical resonance at 17.01 ppm.

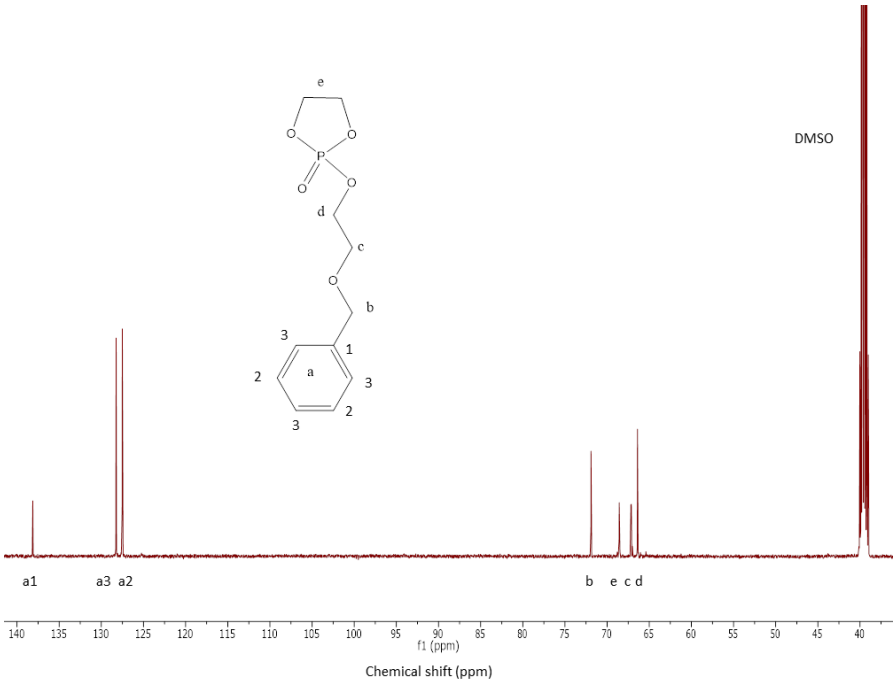


Figure 7: ^{13}C NMR of HEEP

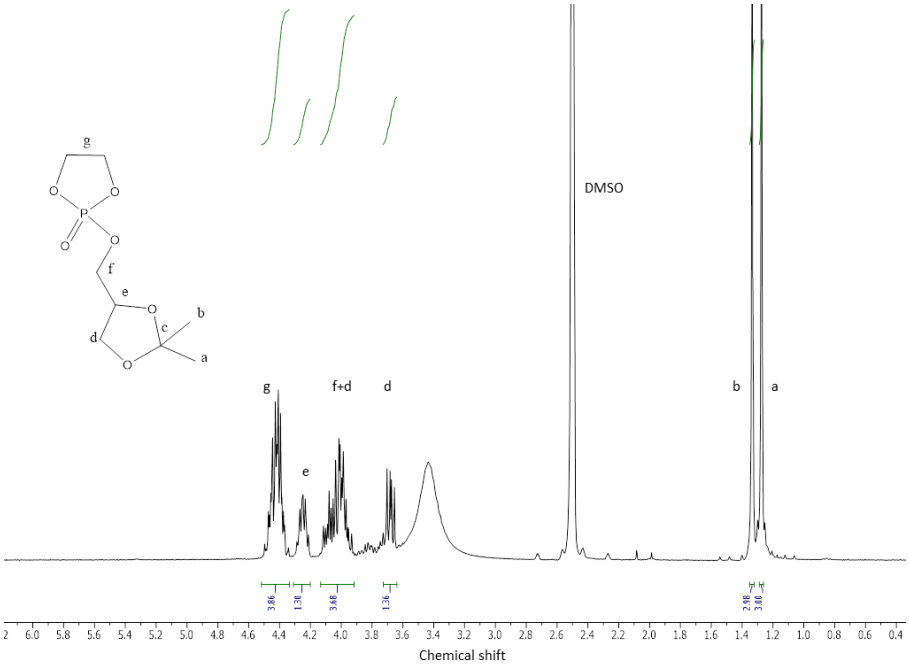


Figure 8: ^{1}H NMR of GEP

In Figure 8 the ^{1}H NMR spectrum of the GEP monomer is shown (300 MHz in DMSO-d_6). The ethylene protons show their typical resonance at 4.3-5.3 ppm. The multiplet with a chemical shift of 4.2-4.3

ppm corresponds to proton **e**. The signal of the two protons **d** is splitted. One signal interfers with the signal originated by the methylene group **f** and has a chemical shift of 3.92-4.15 ppm. The other signal originated by **d** has a chemical shift of 3.59-3.73 ppm. The two methyl groups **b** and **a** were detected as singlets at 1.34 and 1.27 ppm. Between 3.26 and 3.55 occurs a signal that belongs to water in the deuterated solvent.

The ^{13}C NMR spectrum shows resonances at 108.97 ppm for the quaternary carbon **c**, at 73.80 ppm for the tertiary carbon **e**, at 68.03 ppm for the methylene carbon **f** and at 66.50 for the two ethylene carbons **g**. Furthermore, the spectrum shows two more sharp signals in the upfield region for the two methyl carbons **b** and **a** with chemical shifts of 26.49 and 26.25 ppm.

The ^{31}P NMR spectrum of GEP shows the typical single resonance at 16.94 ppm.

3.2 Polymerisation of benzyl protected Hydroxy-ethyl ethylene phosphate (HEEP)

This chapter focusses on the polymerisation of the benzyl protected hydroxyl-ethyl ethylene phosphate monomer (HEEP) via ROP using different catalysts.

Iwasaki and Yamaguchi already reported the ROP of 2-isopropoxy-2-oxo-1,3,2-dioxaphospholane using 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).

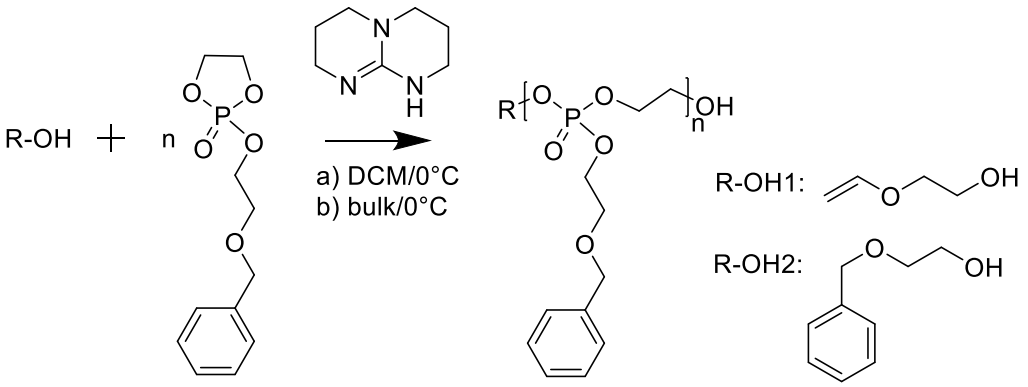
Wang *et al.* used stannous octoate to polymerize 2-ethoxy-2-oxo-1,3,2-dioxaphospholane.

These three different catalysts were used to analyse the polymerisation behaviour of HEEP and to optimize the reaction conditions.

3.2.1 Polymerisation of benzyl protected HEEP with 1,5,7-triazabicyclo[4.4.0]dec-5-ene

The ring-opening polymerisation of HEEP with TBD as a catalyst was conducted in dichloromethane or in bulk at 0°C with different initiators, namely hydroxyl ethyl vinyl ether (initiator 1) and benzyl oxy ethanol (initiator 2). Because of the sensitivity to moisture, the reaction had to be carried out under inert atmosphere with dried glassware and chemicals. After completion, the reaction mixture was quenched with an excess of acetic acid and the product was precipitated into cold diethyl ether. For

purification, the polymer was re-dissolved in dichloromethane and precipitated two more times into diethyl ether.



Scheme 31: Organocatalysed ROP of HEEP with TBD as catalyst to P(HEEP)

Table 1: P(HEEP) synthesis with TBD

entry	initiator	cat	solvent	c	T/°C	time	M/I	M_n(GPC)	PDI	yield/%
1.1	1	5mol%TBD	DCM	1,76	0	15min	15	1200	1,24	26
1.2	1	10mol%TBD	DCM	1,76	0	2h	15	1000	1,17	~40
1.3	1	10mol%TBD	DCM	1,76	0	2h	30	1100	1,15	~40
1.4	2	5mol%TBD	no		0	2h	15	1400	1,41	80
1.5	2	5mol%TBD	no		0	2h	30	2100	1,53	40

As shown in Table 1, full conversion of the monomer was not achieved. This is indicated by the low obtained yields of polymer. One considered explanation could be that precipitation in diethyl ether is not working well. But this idea contradicts with nearly 100% yield of polymer in other samples (see below). Also with extended reaction times no full conversion was achieved which in addition lead to broadened PDIs.

The ^{1}H NMR and GPC data of entry 1.4 are shown exemplary below:

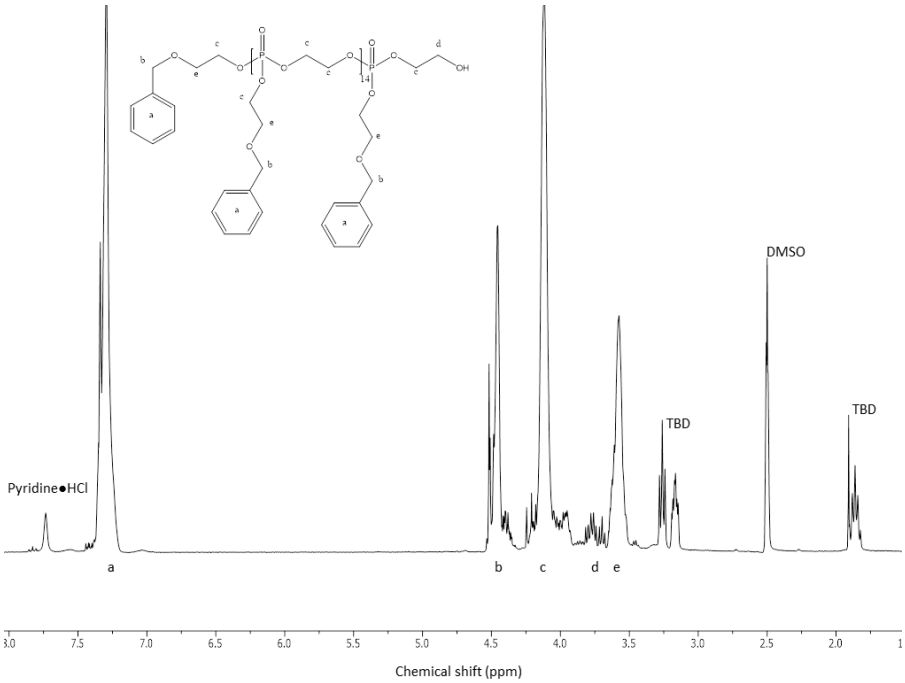


Figure 9: ^{1}H NMR spectrum of entry 4 in DMSO-d_6, 300MHz

The aromatic protons of the initiator and the pendant side chain show resonances as a multiplet at a chemical shift between 7.17-7.44 ppm. The signal at 4.31-4.66 ppm belongs to the methylene groups **b** and the multiplet with a chemical shift of 3.91-4.25 ppm corresponds to the methylene groups **c** of the polymer backbone as well as the side chain. The methylene group **d** next to the hydroxyl end-group shows discrete resonances at 3.65-3.84 ppm as well as the protons **e** of the pendant side chain at 3.52-3.64 ppm.

As shown in Figure 9, the catalyst TBD could not be removed by precipitation.

The GPC elugram measured in DMF shown in Figure 10 is indicating the formation of polymer with a monomodal molecular weight distribution, but also the presence of oligomers at higher elution times.

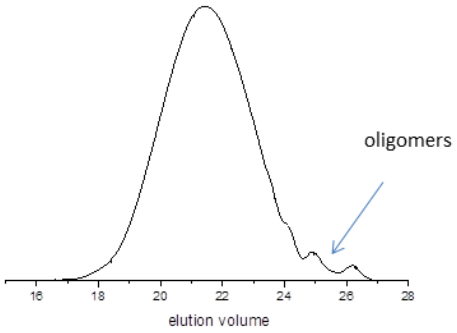
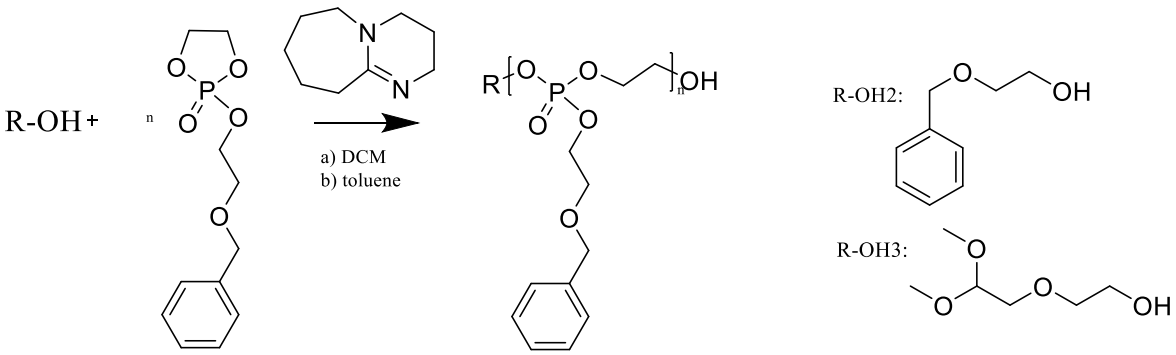


Figure 10: GPC-elugram (in DMF, RI detection) of P(HEEP) (Table 1, entry 1.4)

3.2.2 Polymerisation of benzyl protected HEEP with 1,8-diazabicyclo[5.4.0]-undec-7-ene

The ROP of HEEP using DBU as a catalyst was conducted in different solvents and with different initiators, namely benzyl oxy ethanol (initiator 2) and a protected aldehyde initiator (2-(2,2-dimethoxyethoxy)ethan-1-ol) initiator 3).



Scheme 32: ROP of HEEP with DBU as catalyst to form P(HEEP)

Table 2 sums up the different preparations that were used to polymerize HEEP with DBU as catalyst. Different solvents as well as different reaction temperatures were tested in order to optimize the polymerisation conditions.

Table 2: polymerisation of HEEP using DBU

entry	initiator	cat	solvent	c	T/°C	time	M/I	M_n(GPC)	PDI	yield/%
2.1	2	5eq DBU	DCM	4	RT	17,75h	15	1300	1,81	100
2.2	2	5eq DBU	DCM	4	RT	17,75	30	1500	1,30	87
2.3	2	5eq DBU	Toluene	4	50	20min	15	1500	1,45	100
2.4	2	5eq DBU	Toluene	4	50	20min	30	1400	1,27	88
2.5	3	5eq DBU	Toluene	4	40	19min	15	1300	1,45	100
2.6	3	5eq DBU	Toluene	4	40	22min	30	1400	1,21	85
2.7	3	5eq DBU	Toluene	4	40	20min	15	1500	1,34	98
2.8	3	5eq DBU	Toluene	4	40	30min	30	1500	1,26	97

Full conversion was achieved at higher temperature within 20 till 30 minutes, depending on the monomer to initiator ratio. In analogy to the preparation of the polymer with TBD, the PDIs are still rather broad for a controlled polymerisation; however, the GPC elugrams (an example is shown in Figure 12) show the formation of a monomodal polymer distribution with the presence of some oligomers.

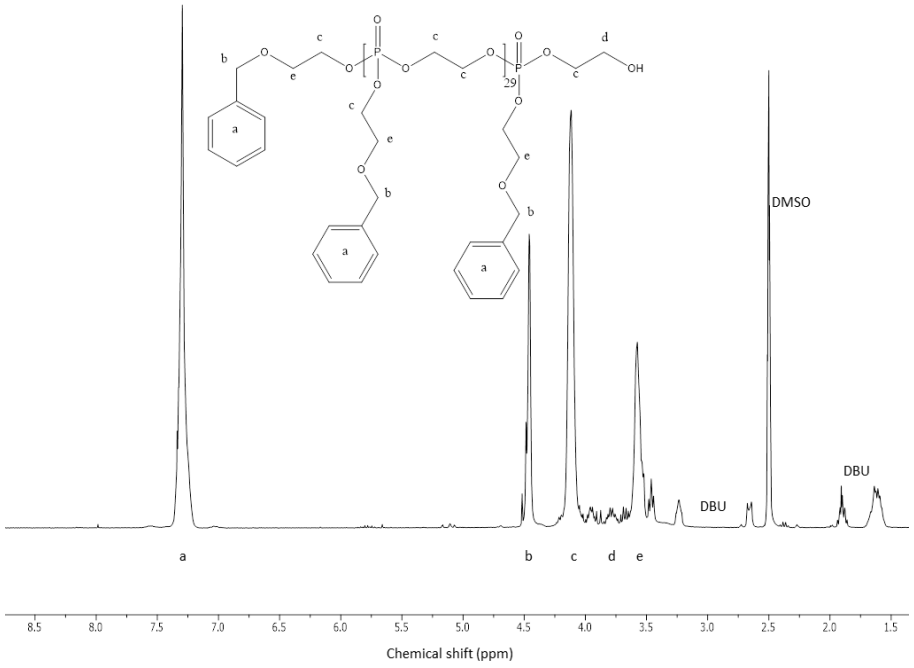


Figure 11: ^{1}H NMR of P(HEEP) (Table 2, entry 2.4) in DMSO-d_6

The ^{1}H NMR spectrum shows the same characteristic peaks as before, also with the catalyst still remaining in the product after precipitation into diethyl ether. To efficiently remove DBU, a further

work-up procedure is necessary. When re-dissolving the polymer in dichloromethane, extraction with slightly acidic (HCl) aqueous solution three times completely removes remaining catalyst, which is important for storing and preventing trans-esterification.

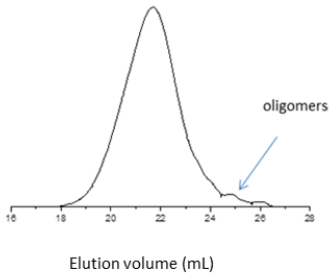
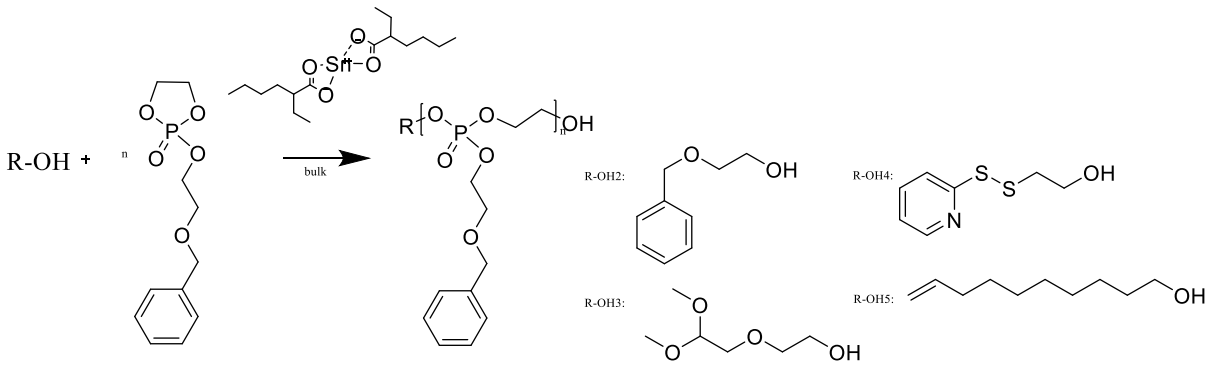


Figure 12: GPC elugram (in DMF, RI detection) of P(HEEP) (table 2, entry 2.4)

3.2.3 Polymerisation of benzyl protected HEEP using stannous octoate

$Sn(Oct)_2$ is a classical catalyst for the ROP of cyclic esters; polymerisations are usually conducted in bulk at elevated temperature (90°C). Different initiators were used, namely benzyl oxy ethanol (initiator 2), 2-(2,2-dimethoxyethoxy)ethan-1-ol (initiator 3), hydroxy-ethyl pyridyl disulfide (initiator 4) and dec-9-en-1-ol (initiator 5).



Scheme 33: polymerisation of HEEP using stannous octoate as a catalyst

Table 3 gives an overview of the different preparation methods that were used to obtain P(HEEP) using stannous octoate as catalyst.

Table 3: P(HEEP) synthesis using stannous octoate

entry	initiator	cat	solvent	T/°C	time	M/I	M_n (GPC)	PDI	yield/%	M/I (NMR)
3.1	2	Sn(Oct)2	no	90	2h	15	700	1,18	39	
3.2	2	Sn(Oct)2	no	90	5,75h	15	1400	1,08	78	
3.3	2	Sn(Oct)2	no	90	4,67h	15	1000	1,21	78	
3.4	2	Sn(Oct)2	no	90	4,67h	30	1000	1,23	77	
3.5	3	Sn(Oct)2	no	90	6h	15	1200	1,18	70	
3.6	3	Sn(Oct)2	no	90	6,25h	30	1200	1,26	~74	
3.7	4	Sn(Oct)2	no	90	6h	15	800	1,31	97	16
3.8	5	Sn(Oct)2	no	90	6h	15	1000	1,36	75	12
3.9	5	Sn(Oct)2	no	90	21h	90	1100	1,56	60	53
3.10	5	Sn(Oct)2	no	90	21h	150	1000	1,60	29	55

For the low molecular weight polymers, full conversion was achieved with low PDIs. The reaction times are however longer compared to the polymerisations that were catalysed with TBD or DBU. A benefit of stannous octoate is that the catalyst can be easily removed by precipitation and NMR studies show, that there is no catalyst remaining in the product after precipitation without further work up (Figure 13).

In addition, some polymers with high molecular weights were targeted (entries 3.9 and 3.10). From this it is clear, that achievable molecular weights are limited with this method and even after 21 hours, only about 50 monomer units build up the polymer chain. In addition, the apparent molecular weights measured by GPC are all very similar indicating interaction with the column material and thus producing underestimated values (in all cases).

The ^{1}H NMR and GPC data of entry 3.7 are shown exemplary below:

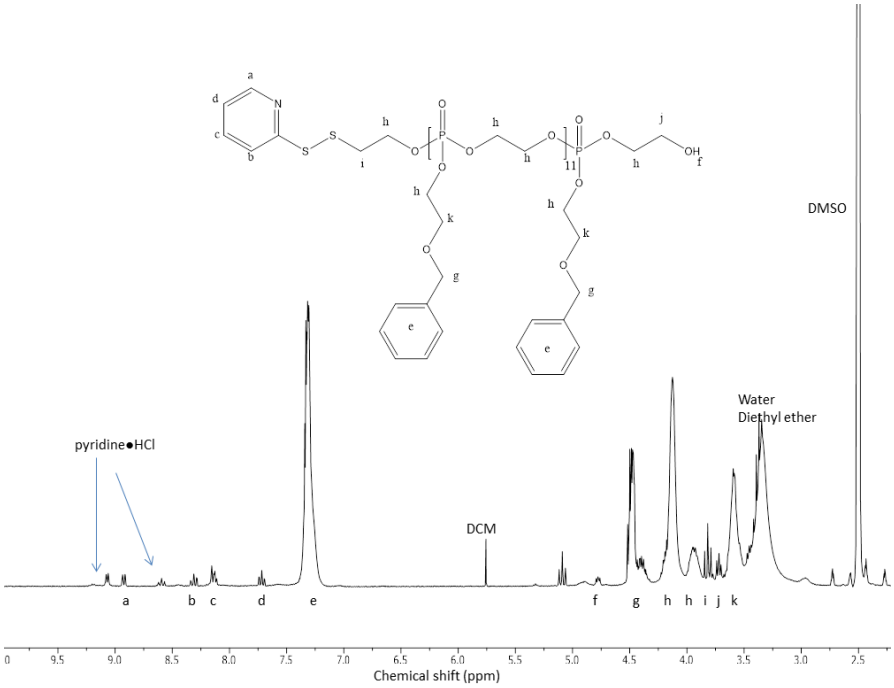


Figure 13: ^{1}H NMR of P(HEEP) polymerised with Sn(Oct)$_2$ (entry 3.7, DMSO-*d*$_6$, 300 MHz)

The resonances of the protons of the polymer backbone as well as the ones of the side chain are similar to previous shown ones. In addition, the terminal hydroxyl group **f** shows a triplet at 4.76-4.80 ppm and each aromatic proton of the initiator shows a signal in the region between 7.65 and 8.91 ppm. There are also some remaining monomer impurities visible in the aromatic region.

The GPC elugram measured in DMF is shown in Figure 14.

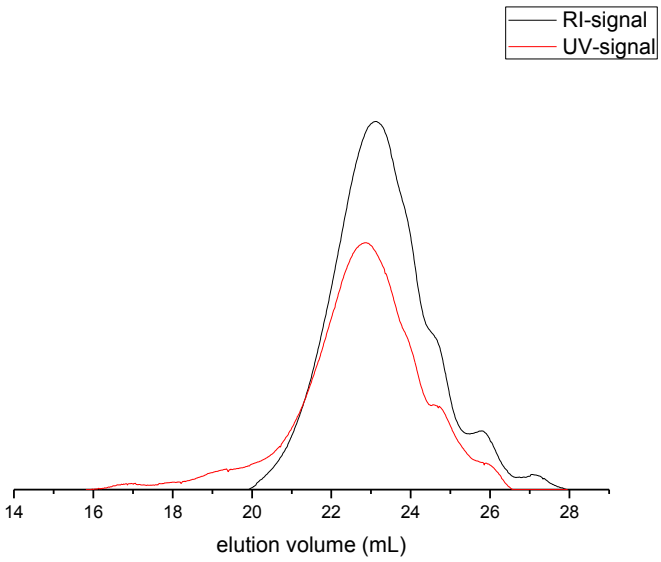


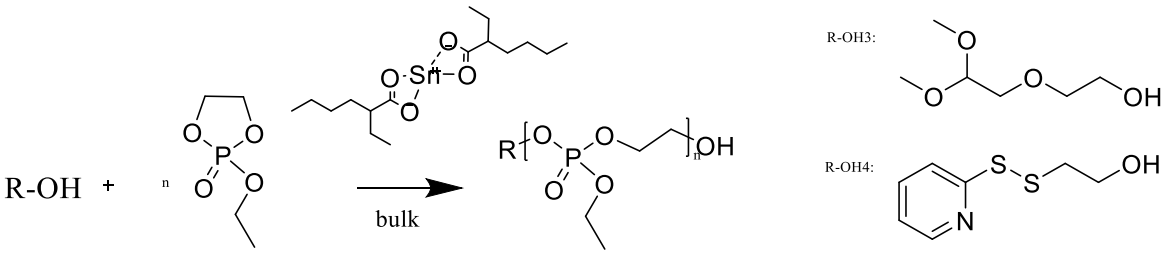
Figure 14: GPC elugram of P(HEEP) (Table 3, entry 3.7)

According to the aromatic groups within the polymer, it is also possible to measure the molecular weight distribution of the polymer with an UV detector. Both elugrams overlap with each other indicating that the initiator is incorporated into the polymer, however smaller oligomers can be detected.

All in all, the polymerisation of the benzyl protected hydroxyl-ethyl ethylene phosphate with molecular weight control has been successful. Stannous octoate is the outstanding catalyst, because the molecular weight distribution of the resulting polymers was very narrow and in addition, the catalyst could be fully removed during precipitation. This is important in regards of the pyridyl-disulfide initiator that is very sensitive.

3.3 Polymerisation of ethyl ethylene phosphate

This chapter focusses on the polymerisation of EEP, which were all done with stannous octoate as catalyst under the same conditions HEEP was polymerised (compare Table 4).



Scheme 34: ROP of EEP using stannous octoate as catalyst to obtain P(EEP)

Table 4: polymerisation of EEP with stannous octoate

Entry	initiator	solvent	T/°C	time	M/I	M_n (GPC)	PDI	Yield/%	M/I (NMR)	M_n (NMR)
4.1	3	no	90	3.3h	15	1300	1.54	96		
4.2	4	no	90	2h	15	1900	1.53	95	23	3700
4.3	4	no	90	3h	30	2600	1.60	87	50	7800
4.4	4	no	90	2h	15	1800	1.77	94	17	2800
4.5	4	no	90	2h	65	1900	1.68	96	50	7800
4.6	4	no	90	1.5h	15	1500	1.66	97	20	3200
4.7	4	no	90	1.5h	15	1400	1.69	95	20	3200

Compared to the HEEP monomer, full conversion was achieved within shorter reaction times, however, the molecular weight distributions are generally higher than for HEEP. This indicates that more side reactions like trans-esterification take place during the polymerisation. Apparently, the sterically demanding side chain of the HEEP monomer hinders nucleophilic attacks on the polymer backbone, while the smaller ethyl group allows transesterification and thus the formation of branched polymers.

^{1}H NMR data, measured in DMSO-d_6 (300 MHz), of entry 4.2 is shown exemplary:

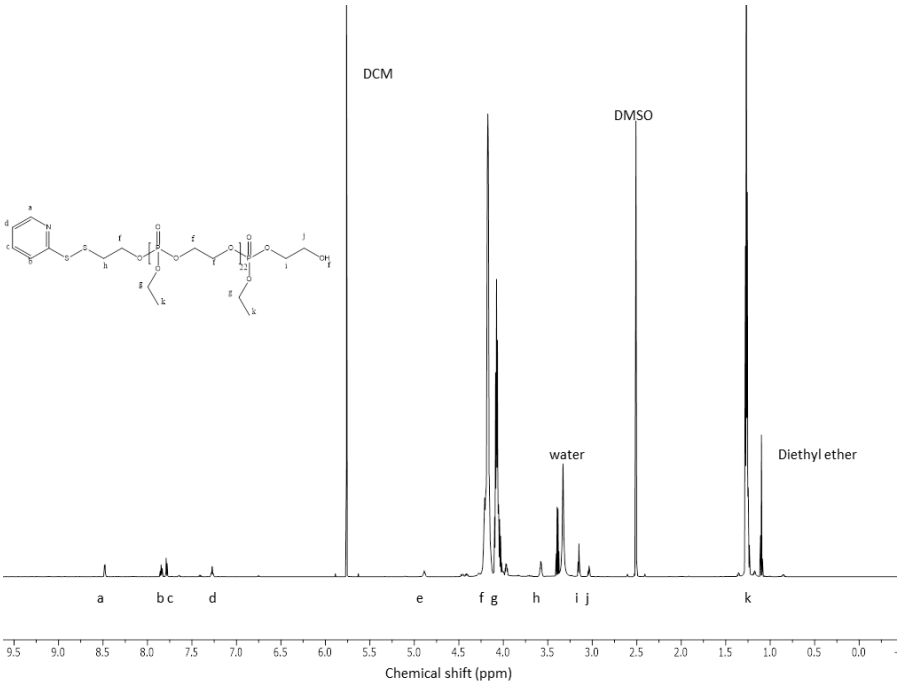


Figure 15: ^{1}H NMR of P(EEP) in DMSO-d_6 (table 4, entry 4.2)

The characteristic signal of the methylene groups (**f**) in the backbone can be found like in previous ^{1}H-NMR spectra as a multiplet at 4.17 ppm. The methylene groups (**g**) of the pendant side chain show resonances at 4.07 ppm as a multiplet due to coupling to phosphorus. The methyl group (**k**) was detected as a multiplet between 1.25 and 1.27 ppm. Again, the hydroxyl group **e** shows resonances at 4.89 ppm.

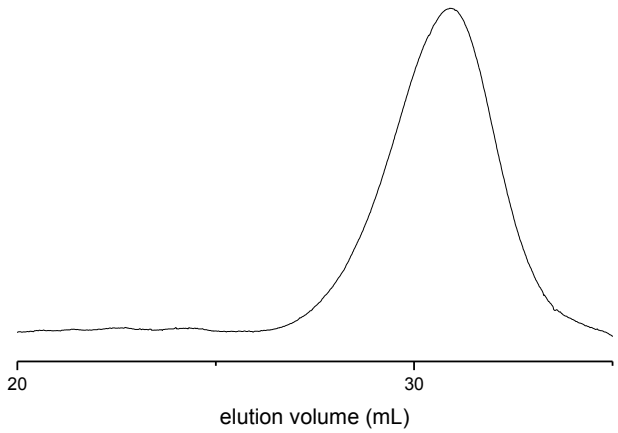


Figure 16: GPC elugram (DMF, RI-detection) of P(EEP) (Table 4, entry 4.2)

The GPC elugram measured in DMF in Figure 16 shows, that there were no oligomers formed during the polymerisation.

To be sure, that the initiator was incorporated intactly, a ^{1}H DOSY NMR in DMSO-d_6 (700MHz) was measured:

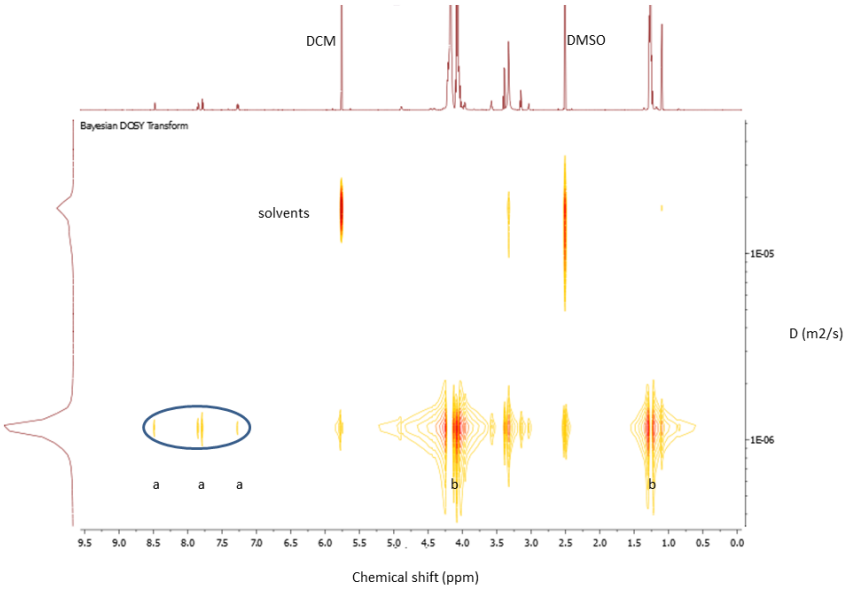
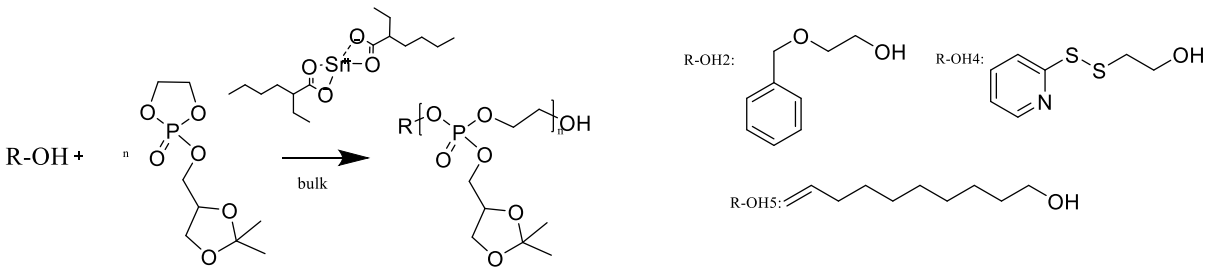


Figure 17: ^{1}H DOSY NMR of P(EEP) in DMSO-d_6 (Table 4, entry 4.2)

The aromatic protons **a** of the initiator have the same diffusion coefficient as the protons **b** of the polymer backbone. This proves, that the initiator is incorporated into the polymer what is required for the conjugation with the peptides.

3.4 Polymerisation of GEP

This chapter focusses on the polymerisation of the GEP monomer using stannous octoate. According to Wang *et al.* all polymerisations were done in bulk at 90°C.[36] Three different alcohols were used as initiators, namely dec-9-en-1-nol (initiator 5), benzyl oxy ethanol (initiator 2) and hydroxy-ethyl pyridyl disulfide (initiator 4).



Scheme 35: polymerisation of GEP with stannous octoate to obtain P(GEP)

The table below gives an overview on the different preparation methods that were tested to optimize the reaction conditions of the polymerisation.

Table 5: P(GEP) preparation methods with stannous octoate

Entry	initiator	M/I	time	M/I (NMR)	M_n (NMR)	PDI	Yield
5.1	5	15	3h	-	-	1.44	72%
5.2	5	30	5h	-	-	1.55	30%
5.3	2	15	3h	20	4900	1.44	54%
5.4	2	30	14h	40	9700	1.75	96%
5.5	4	15	2.5h	20	4900	1.39	63%

Full conversion of the monomer using stannous octoate was achieved within a few hours with a narrow molecular weight distribution. Even for entry 5.4, when the reaction was run for 14 hours, the PDI is in an acceptable range. This indicates again, that the sterically demanding pendant side chain prevents transesterification.

^{1}H NMR data measured in DMSO-d_6 (300 MHz) and GPC elugram measured in DMF of entry 5.3 are shown exemplary below.

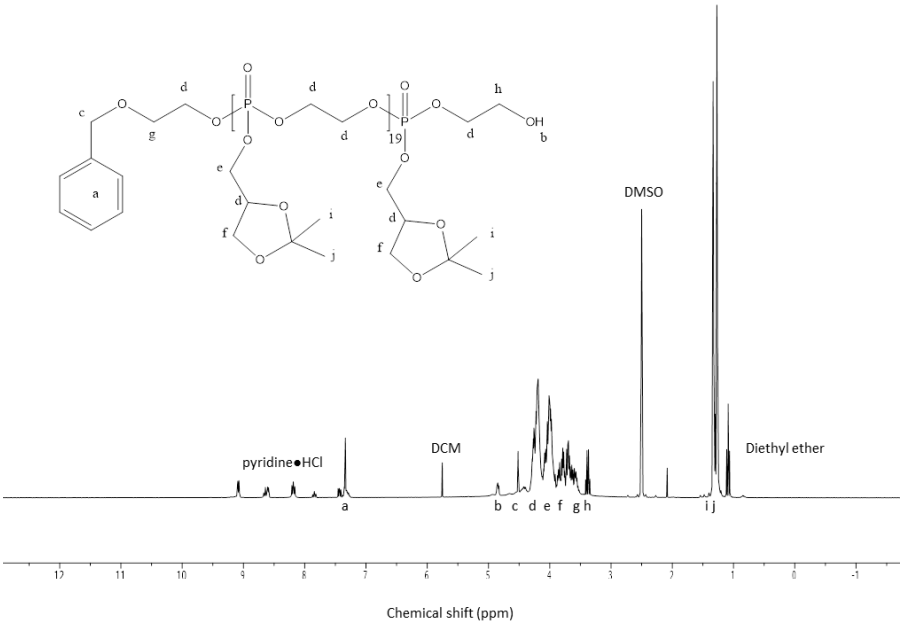


Figure 18: 1H NMR of P(GEP) in DMSO-d_6 (Table 5, entry 5.3)

The characteristic resonances of the methylene group **d** of the polymer backbone can be seen as a multiplet with a chemical shift between 4.29-4.13 ppm and of the methylene group **e** of the pendant side chain as a multiplet due to coupling with phosphorous at a chemical shift between 3.91-4.10 ppm. The signals of the initiator appear in the aromatic region with a shift between 7.23-7.32 ppm (**a**), at 4.52 ppm as a singlet (**c**) and at 3.54-3.64 ppm as a multiplet. The hydroxyl group **b** can be seen as a triplet at 4.83 ppm and the methylene group **h** next to the hydroxyl group between 3.35-3.37 ppm. The two methyl groups of the side chain can be seen as two singlets at 1.33 ppm (**i**) and 1.27 ppm (**j**). Again, there are no signals that can be related to the catalyst.

The high elution volume of the polymer in the GPC elugram indicates that there are strong polar interactions with the column material.

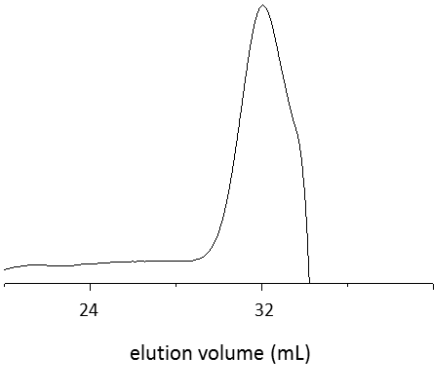
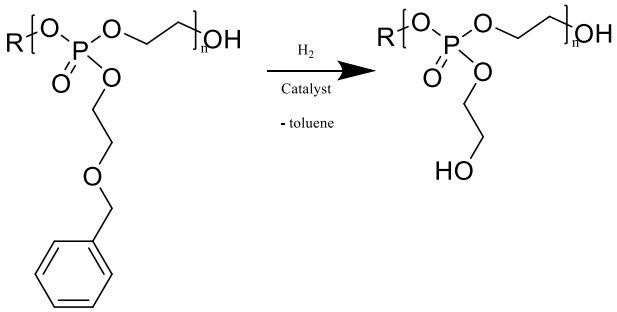


Figure 19: GPC elugram (in DMF, RI-detection) of P(GEP) (Table 5, entry 5.3)

3.5 Hydrogenation of benzyl protected P(HEEP)

To release pendant hydroxyl functionalities along the PPE, the benzyl protecting group was removed of different P(HEEP) polymers with different catalysts under different conditions – the effect on stability of the main chain and the efficiency in deprotection should be monitored.



Scheme 36: removal of the protective benzyl group via hydrogenation in order to release pendant hydroxyl functionalities

Table 6: P(HEEP) that were used for hydrogenation with different catalysts

entry	initiator	cat	solvent	c	T/°C	time	M/I	M_n(GPC)	PDI	yield/%
6.1	2	5eq DBU	DCM	4	RT	17,75	30	1500	1,30	87
6.2	2	5eq DBU	Toluol	4	50	20min	30	1400	1,27	88
6.3	3	Sn(Oct)2	no		90	6h	15	1200	1,18	70
6.4	3	Sn(Oct)2	no		90	6,25h	30	1200	1,26	~74
6.5	2	Sn(Oct)2	no		90	4,67h	15	1000	1,21	78

To find the best conditions for the hydrogenation, different reaction conditions were tested. As catalysts, palladium, nickel, platinum and palladium hydroxide were used. Also the solvent plays an important role in the hydrogenation as a rather hydrophobic polymer is transformed into a highly hydrophilic material during the process – so the solvent should probably dissolve both (THF and methanol). As it is also known from the mechanism that acid can catalyse deprotection,[37,38] also hydrochloric acid (3 µL HCl conc. in 3 mL solvent) was added to both solvents in some experiments to study the effects. The yield of each reaction was about 100%. When deprotection was not complete, even more than the theoretical possible 100% was obtained due to remaining starting material.

The results are listed in Table 7. The hydrogenation and removal of the benzyl group with palladium on activated charcoal as a catalyst in THF or methanol requires very long reaction times. When acid was introduced into the system, the reaction times are shortened, but also during the hydrogenation the formation of ethylene glycol (EG) was detected, indicating degeneration of the polymer. The ^{1}H NMR spectrum of entry 7.8 (Table 7) is exemplary for that shown (Figure 20).

Table 7: hydrogenation samples of P(HEEP) with different catalysts as well as solvents

Entry	Polymer	cat	solvent	c (PHEEP) g/L	p/bar	time	% of removed benzyl group	Note
7.1	1	Pd/C 10%	THF	8	50	2h	0%	
7.2	1	Pd/C 10%	THF	13	50	6h	0%	
7.3	1	Pd/C 1%	THF	10	50	16,33h	16%	
7.4	2	Pd/C 1%	MeOH	10	50	20,33h	100%	EG
7.5	2	Pd/C 1%	THF	12	100	22,5h	10%	
7.6	2	Pt/C 5%	MeOH/HCl	10	85	15,67h	6%	EG
7.7	2	Ni	MeOH/HCl	14	85	15,67h	36%	EG
7.8	**2**	**Pd/C 1%**	**THF/HCl**	**10**	**80**	**20,8h**	**99%**	**EG**
7.9	2	Ni	THF/HCl	10	80	20,8h	100%	EG
7.10	2	Pt/C 10%	THF/HCl	10	80	20,8h	0%	EG
7.11	**3**	**Pd(OH)2**	**THF**	**11**	**75**	**20,5h**	**100%**	
7.12	3	Pd(OH)2	MeOH	11	75	20,5h	100%	
7.13	3	Pd(OH)2	DCM/MeOH	12	75	20,5h	100%	EG

The last column of Table 7 describes whether ethylene glycol (EG) was formed during the hydrogenation or not, indicating whether degeneration took place or could be avoided.

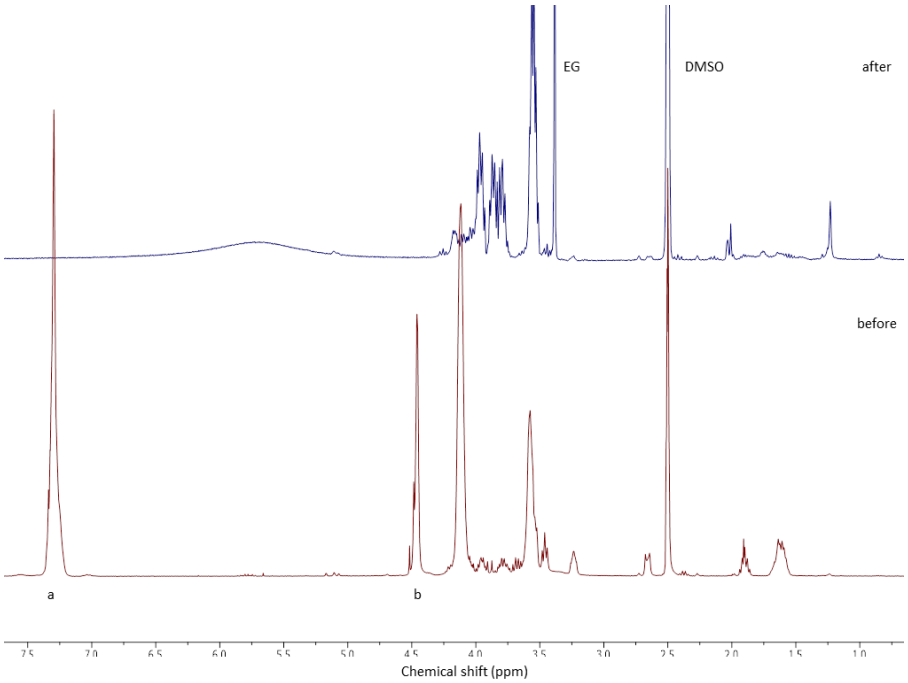


Figure 20: hydrogenation of P(HEEP) that lead to the formation of ethylene glycol and thus already started to degenerate (Table 7, entry 7.8)

It is obvious, that the aromatic signal **a** belonging to the protons of the benzyl ring as well as the signal **b** belonging to the methylene carbon of the protecting group completely disappeared. However, a singlet with a shift of 3.34 is also detected and belongs to the ethylene glycol from degenerated PPE backbone.

Degeneration can also be observed in the GPC elugrams as almost no high molecular weight material can be detected, only oligomers stay behind.

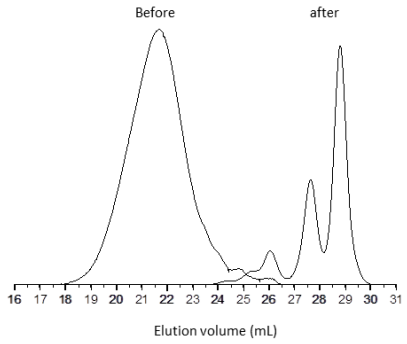


Figure 21: GPC elugram (in DMF, RI-detection) of P(HEEP) before and after hydrogenation including degeneration (Table 7, entry 7.8)

Fragmentation of the polymer is also confirmed by the formation of divers peaks in the elugram.

When nickel or platinum was used as the respective catalyst also degeneration, i.e. the observation of ethylene glycol formation was made. Another disadvantage of platinum is the ability to catalyse the further hydrogenation of the toluene leaving group to cyclohexane derivatives.

Efficient hydrogenation without chain scission was achieved with palladium hydroxide. To illustrate that, GPC and ^{1}H NMR data of entry 7.11 (Table 7) are shown below.

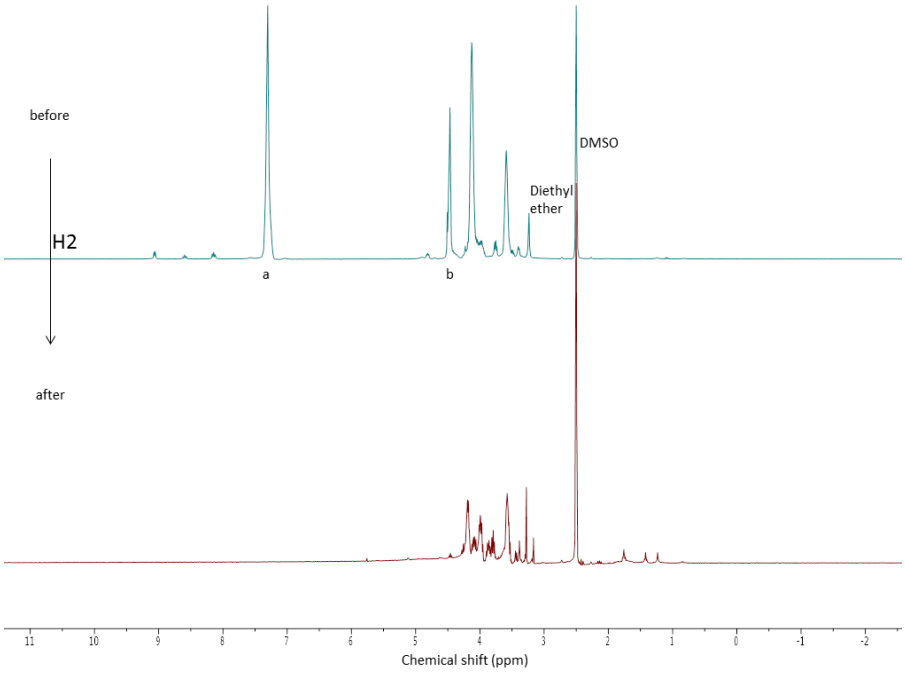


Figure 22: ^{1}H NMR spectrum of P(HEEP) before and after successful hydrogenation in DMSO-d_6 (Table7, entry 7.11)

The signal of the aromatic protons **a** of the benzyl protecting group as well as signal **b** disappeared and there is no signal at 3.34 ppm belonging to ethylene glycol after hydrogenation. In addition, the signals belonging to the polymer backbone are quite similar before and after deprotection.

When looking at the GPC data, there is of course according to the loss of molecular weight a shift to longer elution times, but the shape of the signal still indicates the presence of a polymer (which has strong polar interactions with the column material) but no fragmentation.

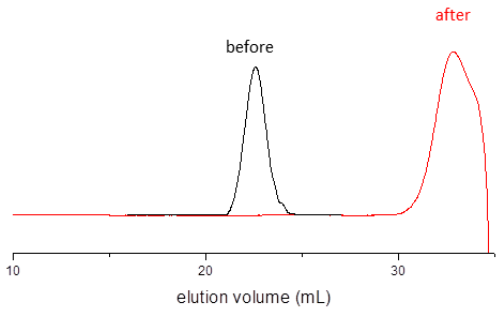


Figure 23: GPC elugrams (DMF, RI-detection) of P(HEEP) before and after successful hydrogenation (Table 7, entry 7.11)

Because of the convincing results, a closer look was taken on the hydrogenation with palladium hydroxide. Table 8 sums up the results.

Table 8: kinetic studies oft he hydrogenation of P(HEEP) with palladium hydroxide in THF

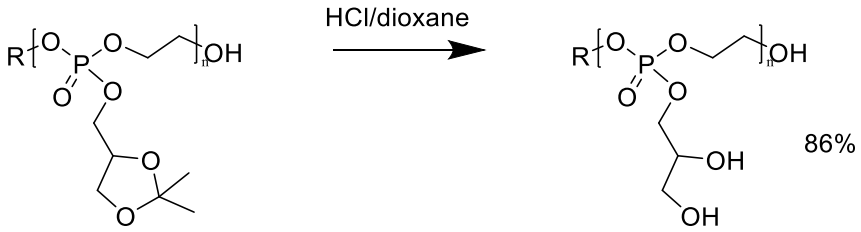
Entry	Polymer	cat	solvent	c (PHEEP) g/L	p/bar	time	dAr-H	m(P)/m(cat)
8.1	4	Pd(OH)2	THF	10	40	2h	9%	3
8.2	4	Pd(OH)2	THF	10	40	4h	10%	3
8.3	4	Pd(OH)2	THF	10	40	6h	15%	3
8.4	4	Pd(OH)2	THF	10	40	13,5h	11%	3
8.5	4	Pd(OH)2	THF	10	40	8h	14%	3
8.6	4	Pd(OH)2	THF	10	40	66,5h	43%	3
8.7	5	Pd(OH)2	THF	10	40	2h	95%	1
8.8	5	Pd(OH)2	THF	10	40	4h	100%	1
8.9	5	Pd(OH)2	THF	10	40	6h	100%	1
8.10	5	Pd(OH)2	THF	10	40	14.5h	100%	1
8.11	5	Pd(OH)2	THF	10	40	8h	100%	1

As shown in Table 8, two series of samples were hydrogenated with $Pd(OH)_2$. For the first one 30 mg of polymer were diluted in 3 mL of the solvent. Afterwards, 10 mg of catalyst were added and hydrogenated with 40 bar of hydrogen. Nearly no change of the aromatic signal in ^1H NMR was observed within 14 hours. So for the second series, the amount of added catalyst was increased to 30 mg under equal conditions. The benzyl protecting group was completely removed within 4 hours.

These experiments render the monomer HEEP as a novel and potential monomer to introduce hydroxyl groups along the PPE backbone. The OH groups can further be used for functionalization and will have an influence on degradation of the polymers.

3.6 Acidic hydrolysis of P(GEP)

To release pendant hydroxyl functionalities along the PPE, the acetal protecting group was removed from P(GEP) via acidic hydrolysis according to Wang and co-workers.[36] Therefore, aqueous hydrochloric acid (1 mol/L) was added dropwise to a solution of P(GEP) in 1,4-dioxane. After stirring the solution for 3 hours, the solution was concentrated in vacuum and the product precipitated into cold diethyl ether with reasonable yields compared to literature (90%).[36]



Scheme 37: acidic hydrolysis of P(GEP) in order to obtain pendant hydroxyl functionalities

In order to characterize the deprotected polymer, ^{1}H NMR and IR spectroscopy were used. The ^{1}H NMR spectra before and after acidic hydrolysis are shown below (Figure 24).

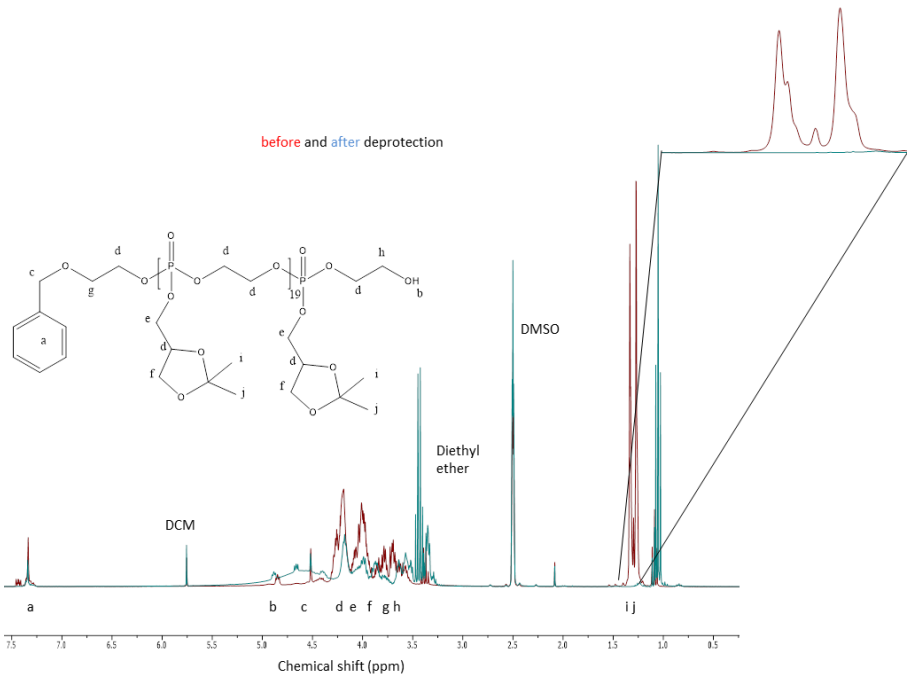


Figure 24: ^{1}H NMR of P(GEP) before (red) and after (blue) acidic hydrolysis

In Figure 24 the ^{1}H NMR spectrum of P(GEP) before deprotection is shown in red and after deprotection in blue. It is obvious, that the two resonances with chemical shifts of 1.27 ppm and 1.33 ppm that belong to the two methyl groups of the acetal protecting group completely disappeared after acidic hydrolysis. In addition, the aromatic protons of the initiator with a chemical shift between 7.23 and 7.32 ppm were detected in both cases. This indicates, that orthogonal deprotection of P(HEEP)-*co*-P(GEP) should be possible according to the fact, that the initiator is similar to the pendant side chain of P(HEEP).

To confirm, that the deprotection was successful, also an IR spectrum was measured before and after acidic hydrolysis (Figure 25).

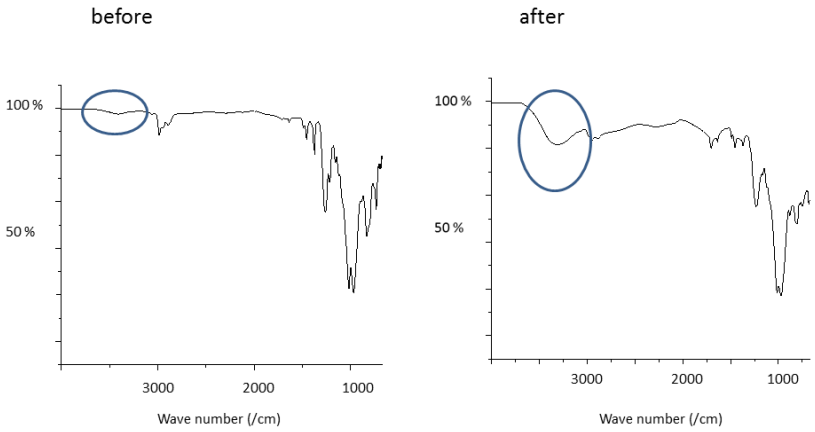


Figure 25: IR spectra of P(GEP) before and after acidic hydrolysis

The spectra before and after acidic hydrolysis are rather similar, despite the signal at a wave number of about 3500 cm^{-1}. This signal belongs to absorbance caused by hydroxyl groups within the polymer. It is clear, that this signal is much stronger after acidic hydrolysis and proves the previous conclusion, that the reaction has been successful.

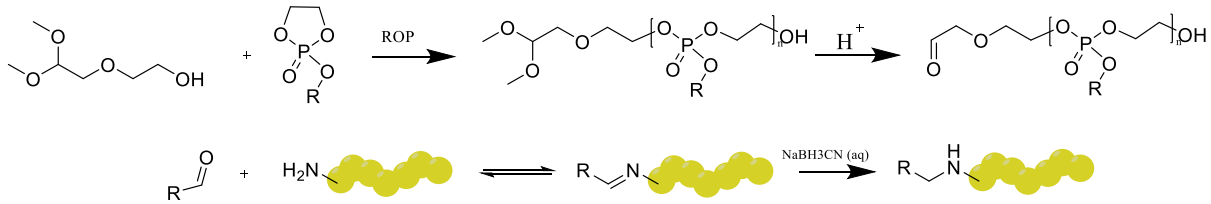
3.7 Conjugation of P(EEP) with different peptides

This chapter focusses on the linking of previous synthesized P(EEP) polymers with different peptides. All peptides carry additional cysteines or amines in order to establish a potential linkage to the pyridyl-disulfide carrying PPEs or to aldehyde functionalized PPEs (see chapter 1.4). As a proof of concept, the thio-reactive polymer was first conjugated with *N*-acetyl-L-cysteine-methyl-ester.

Different peptides were studied in this work, such as tri-peptide glutathione and a cell-penetrating peptide (see chapter 3.7.2.3) that was synthesized by Dr. Kalina Peneva (MPI Mainz). The first one was chosen because of the absence of secondary and tertiary structures, the second one because of the already mentioned cell-penetrating properties which could be used in future nanomedicine.

3.7.1 Amine formation

By functionalising the polymer with an acetal protected initiator, amine formation with the *N*-terminus of the peptide should be possible.



Scheme 38: amine formation as covalent linkage between PPE and peptide for bioconjugation

After polymerisation, the acetal protecting group can be removed under acidic conditions to release the according aldehyde group at the position of the PPE. Afterwards, an imine can be formed by reaction with the *N*-terminus of a peptide via a nucleophilic attack at neutral pH. The labile Schiff base can be further stabilized towards an amine bond after reduction.

3.7.1.1 Deprotection of acetal functionalised P(EEP)

In order to find out the conditions for the removal of the acetal protecting group, aldehyde initiated polymers were diluted in different acidic aqueous solutions and stirred for several hours. Table 9 sums up the reaction conditions that were tested:

Table 9: reaction conditions in order to deprotect the acetal protected PPE and to obtain the aldehyde functionality

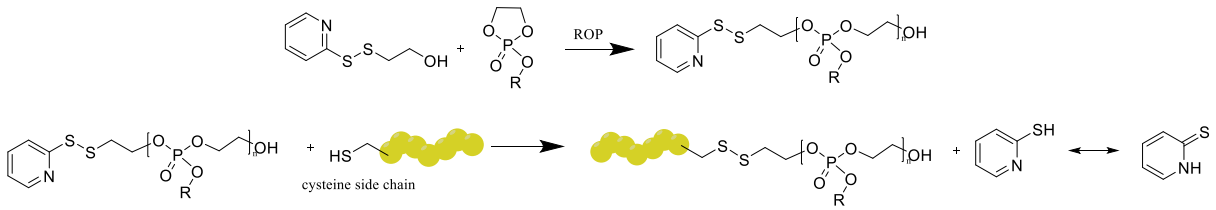
solvent	time	% aldehyde	PDI
0.1M HCl	4h	0.10	2.0
0.1M HCl	16h	0.06	-
1M HCl	16h	0.33	-
TFA/H_2O 1/1	24h	0.35	-

^{1}H NMR spectroscopy in DMSO-d_6 was used to determine the aldehyde formation. The proton shows resonances at a chemical shift of 9.57 ppm as a singlet. As shown in Table 9, all reaction conditions lead to polymers that show the characteristic signal, but conversion was incomplete. In addition, the acidic conditions lead to crosslinking, so that most polymers were not soluble very well anymore. The NMR spectra were therefore just measured of the soluble part.

Due to crosslinking, these methods are not suitable to synthesize polymers for amine formation. The work further on therefore concentrates on the conjugation via disulfide formation.

3.7.2 Polymer-Peptide Conjugates by disulfide formation

By using hydroxyl-ethyl pyridyl disulfide as an initiator for polymerisation, the formation of a dislufide with the thiol side chain of cysteine within a peptide is possible:



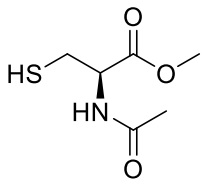
Scheme 39: conjugation od pelyposphate and cysteine containing peptide via disulfide linkage

Important for the conjugation of cysteine modified peptides with polyphosphates via disulfide linkage are mild reaction conditions that keep the polymer backbone intact. Usually, the reaction is carried out at room-temperature in water. During the disulfide exchange, 2-mercaptopyridine is formed as a side product. This pyridine derivative absorbs light of the wavelength of 343 nm. This

offers the opportunity, to follow the conversation of educts via UV-VIS-spectroscopy or HPLC with an UV-detector.

3.7.2.1 Proof of concept

Before conjugating the synthesized polymers containing the disulfide functionality with peptides, it was ensured to be possible with the present polyphosphates. On that account the protected amino acid *N*-acetyl-L-cysteine-methyl-ester was used for a first trial.



Scheme 40: *N*-acetyl-L-cysteine-methyl-ester

To a stirred solution of the amino acid in degassed Milli-Q water was added a polymer solution in dimethyl sulfoxide. After stirring at room temperature for one day, the solvents were evaporated. The ^{1}H NMR spectrum and the ^{1}H DOSY NMR spectrum of the corresponding product are shown below.

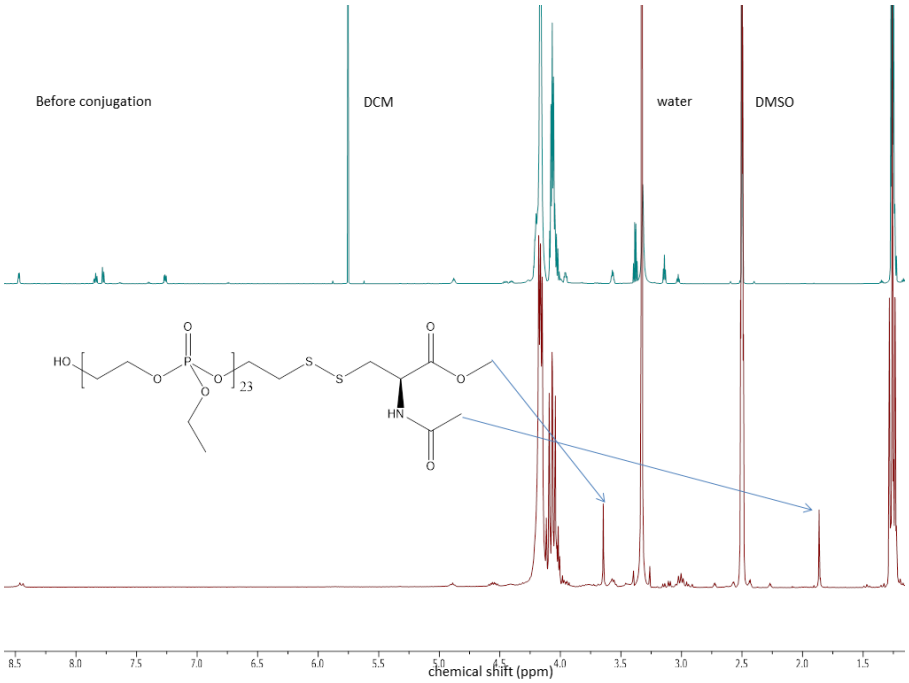


Figure 26: ^{1}H NMR spectrum of the conjugation trial in DMSO-d_6

On top the ^{1}H NMR spectrum belonging to the disulfide functionalized polymer is shown, while on the bottom the one of the corresponding conjugation product is shown. After the reaction with *N*-acetyl-L-cysteine-methyl-ester, two singlets with shifts of 1.86 and 3.66 ppm can be detedted in the proton NMR spectrum, which belong to the two methyl groups within the cysteine derivative. In addition, the resonances of the aromatic protons belonging to the initiator completely disappeared after conjugation. To make sure that the coupling indeed was successful, a ^{1}H DOSY NMR was measured.

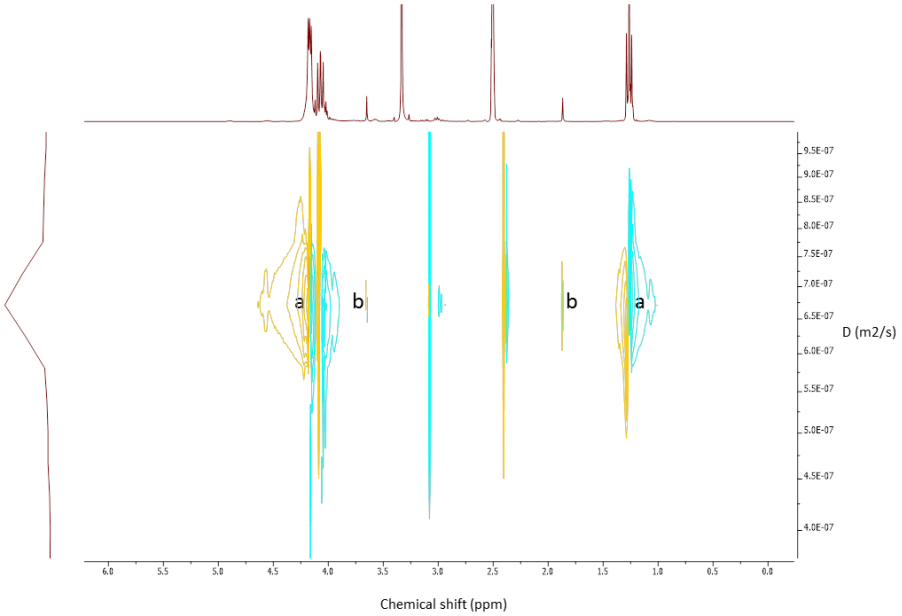
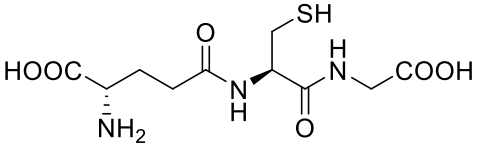


Figure 27: ^{1}H DOSY NMR spectrum of conjugation trial in DMSO-d_6, 700MHz

Figure 27 shows a zoom into the ^{1}H DOSY NMR of the modified polymer, showing that the methyl groups **b** of the cysteine derivatives have the same diffusion rate as the protons **a** of the polymer chain. This proves that the coupling of the polyphosphate with *N*-acetyl-L-cysteine-methyl-ester was successful and opens the possibility of conjugating polyphosphates with peptides via the disulfide formation. In addition, there is just one signal for the diffusion rate, which indicates, that the polymer was fully converted.

3.7.2.2 Conjugation of polyphosphates with L-glutathion

The tripeptide L-glutathion was chosen for conjugation, because of the absence of secondary or tertiary structures due to the small size of the peptide.



Scheme 41: L-glutathion

To a stirred solution of the peptide in degassed Milli-Q water was added a solution of the polymer in dimethyl sulfoxide under inert gas atmosphere. The mixture was stirred for 3 hours and afterwards dialysed in water to remove the excess of L-glutathion.

Because of the low molecular weight of the tripeptide (307 g/mol) compared to the polymer (3200 g/mol) HPLC was no adequate method to proof, whether the conjugation was successful or not, due to the fact, that the behaviour in HPLC is mostly determined by the polymer and PPEs show a strong interaction with any column materials that was investigated. Therefore, NMR-spectroscopy was used for characterisation of the product.

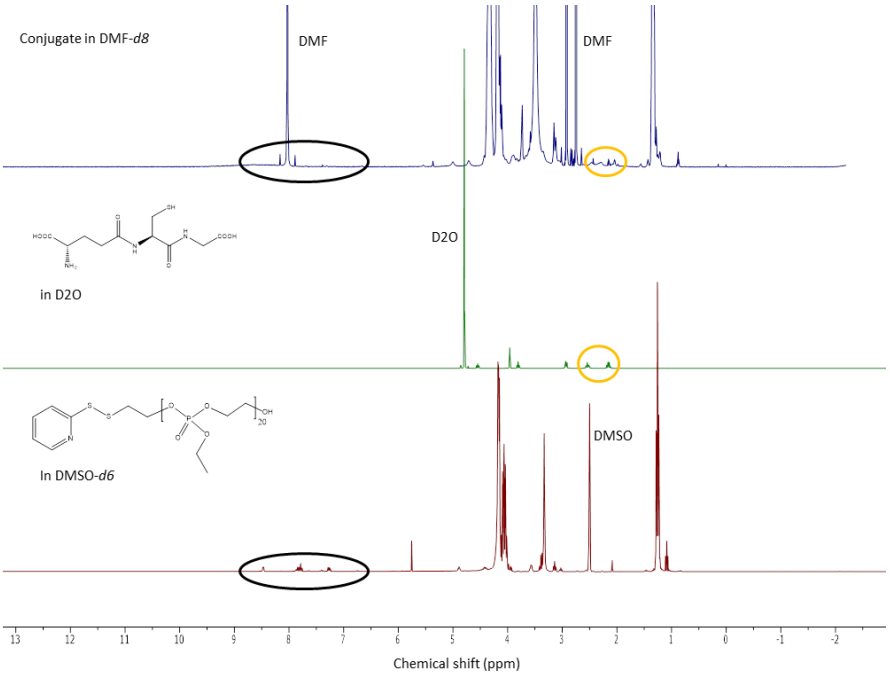


Figure 28: ^{1}H NMR spectrum of glutathion conjugate in DMF-d_8 compared to the ^{1}H NMR spectra of the educts

In Figure 28 the ^{1}H NMR spectrum of the conjugate with glutathione as well as the ones of the educts are shown. Apparently, the signals belonging to the aromatic protons of the polymers initiator (in black) disappeared after the reaction. In addition, there are resonances with chemical shifts between 1.5 and 2.5 ppm (in orange) belonging to L-glutathion indicating a positive coupling of glutathion to the polymer. Final proof can be gained from ^{1}H DOSY NMR which was measured in DMF-d_8 (700 MHz).

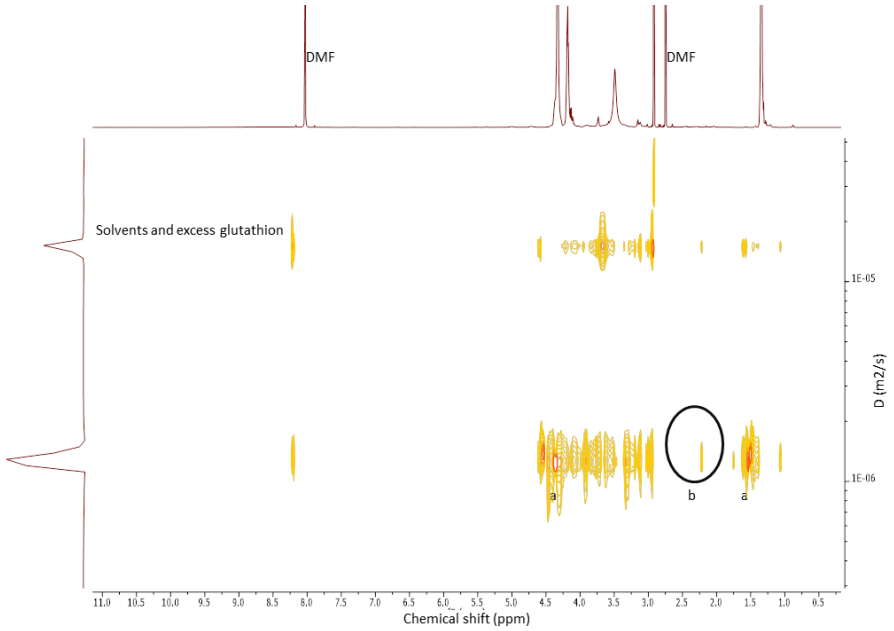


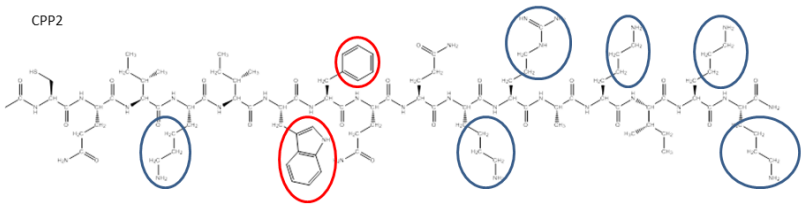
Figure 29: ^{1}H DOSY NMR of the PEEP-glutathion conjugate

In Figure 29 the ^{1}H DOSY NMR spectrum of the glutathione conjugate with PEEP is shown. The protons **b** with a chemical shift between 1.5 and 2.5 ppm belong to L-glutathione and have the same diffusion coefficient as the protons **a** of the polyphosphate chain. This proofs, that the conjugation was successful. Due to the fact, that there is just one sharp peak for the diffusion rate of the polymer, the conversion was complete.

On top of the spectrum an excess of L-glutathion with faster diffusion rate is visible. This excess was tried to be removed by dialysing the conjugate against DMF, but was not successful.

3.7.2.3 Conjugation with cell-penetrating peptides

This chapter focusses on the conjugation of different polyphosphates with a cell-penetrating peptide that was synthesized by Dr. Kalina Peneva (MPI Mainz). The structure of the peptide is shown below. Blue parts identify side chains that are positively charged under physiologically conditions and red parts identify hydrophobic side chains.



Scheme 21: cell penetrating peptide 2

Table 10 sums up the different reaction conditions for the conjugation of CPP2 to polyphosphates:

Table 10: conjugation of CPP2

Entry	MW (polymer)	PDI (polymer)	solvent	Reaction time
10.1	2800	1.70	DPBS/DMF 2/1	2h
10.2	7800	1.68	DPBS*	3h
10.3	7800	1.68	Milli-Q	2.5h

DPBS = dulbecco´s phosphate buffered saline (pH 7.2)

For entry 10.1, the low molecular weight polyphosphate was dissolved in DMF and dropped to a solution of CPP2 in phosphate buffered saline. The solution became immediately turbid indicating unwanted side reactions or other aggregation phenomena. This could be rationalized by the generated heat while mixing water and DMF. Therefore, the solvent was changed to phosphate buffered saline only, which requires higher molecular weight of the polyphosphate to ensure water solubility. When adding a solution of polyphosphate to a solution of CPP2 (entry 2) the mixture became cloudy again, but when adding the peptide-solution to a stirred solution of polymer, the reaction mixture stayed clear (entry 3).

After 2.5 hours, the solvent was evaporated and high pressure liquid chromatography was used for characterisation.

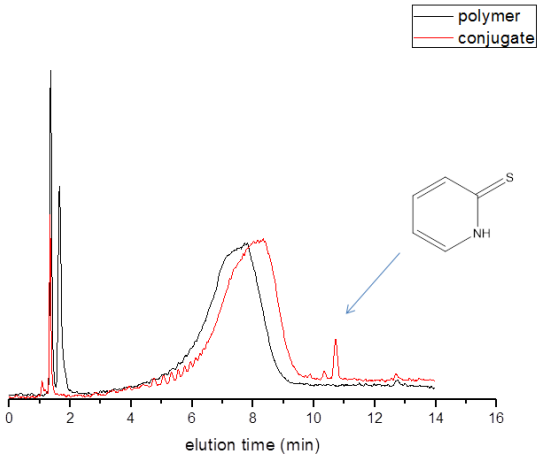


Figure 30: chromatogram CPP2-PEEP-conjugate

As stationary phase, a reversed phase with a diameter of 5 µm (Merck RP-CN) was used. The measurement was run with a solvent gradient starting with 20% acetonitrile with 0.01% TFA and 80% water with 0.001% TFA as mobile phase. Within 20 minutes, the mobile phase changed into 100% acetonitrile with 0.01% TFA. This way, the more polar analytes elute first, detection was done via light scattering.

In Figure 30 the elugrams of the polymer before (black) and after (red) conjugation are shown. Apparently, elution times of the conjugate shifted a little bit to longer ones. This indicates, that conjugation took place and as already mentioned, that elution times are mostly determined by the polyphosphate. Another hint for a successful reaction is the fact, that the oligomers in the conjugate mixture can be separated in a better way, than in the polymer itself. After 11 minutes 2-mercaptopyridine elutes, which was also confirmed by an UV-detector and confirms the assumption of a successful conjugation.

In order to proof the results, MALDI-TOF mass spectrometry was used with 1,8-dihydroxy-10*H*-anthracene-9-one and potassium ions as matrix. In Figure 31 the mass spectrum of the polymer only detected in positive mode is shown.

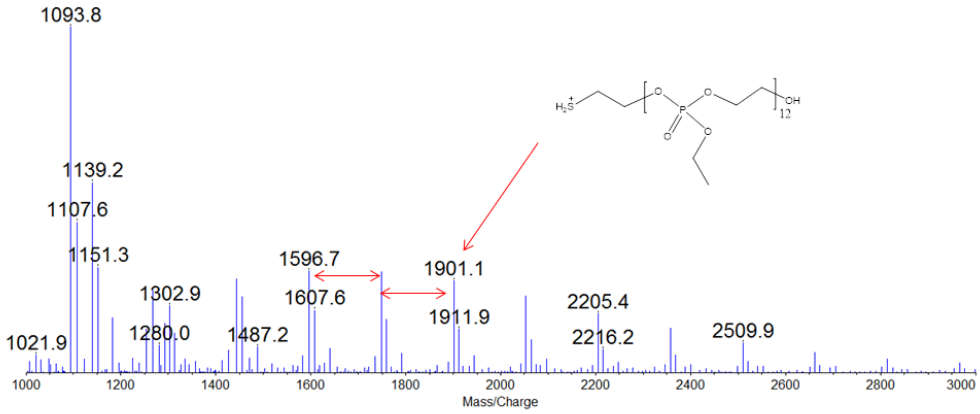


Figure 31: MALDI-TOF-MS-spectrum of polyphosphate

Apparently, the mass to charge ratios are in a smaller size range than expected. The red double arrows show mass to charge differences of 152, which correlate exactly to the mass of one monomer unit. This means, that the polymer was fragmented in a great manner during the ionisation process and explains the shift of mass to charge ratio.

The mass spectrum shows a sharp peak at a mass to charge ratio of 1901. This peak can be related to a polymer chain consisting out of 12 polymer units that is terminated by a protonated thiol, which means, that the disulfide initiator was cleaved between the two sulphur atoms during the ionisation process. In order to characterise the polymer-peptide-conjugates via mass spectroscopy, an intact disulfide bond is required. Matrix assisted laser desorption and ionisation is therefore no suitable ionisation method. Electro-spray ionisation is another mild method for mass spectrometry, but the same phenomenon was observed for the polyphosphates.

All in all, mass spectroscopy is no suitable method to characterise the disulfide linked conjugates, but high pressure liquid chromatography lead to promising results.

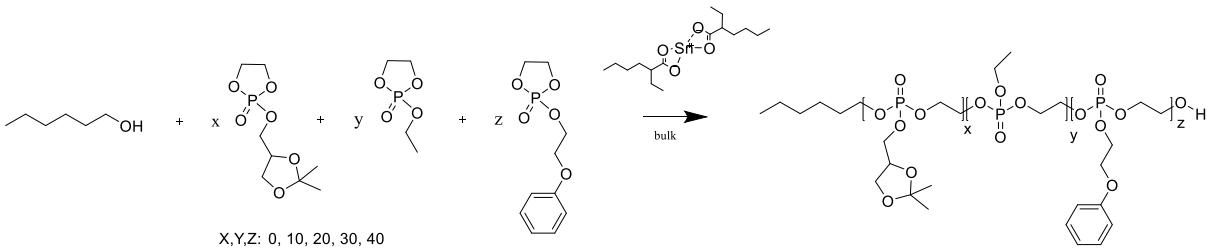
3.7.3 Resume

Altogether, the conjugation of polyphosphates to peptides via disulfide formation is possible and can be proofed by linking polymer to *N*-acetyl-L-cysteine-methyl-ester. Mass spectrometry is no suitable method for the characterisation of formed conjugates because of the instability of the disulfide linker, but HPLC gives promising results so far.

The conjugation of polyphosphates to peptides via amine formation with their *N*-terminus could not be investigated due to the instability of the polymers while deprotecting the initiator.

3.8 Copolymerisation of the different monomers

This chapter focusses on the synthesis of various co-polymers with stannous octoate as catalyst. This way, polymers are obtained, that can be deprotected by hydrogenation or under acidic conditions in an orthogonal way to fine tune hydrophilicity and degradation times or to introduce selectively various functional groups. As initiator hexanol was used, because the terminal methyl group can be detected in ^{1}H NMR spectroscopy without any overlapping with signals of all three monomers.



Scheme 43: reaction scheme for homo- and co-polymerisations

Table 11: composition of synthesized copolymers

entry	N(EEP)	N(HEEP)	N(GEP)	N(EEP)/ NMR	N(HEEP)/ NMR	N(GEP)/ NMR	PDI	Reaction time
11.1	40	-	-	32	-	-	1.58	2h
11.2	-	-	40	-	-	39	1.62	6h
11.3	-	40	-	-	36	-	1.57	16.3h
11.4	20	-	20	15	-	15	1.61	4.75h
11.5	20	20	-	15	15	-	1.91	6h
11.6	20	20	-	18	19	-	1.50	19.25h
11.7	-	20	20	-	18	15	2.47	14.5
11.8	-	20	20	-	22	19	1.56	19h
11.9	-	30	10	-	24	7	2.54	14.5h
11.10	-	30	10	-	30	10	1.60	19.3h
11.11	-	10	30	-	9	23	2.13	14.5h
11.12	-	10	30	-	10	27	1.72	19.17h
11.13	10	30	-	11	34	-	2.39	14.5h
11.14	10	30	-	11	32	-	1.47	19.25h
11.15	30	10	-	20	9	-	1.81	3.5h
11.16	30	10	-	27	10	-	1.34	19.25h
11.17	30	-	10	26	-	10	2.75	4.17h
11.18	30	-	10	27	-	9	1.72	18.25h
11.19	10	-	30	6	-	23	1.93	5h
11.20	10	-	30	11	-	22	1.66	18.25h

Table 11 sums up the compositions of the synthesised polymers. Of each monomer, a homo-polymer was synthesised. In addition, co-polymers with equal amounts of each monomer as well as co-polymers with ratios of 30/10 and 10/30 monomer units in every possible combination of monomers were synthesised.

All copolymers were synthesized with almost desired monomer-ratios and molecular weight distributions in a reasonable range. The GPC elugrams of homo- and copolymers look like those of the homopolymers with a monomodal shape and overlap completely in most cases. Therefore, they are not shown again.

The ^{1}H NMR spectra of the copolymers are built up of the ones of the homopolymers. For demonstration, the spectra of the three copolymers with equal monomer units (Table 11, entry 11.4/6/8) are shown below.

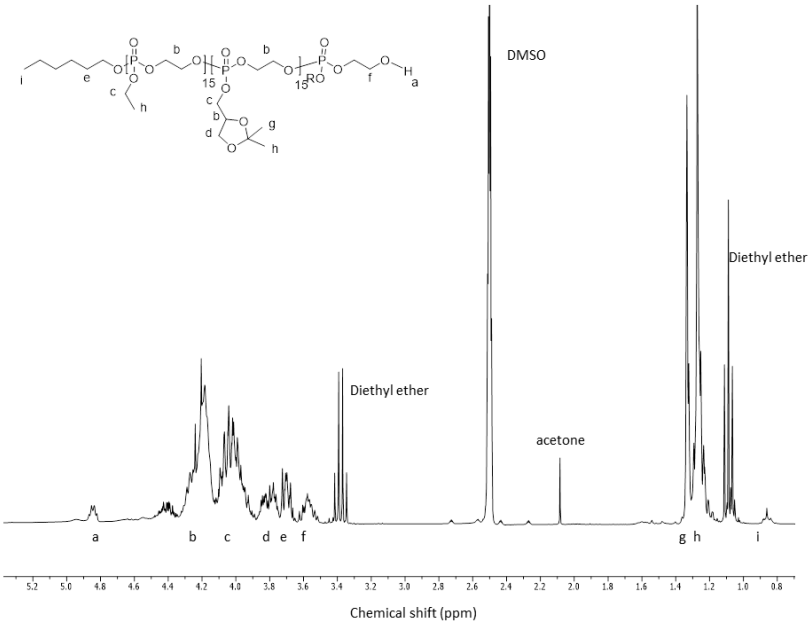


Figure 32: ^{1}H NMR spectrum of P(EEP$_{15}$-*co*-GEP$_{15}$)

In Figure 32, the ^{1}H NMR spectrum of P(EEP$_{15}$-*co*-GEP$_{15}$) is shown. The protons of the polymer backbone were detected like in previous cases in the region between 3.8-4.4 ppm. The terminal hydroxyl group **a** was detected as a triplet with a chemical shift of 4.84 ppm. The terminal methyl group **i** can be seen at 0.86 ppm as a multiplet. The signal of the methyl group **h** of the EEP pendant side chain coincides with the one of the methyl group **h** of the GEP pendant side chain at 1.23-1.32 ppm.

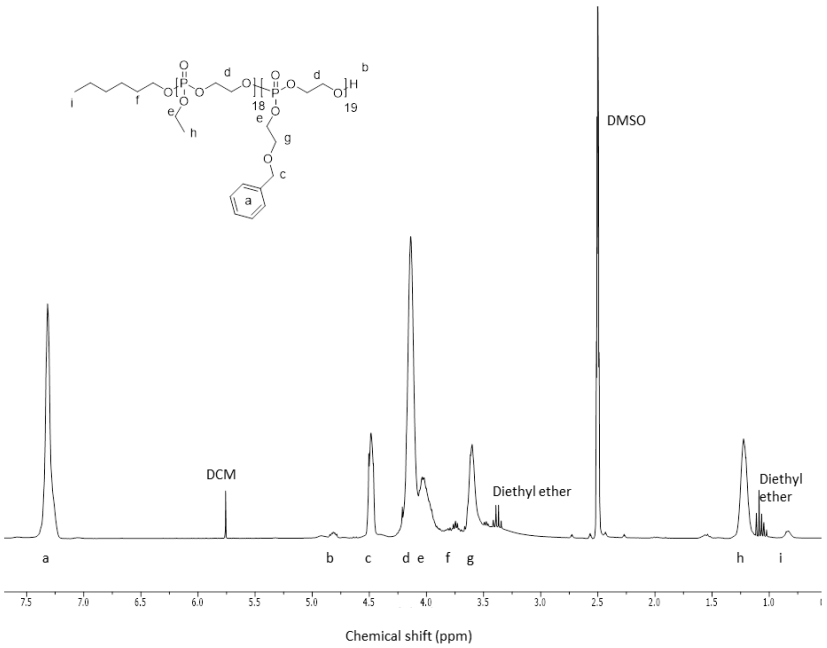


Figure 33: ^{1}H NMR spectrum of P(EEP$_{18}$-*co*-HEEP$_{19}$)

In Figure 33, the ^{1}H NMR spectrum of P(EEP$_{18}$-*co*-HEEP$_{19}$) is shown. The significant protons of each monomer (**a** and **h**) can be detected as well as the ones of the polymer backbone with the typical shift between 3.8-3.25 ppm.

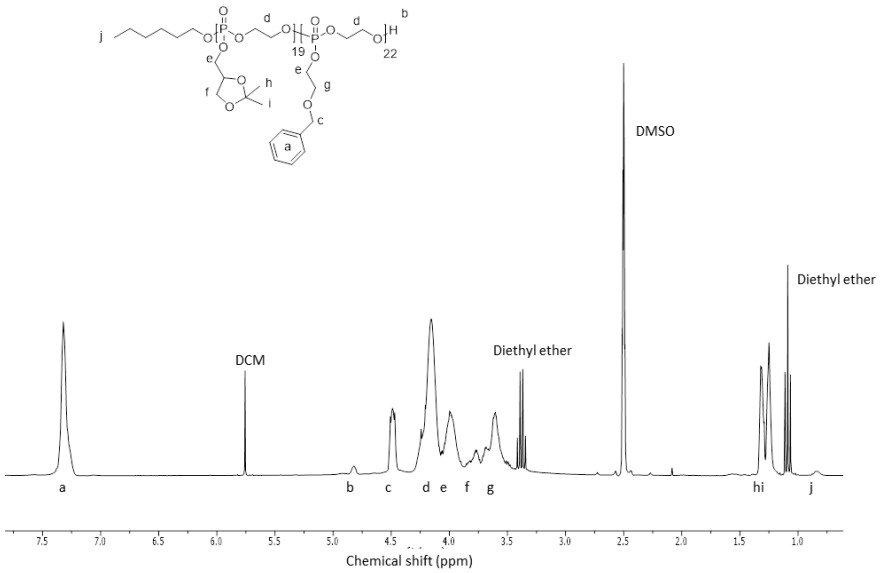


Figure 34: ^{1}H NMR spectrum of P(HEEP$_{22}$-*co*-GEP$_{19}$)

In Figure 34, the ^{1}H NMR spectrum of P(HEEP$_{22}$-*co*-GEP$_{19}$) is shown. Again, all significant signals were detected.

Due to a statistic composition of monomers within the polymer chain, the phosphorous atoms in the polymer backbone have different chemical environments, resulting in several signals detected by ^{31}P NMR spectroscopy. This is exemplary shown for entry 11.6 in Table 11.

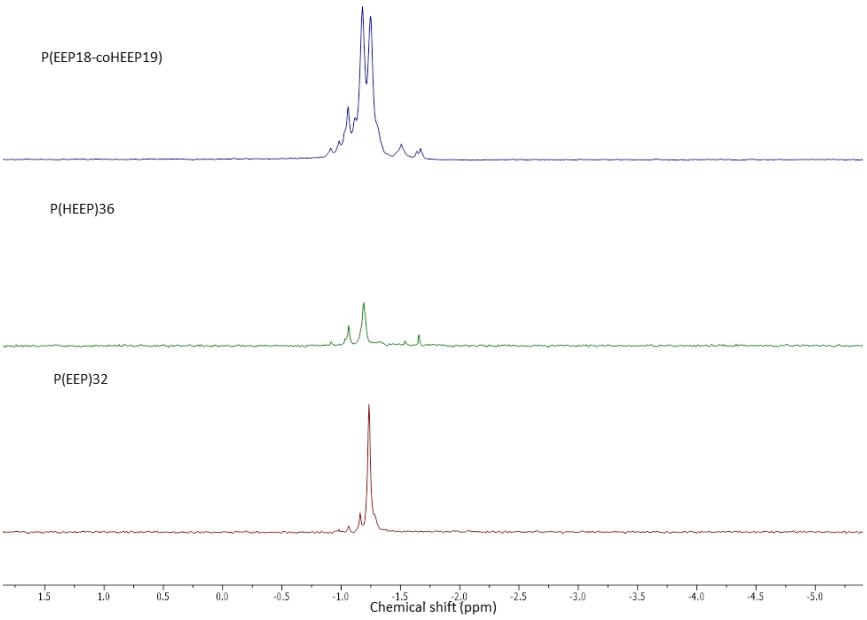


Figure 35: ^{31}P NMR spectra of homopolymers compared with copolymer consisting out of an equal amount of both monomers

In the bottom the two NMR spectra of the homopolymers P(EEP) and P(HEEP) are shown. On top, the ^{31}P NMR spectrum of the copolymer existing out of 20 units of each monomer is shown. The spectrum becomes more complex due to the statistical composition. The two ^{31}P NMR spectra of the homo-polymers also show more than one single peak. This phenomen is originated in the different chemical environment of the phosphorous atoms near the chain ends compared to the ones in the middle of the backbone.

The glass transition temperature of all polymers was measured in order to investigate how monomer composition effects the properties of the copolymers. Table 12 shows the results.

Table 12: glass-transition temperature of different homo- and co-polymers

Polymer-compostion	glass-transition temperature in °C
$P(EEP)_{32}$	-62
$P(HEEP)_{36}$	-39
$P(GEP)_{39}$	-35
$P(EEP_{18}\text{-co-}HEEP_{19})$	-45
$P(EEP_{11}\text{-co-}HEEP_{32})$	-46
$P(EEP_{27}\text{-co-}HEEP_{10})$	-52
$P(GEP_{19}\text{-co-}HEEP_{22})$	-34
$P(GEP_{27}\text{-co-}HEEP_{10})$	-38
$P(GEP_{10}\text{-co-}HEEP_{30})$	-47
$P(EEP_{15}\text{-co-}GEP_{15})$	-45
$P(EEP_{11}\text{-co-}GEP_{22})$	-40
$P(EEP_{27}\text{-co-}GEP_{9})$	-46

Arising from Table 12, the more ethyl ethylene phosphate the polymer contains, the lower its glass-transition temperature is. This phenomenon is caused by a lack of inter- and intra-molecular interactions. The aromatic side-chains as well as the acteal protected side chain of the other monomers allow electrostatic as well as hydrophilic interactions and thus limit flexibility of the polymer chains resulting in higher glass-transition states. This phenomenon is also shown in Figure 36 on grounds of illustration.

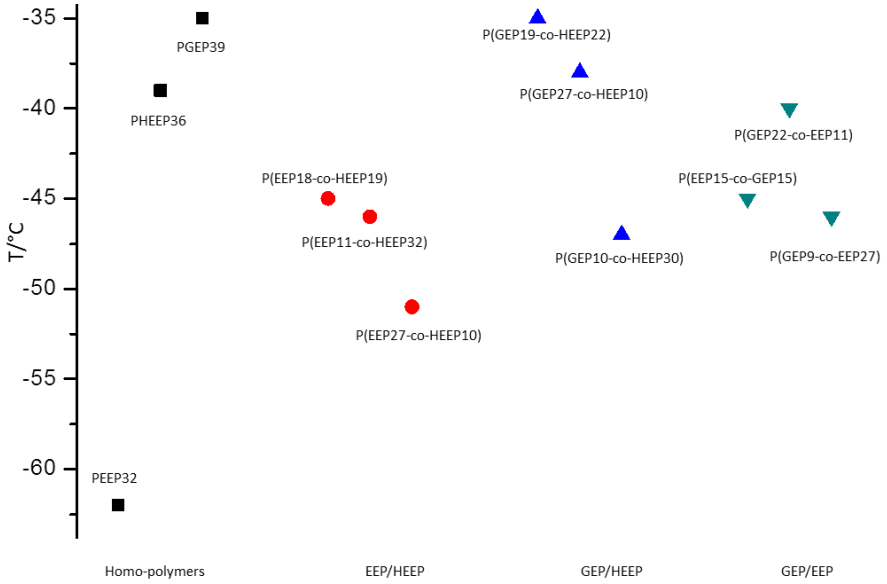


Figure 36: glass-transition temperatures of the different homo- and co-polymers

4. Summary

This thesis was devoted to investigate a new class of drug-delivery systems by combining bio-degradable polyphosphates with cell-penetrating peptides. Peptides that are positively charged are in addition able to bind non-covalently to *si*RNA or plasmid-DNA and therefore avoid covalent modification of the bioactive compound.

In this thesis, several polyphosphates that vary in terms of initiator, molecular weight and pendant side chain have been synthesized and characterised.

The cyclic monomers ethyl ethylene phosphate (EEP) and 2-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)-2-oxo-1,3,2-dioxaphospholane (GEP) were synthesized within three steps according to literature and afterwards characterised by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. These two monomers were polymerised with stannous octoate under many conditions in order to control molecular weights and molecular weight distribution. In addition, the monomer 2-(2-(benzyloxy)ethoxy)-1,3,2-dioxaphospholane-2-oxide (HEEP) that was synthesized by Tobias Steinbach (Johannes Gutenberg-Universität Mainz) was polymerised with DBU, TBD and stannous octoate. The polymers were characterised by GPC and ^{1}H NMR spectroscopy. ^{1}H DOSY NMR spectra proved that functionalities within different initiators were incorporated intactly and therefore could be used for bio-conjugation with different peptides.

The homo-polymer P(HEEP) was deprotected via hydrogenation in order to release pendant hydroxyl functionatilies. Best catalyst turned out to be palladium hydroxide in THF. The acetal protecting group within the side chains of P(GEP) was removed via acidic hydrolysis. Results were confirmed via ^{1}H NMR and IR spectroscopy.

Many copolymers consisting out of the three different monomers were synthesized with stannous octoate as catalyst in bulk in order to obtain polyphosphates that can be adjusted in terms of hydrophilicity and degradation time via orthogonal deprotection. Acidic hydrolysis of P(GEP) with benzyl oxy ethanol as initiator already indicated that this should be possible. The different copolymers were characterized by GPC and ^{1}H NMR spectroscopy as well as differential scanning calorimetry (DSC).

In order to proof that conjugation of polyphosphates containing a pyridyl-disulfide functionality with peptides is possible, poly(ethyl ethylene phosphate) (PEEP) was linked to the protected amino acid *N*-acetyl-L-cysteine-methyl-ester. The positive conjugation was proofed by ^{1}H DOSY NMR spectroscopy.

Afterwards, P(EEP) was conjugated to the tri-peptide L-glutathion and to a cell-penetrating peptide that was synthesized by Dr. Kalina Peneva. The conjugates were characterized by high pressure liquid chromatography. Mass spectroscopy turned out to be no suitable method for that, because the disulfide linkage is not stable during the ionisation process.

Altogether one could say that conjugation of bio-degradable polyphosphates with cell-penetrating peptides via disulfide linkage has been successful.

5. Outlook

The results of this work strongly suggest that conjugation of polyphosphates with cell-penetrating peptides in order to obtain a new class of drug delivery systems is possible. The conjugates therefore need to be investigated in terms of drug load with *si*RNA or plasmid-DNA and cell viability.

Different homo- and copolymers were successfully synthesized with stannous octoate and for future investigations it would be interesting to release pendant hydroxyl functionalities by orthogonal deprotection within the different copolymers. For homopolymers, it was already investigated that hydrogenation and acidic hydrolysis are suitable methods for this purpose. Now it would be interesting to have a closer look on how those methods affect the different monomers within the copolymers and whether they are stable under given conditions for deprotection.

Moreover, kinetic studies via ^{1}H and ^{31}P spectroscopy of the polymerisation of the new monomer 2-(2-(benzyloxy)ethoxy)-1,3,2-dioxaphospholane-2-oxide (HEEP) would be interesting to examine. Those methods would also be suitable in order to obtain the degradation rates of various synthesized homo- and copolymers that differ in number of hydroxyl functionalities.

Now that conjugation of P(EEP) with the investigated cell-penetrating peptide via disulfide linkage was possible, it would be interesting to concentrate on other conjugation techniques like the amine formation that was already discussed. Therefore, suitable acetal-deprotection methods for the given polyphosphates need to be investigated, that do not attack the polymer backbone.

6. Experimental section

6.1 Methods

Nuclear magentic resonance (NMR) spectroscopy

^{1}H, ^{13}C and ^{31}P NMR spectra of the monomers were measured by a Bruker AVANCE III 500 spectrometer. ^{1}H NMR spectra of the different polymers and conjugates were recorded on a Bruker AVANCE 300 spectrometer. ^{1}H and ^{31}P DOSY spectra of polymers and conjugates were recorded by a Bruker AVANCE III 700 spectrometer with a gradient strength of 5.535 G/mm. As deuterated solvent DMSO-d_6, $CDCl_3$, D_2O or DMF-d_8 were used. For analysis of all measured spectra MestReNova 8 from Mestrelab Research S.L. was used. The spectra were calibrated against the solvent signal.

Gel permeation chromatography (GPC)

All GPC elugrams of PEEP were measured in DMF containing 1 g/L LiBr. An Agilent 1100 Series integrated instrument with autosampler, Agilent G1310A pump and a PSS HEMA column ($10^6/10^5/10^4$ g/mol) was used. The flow rate amounted 1 mL/min at a temperature of 50°C. The RI-detector G1362A RID and the UV detector Agilent G131 were used for detection. Poly (ethylene glycol) standards provided by Polymer Standards Service were used for calibration and the software PSS-WinGPC UniChrom (PSS) was used for recording and analysis of the data.

GPC measurement of PGEP and P(HEEP) was also done in DMF but with an Agilent Technologies 1260 Infinity instrument. A PSS SecCurity autosampler, a 1260 IsoPump and a GRAM (PSS) 0.8x30 cm column (particle size 10 µm and pore sizes 10000, 1000, 100 Å) was used. For detection, an UV-detector S-3702 from SOMA (270 nm) and a RI-detector RI-101 from Erc came into operation.

High pressure liquid chromatography (HPLC)

The high pressure liquid chromatograms of the polyphosphates as well as of the conjugates were measured by using an Agilent Series 1100 quarternary gradient-pump and a Varian 385-LC evaporative light-scattering detector. The column that was used was a Macherey-Nagel reversed-phase column with nitrile groups on the surface (125/4/5 µm). As mobile phase two systems were used. Mobile phase A consisted out of aceto nitrile with 0.01% triflouroacetic acid. Mobile phase B consisted out of water with 0.001% triflouroacetic acid. The solvent gradient started at 20% A and 80% B and changed to 100% A within 20 minutes. The measurement was run at 20°C and a flow-rate of 1 mL/min.

MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption and ionization time-of-flight (MALDI-ToF) measurements were obtained with a Shimadzu Axima CFR MALDI-ToF mass spectrometer, equipped with a nitrogen laser delivering 3 ns laser pulses at 337 nm. Dithranol (1,8,9-tris-hydroxy-anthracene) was used as a matrix. Samples were prepared by dissolving the analyte in $CHCl_3$ at a concentration of 10 g L^{-1}. A 10 mL aliquot of this solution was added to 10 mL of a 10 g L^{-1} matrix solution and 1 mL of a potassium trifluoroacetic acid (KTFA) solution (0.1 M in methanol as cationization agent). A 1 mL aliquot of the resulting mixture was applied to a multistage target, the thin matrix/analyte film was obtained by evaporation of the solvent. The samples were measured in positive ion- and in linear mode of the spectrometer.

Infrared-Spectroscopy

FT-IR measurement was done with a Nicolet 5DXC FT-IR

6.2 Materials

Except of ethanol and solketal, all solvents were merchandised from Sigma Aldrich or Acros Organics and used as received. Ethanol was dried with sodium and distilled freshly before use, solketal was dried with benzene, distilled and stored over a molecular sieve (3Å).

Deuterated solvents were purchased from Deutero GmbH (DMSO-d_6) or Sigma Aldrich (D_2O, DMF-d_8, $CDCl_3$).

All other chemicals were purchased from Sigma Aldrich and used as received.

Cell Penetrating Peptide 2 was synthesized by the group of Dr. Kalina Peneva (MPIP Mainz) and benzyle protected HEEP monomer was prepared by Tobias Steinbach (Johannes Gutenberg-University, Mainz).

6.3 Monomers

2-Chloro-1,3,2-dioxaphospholane

Ethylene glycol (62.07 g, 1mol) was dropped into phosphorous trichloride (137.33 g, 1mol) in dry dichloromethane (150 mL) in a 500-mL three-neck round bottom flask using a Dimroth condenser with connected sodium hydroxide bath to neutralize formed HCl and a dropping funnel. Argon was conducted to remove formed HCl. The mixture was stirred for further two hours. The crude product was purified by distillation under vacuum using a column. The product was obtained as a colorless liquid at 68-72°C/63-68 mbar (yield: 67.67 g, 0.54 mol, 54%).

2-Chloro-2-oxo- 1,3,2-dioxaphospholane (COP)

A 1000 mL three-neck round bottom flask with an intensive condenser and a septum with a Teflon flexible tube linked with a three-way cock on top of the condenser and a balloon (closed apparatus!) was filled with 2-chloro-1,3,2-dioxaphospholane (156.2 g, 1.24 mol) and benzene (450 mL). After freezing the solution, the apparatus was evaporated and filled with oxygen. A flexible tube pump was used to let the oxygen rotate in the closed apparatus and bubble through the solution. While stirring,

the solution was heated up to 80°C for 18 hours. The crude product was purified by distillation under vacuum. The product was obtained as colorless liquid at 88-106°C/0.04-0.05 mbar (yield: 78.2 g, 0.55 mol, 44%)

^{1}H-NMR (500 MHz, $CDCl_3$): δ 4.64-4.44 (m, 4H, O-C*H*$_2$-C*H*$_2$-O).

2-Ethoxy-2-oxo-1,3,2-dioxaphospholane (EEP)

A 1000 mL three-necked round bottom flask was equipped with a dropping funnel. To a solution of COP (78.2 g, 0.55 mol) in 50 mL dry THF was dropped a mixture of pyridine (43.43 g, 0.55 mol) with 230 mL dry THF and dry ethanol (25.29 g, 0.55 mol) while cooling to -20°C. The mixture was stirred over night at -4°C. The formed pyridine hydrochloride was removed by filtration under inert conditions and afterwards, the solvent was evaporated *in vacuo*. The crude product was purified by distillation under vaccum. The product was obtained as a colorless liquid at 95-98°C/0.003 mbar (yield: 53.71 g, 0.35 mol, 64%).

^{1}H-NMR (300 MHz, DMSO-d_6): δ 4.48-4.32 (m, 4H, O-C*H*$_2$-C*H*$_2$-O), 4.22-4.01 (m, 2H, O-C*H*$_2$-CH_3), 1.25 (t, 3H, O-CH_2-C*H*$_3$).

^{13}C-NMR (125 MHz, DMSO-d_6): δ 66.35 (s, 2C, O-*C*H_2-*C*H_2-O), 64.18 (s, 1C, O-*C*H_2-CH_3), 15.96 (s, 1C, O-CH_2-*C*H_3).

^{31}P-NMR (202 MHz, DMSO-d_6): δ 16.83.

2-(2,2-dimethyl-1,3-dioxolan-4-yl-methoxy)-2-oxo-1,3,2-dioxaphospholane

A 1000 mL three-necked round bottom flask was equipped with a dropping funnel. To a solution of COP (59.0 g, 0.35 mol) in 32 mL dry THF was dropped a mixture of pyridine (27.76 g, 0.35 mol), 150 mL dry THF and dry solketal (46.38 g, 0.35 mol) while cooling to -20°C. The mixture was stirred over night at -4°C. The formed pyridine hydrochloride was removed by filtration under inert conditions and afterwards, the solvent was evaporated *in vacuo*. The product was collected as colorless solid with a melting point of 65°C and was pure enough for further studies (yield: 83.3 g, 0.35 mol, 100%).

^{1}H-NMR (300 MHz, DMSO-d_6): δ 5.30-4.30 (m, 4H, O-C*H*$_2$-C*H*$_2$-O), 4.20-4.30 (m, 2H, O-C*H*$_2$-CH_3), 3.92-4.15 (m, 2H, O-C*H*$_2$-CH-$O_2C_4H_8$, 1H, CH-C*H*$_2$-O-C), 3.59-3.73 (m, 1H, CH-C*H*$_2$-O-C), 1.34 (s, 3H, C-CH_3), 1.27 (s, 3H C-CH_3).

^{13}C-NMR (125 MHz, DMSO-d_6): δ 108.97 (s, 1C, *C*), 73.80 (s, 1C, *C*H_1), 68.03 (s, 1C, O-*C*H_2-CH-$O_2C_4H_8$), 66.50 (s, 2C, O-*C*H_2-*C*H_2-O), 26.49 (s, 1C, *C*H_3), 26.25 (s, 1C, *C*H_3).

^{31}P-NMR (202 MHz, DMSO-d_6): δ 16.94.

6.4 Homopolymers

The polymerization reactions were carried out in 25 mL Schlenk tubes. The tubes were flame-dried under vacuum and purged with argon three times prior to use.

Polymerisation of benzyl protected HEEP with 1,5,7-triazabicyclo[4.4.0]dec-5-ene in DCM

Hydroxyl ethyl vinyl ether was used as initiator and the polymerisation was conducted at 0°C in dichloromethane. HEEP (0.5045 g, 1.93 mmol) and hydroxyl ethyl vinyl ether (0.0160 g, 0.13 mmol) were introduced into a tube via syringe and diluted with 0.9 mL DCM. After freeze drying, a stock solution of TBD (25 mg in 0.19 mL DCM) was prepared. Both tubes were cooled to 0°C and the polymerisation was initiated by adding 0.1 mL of the TBD stock solution (0.0135 g, 0.10 mmol) to the monomer/initiator solution. After 2 hours, the polymerisation was quenched with an excess of acetic acid. The polymers were purified by precipitation in diethyl ether three times and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colorless polymer dried *in vacuo*.

Yields:

$P(HEEP)_{15}$ (entry 1.1): 0.1358 g (4,000 g/mol; 26%)

$P(HEEP)_{15}$ (entry 1.2): 0.1904 g (4,000 g/mol; 40%)

$P(HEEP)_{30}$ (entry 1.3): 0.2041 g (7,800 g/mol; 40%)

Polymerisation of benzyl protected HEEP with 1,5,7-triazabicyclo[4.4.0]dec-5-ene in bulk

Benzyl oxy ethanol was used as initiator and the polymerisation was conducted at 0°C in bulk. HEEP (0.5045 g, 1.93 mmol) and benzyl oxy ethanol (0.0276 g, 0.13 mmol) were introduced into a tube via syringe. After freeze drying, a stock solution of TBD (25 mg in 0.19 mL DCM) was prepared. Both tubes were cooled to 0°C and the polymerisation was initiated by adding 0.1 mL of the TBD stock

solution (0.0135 g, 0.10 mmol) to the monomer/initiator mixture. After 2 hours, the polymerisation was quenched with an excess of acetic acid. The polymers were purified by precipitation in diethyl ether three times and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colorless polymer dried *in vacuo*.

Yields:

$P(HEEP)_{15}$ (entry 1.4): 0.4209 g (4,000 g/mol; 80%)

$P(HEEP)_{30}$ (entry 1.5): 0.1876 g (7,900 g/mol; 40%)

Polymerisation of benzyl protected HEEP with 1,8-diazabicyclo[5.4.0]-undec-7-ene

The polymerization was conducted in dichloromethane or toluene depending on the different temperatures that were tested. In DCM, the reaction was carried out at room-temperature and in toluene at 40°C, or rather 50°C. Due to the fact, that polymerization at 40°C in toluene gave the best results, the according procedure is described.

HEEP (0.5010 g, 1.94 mmol) and benzyl oxy ethanol as initiator (0.0115 g, 0.06 mmol) were introduced into the schlenk tube via syringe and dissolved in 0.38 mL toluene afterwards. DBU was freeze dried once and a stock solution in toluene (98 mg in 1 mL toluene) was prepared. After cooling down both tubes to 0°C, the polymerization was initiated by adding 0.1 mL of the catalysts stock solution (49 mg, 0.32 mmol) to the stirred monomer solution. The ice bath was removed and the reaction mixture heated up to 40°C. After 2 hours, an excess of acetic acid was added. The polymers were purified by precipitation in diethyl ether three times and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colorless polymer dried *in vacuo*.

Yields:

$P(HEEP)_{15}$ (entry 2.1): 0.5272 g (4,000 g/mol; 100%)

$P(HEEP)_{30}$ (entry 2.2): 0.4501 g (7,900 g/mol; 87%)

$P(HEEP)_{15}$ (entry 2.3): 0.5809 g (4,000 g/mol; 100%)

$P(HEEP)_{30}$ (entry 2.4): 0.4513 g (7,900 g/mol; 88%)

$P(HEEP)_{15}$ (entry 2.5): 0.2098 g (4,000 g/mol; 100%)

$P(HEEP)_{30}$ (entry 2.6): 0.4226 g (7,900 g/mol; 85%)

$P(HEEP)_{15}$ (entry 2.7): 0.5570 g (4,000 g/mol, 98%)

$P(HEEP)_{30}$ (entry 2.8): 0.5020 g (7,900 g/mol; 97%)

Polymerisation of benzyl protected HEEP using stannous octoate

The polymerisation was conducted in bulk at 90°C. HEEP (0.6740 g, 2.61 mmol) and dec-9-en-1-ol as initiator (0.0260 g, 0.17 mmol) were introduced into the schlenk tube via syringe. After cooling down the reaction tube to 0°C, the polymerization was initiated by adding three drops of stannous octoate to the stirred monomer solution. The ice bath was removed and the reaction mixture heated up to 90°C. After 6 hours, the polymerization was stopped and 2 mL of DCM were added. The polymers were purified by precipitation in diethyl ether three times and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colorless polymer dried *in vacuo*.

Yields:

$P(HEEP)_{15}$ (entry 3.1): 0.2098 g (4,000 g/mol; 39%)

$P(HEEP)_{15}$ (entry 3.2): 0.3978 g (4,000 g/mol; 78%)

$P(HEEP)_{15}$ (entry 3.3): 0.4147 g (4,000 g/mol; 78%)

$P(HEEP)_{30}$ (entry 3.4): 0.4051 g (7,900 g/mol; 77%)

$P(HEEP)_{15}$ (entry 3.5): 0.3082 g (4,000 g/mol; 70%)

$P(HEEP)_{30}$ (entry 3.6): 0.3750 g (7,900 g/mol; 74%)

$P(HEEP)_{16}$ (entry 3.7): 0.5508 g (4,100 g/mol; 97%)

$P(HEEP)_{12}$ (entry 3.8): 0.1477 g (3,300 g/mol; 75%)

$P(HEEP)_{53}$ (entry 3.9): 0.7213 g (13,800 g/mol; 60%)

$P(HEEP)_{55}$ (entry 3.10): 1.1477 g (14,400 g/mol; 29%)

Polymerisation of ethyl ethylene phosphate

All polymerisations were done with stannous octoate as catalyst in bulk at 90 °C. EEP (0.5010 g, 3.29 mmol) and hydroxy-ethyl pyridyl disulphide as initiator (0.0367 g, 0.22 mmol) were introduced into the schlenk tube via syringe. After cooling down the reaction tube to 0°C, the polymerization was initiated by adding three drops of stannous octoate to the stirred monomer solution. The ice bath was removed and the reaction mixture heated up to 90°C. After two hours, the polymerization was stopped and 2 mL of DCM were added. The polymers were purified by precipitation in diethyl ether once and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the slightly yellow polymer dried *in vacuo*.

Yields:

$P(EEP)_{15}$ (entry 4.1): 0.5762 g (2,400 g/mol; 96%)

$P(EEP)_{23}$ (entry 4.2): 0.4992 g (3,700 g/mol; 95%)

$P(EEP)_{50}$ (entry 4.3): 0.5209 g (7,800 g/mol; 87%)

$P(EEP)_{17}$ (entry 4.4): 0.6805 g (2,800 g/mol; 96%)

$P(EEP)_{50}$ (entry 4.5): 0.8223 g (7,800 g/mol; 96%)

$P(EEP)_{20}$ (entry 4.6): 0.5012 g (3,200 g/mol; 97%)

$P(EEP)_{20}$ (entry 4.7): 1.1580 g (3,200 g/mol; 95%)

Polymerisation of GEP

All polymerisations were done in bulk at 90°C using stannous octoate as a catalyst. GEP (0.6452 g, 2.71 mmol) and benzyl oxy ethanol as initiator (0.0302 g, 0.19 mmol) were introduced into the schlenk tube via syringe. The mixture was heated up to 90°C and stirred for 10 minutes. The polymerization was initiated by adding three drops of stannous octoate to the stirred monomer solution. After 3 hours, the polymerization was stopped and 2 mL of DCM were added. The polymers were purified by precipitation in diethyl ether three times and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colour less polymer dried *in vacuo*.

Yields:

$P(GEP)_{15}$ (entry 5.1): 0.2854 g (3,700 g/mol; 72%)

$P(GEP)_{30}$ (entry 5.2): 0.2888 g (7,300 g/mol; 30%)

$P(GEP)_{20}$ (entry 5.3): 0.3642 g (4,900 g/mol; 54%)

$P(GEP)_{40}$ (entry 5.4): 0.5701 g (9,700 g/mol; 96%)

$P(GEP)_{20}$ (entry 5.5): 0.5850 g (4,900 g/mol; 63%)

PEEP ^{1}H NMR (entry 4.2): (300 MHz, DMSO-d_6): δ 8.48 (d, 1H, N=C*H*-CH), 7.84-7.86 (m, 1H, C-C*H*-CH) 7.50-7.80 (m, 1H, C=CH-C*H*=CH), 7.25 (m, 1H, N=CH-C*H*=CH), 4.89 (t, 1H, P-O-CH_2-CH_2-O*H*), 4.18-4.15 (m, 62 H, O-CH_2-CH_2-O), 4.07 (m, 30H, O-CH_2-CH_3), 3.57 (m, 2H, P-O-CH_2-CH_2-OH), 1.25-1.27 (t, 132H, O-CH_2-CH_3).

PHEEP ^{1}H NMR (entry 3.7): (300 MHz, DMSO-d_6): δ 8.48 (d, 1H, N=C*H*-CH), 7.84-7.86 (m, 1H, C-C*H*-CH), 7.50-7.80 (m, 1H, C=CH-C*H*=CH), 7.25 (m, 1H, N=CH-C*H*=CH), 7.17-7.44 (m ,75H, *Ar*), 4.76-4.80 (t, 1H, P-O-CH_2-CH_2-O*H*), 3.91-4.25 (m, 90 H, O-CH_2-CH_2-O), 3.65-3.84 (m, 2H, P-O-CH_2-CH_2-OH), 3.52-3.64 (m, 30H, Ar-CH_2-O).

P(GEP) ^{1}H NMR (entry 5.3): (300 MHz, DMSO-d_6): δ 7.23-2.32 (m, 5H, *Ar*), 4.83 (t, 1H, O*H*), 4.52 (s, 2H, Ar-CH_2-O), 4.13-4.29 (m, 100H, O-CH_2-CH_2-O), 3.91-4.10 (m, 40H, O-CH_2-CH), 3.54-3.64 (m, 2H, Ar-CH_2-O-CH_2-CH_2), 3.35-3.37 (m, 2H, P-O-CH_2-CH_2-OH), 1.33 (s, 60H, CH_3), 1.27 (s, 60H, CH_3).

6.5 Initiator

Hydroxy-ethyl pyridyl disulfide

A two-necked round bottom flask, that was flame-tried under vacuum and purged with argon three times, was equipped with a dropping funnel an a septum. Aldrithiol-2 (5.083 g, 23 mmol) was dissolved in degassed methanol (25 mL) in that flask. 0.76 mL degassed acetic acid were added. Afterwards, 2-mercaptoethanol (1.2 g, 15 mmol) was dropped into that solution very slowly. The solution was stirred overnight. The solvents were evaporated and the crude product purified by column chromatography (petroleum ether/ethyl acetate 4/3). The product was received as colour less liquid with an R_f-value of 0.23 (yield: 1.35 g, 7.19 mmol, 47%).

^{1}H NMR: (300 MHz, $CDCl_3$): δ 8.50 (m, 1H, N=C*H*-CH), 7.61 (m, 1H, C=C*H*-CH), 7.41 (m, 1H, C=CH-C*H*=CH), 7.31 (m, 1H, N=CH-C*H*-CH), 5.77 (t, 1H, O*H*), 3.83 (m, 2H, S-CH_2-CH_2), 2.95 (t, 2H, CH_2-CH_2-O).

6.6 Deprotection of polymers

Deprotection of benzyl protected P(HEEP) using hydrogenation

In a reactor, 30 mg of polyphosphate were dissolved in 3 mL dry THF. 30 mg of $Pd(OH)_2$ were added and the solution stirred for 4 hours under 40 bars of hydrogen pressure. After completition of the hydrogenation, the catalyst was removed by centrifugation (10 min, 4500 rpm, 0°C) and decantation of the supernatant. THF was evaporated and the deprotected polymer dried *in vacuo* (yield: 0.2128 g; 0.07 mmol; 100%).

^{1}H NMR (entry 8.8): (300 MHz, DMSO-d_6): δ 4.76-4.80 (t, 16H, P-O-CH_2-CH_2-O*H*), 3.91-4.25 (m, 90 H, O-C*H*$_2$-C*H*$_2$-O), 3.65-3.84 (m, 2H, P-O-CH_2-C*H*$_2$-OH).

Deprotection of P(GEP) using acidic hydrolysis

In a 50 mL round-bottom flask, the polymer (0.0895g; 0.018 mmol) was dissolved in 15 mL 1,4 dioxane. Aqueous hydrochloric acid (1 mol/L; 5 mL) was added dropwise via a syringe. After stirring the solution for additional 3 hours, the solution was concentrated in vacuum and the product precipitated into cold diethyl ether with reasonable yields compared to literature (yield: 0.0749 g, 0.018 mmol, 90%).

^{1}H NMR P(HEP): (300 MHz, DMSO-d_6): δ 7.23-2.32 (m, 5H, *Ar*), 4.83 (t, 41H, O*H*), 4.52 (s, 2H, Ar-C*H*$_2$-O), 4.13-4.29 (m, 100H, O-C*H*$_2$-C*H*$_2$-O), 3.91-4.10 (m, 40H, O-C*H*$_2$-CH), 3.54-3.64 (m, 2H, Ar-CH_2-O-C*H*$_2$-CH_2), 3.35-3.37 (m, 2H, P-O-CH_2-C*H*$_2$-OH).

6.7 Conjugation of P(EEP) with different peptides

Conjugation of P(EEP) with N*-acetyl-L-cysteine-methyl-ester*

$P(EEP)_{23}$ (0.0480 g; 0.013 mmol) was dissolved in 0.5 mL of degassed DMSO and *N*-acetyl-L-cysteine-methyl-ester (0.0030 g; 0.017 mmol) was dissolved in 2.5 mL of degassed MilliQ-water. Using Argon as inert gas, the polymer solution was added dropwise under vigorous stirring to the amino acid solution and the mixture was stirred for additional 18.5 hours at room-temperature. The solution

was dialysed against MilliQ-water for 1 day (MW: 14000 g/mol). The solvent was evaporated *in vacuo* and the pure product obtained as colorless oil (yield: 0.1438 g; 0.013 mmol; 100%).

^{1}H DOSY NMR (700 MHz, DMSo-d_6): δ 4.89 (t, 1H, P-O-CH_2-CH_2-O*H*), 4.18-4.15 (m, 62 H, O-CH_2-CH_2-O), 4.07 (m, 30H, O-CH_2-CH_3), 3.66 (s, 3H, O-CH_3), 3.57 (m, 2H, P-O-CH_2-CH_2-OH), 1.86 (s, 3H, CO-CH_3), 1.25-1.27 (t, 132H, O-CH_2-CH_3), D=6.72x10^{-11}m^2/s.

Conjugation of polyphosphate to L-glutathion

To a stirred solution of the peptide (0.01 g, 0.33 mmol) in 1 mL degassed Milli-Q water was added a solution of the polymer (0.1050 g; 0.24 mmol) in dimethyl sulfoxide under inert gas atmosphere. The mixture was stirred for 3 hours at room-temperature and afterwards dialysed in water to remove the excess of L-glutathion (MW = 14000 g/mol). The solvent was evaporated and the procuct obtained as colorless oil (yield: 0.2056 g; 0.24 mmol; 100%).

Conjugation of polyphosphate to cell-penetrating peptide 2

The cell-penetrating peptide (0.0021 g; 0.001 mmol) was dissolved in 0.5 mL of degassed MilliQ-water. The polyphosphate (0.04 g; 0.051 mmol) was also dissolved in 0.5 mL of degassed MilliQ-water. Afterwards, the peptide solution was added dropwise to the stirred polymer solution under inert gas atmosphere. After 2.5 hours of stirring the solution at room-temperature, the solvent was removed and the product obtained as colorless oil (0.01 g, 100%).

HPLC (RP-CN; 20°C; 1 mL/min): entry 10.3

Solvents: A: acetonitrile with 0.01% TFA and B: water with 0.001% TFA

Gradient: starting with 20% of A and 80% of B becoming 100% of A within 20 minutes

Elution time: 8.25 min

6.8 Copolymerisation of the different monomers

The polymerization reactions were carried out in 25 mL Schlenk tubes. The tubes were flame-dried under vacuum and purged with argon three times prior to use. All polymerisations were done with

stannous octoate as catalyst in bulk at 90 °C. The monomers as well as initiator were introduced into the schlenk tube via syringe. After cooling down the reaction tube to 0°C, the polymerization was initiated by adding three drops of stannous octoate to the stirred monomer solution. The ice bath was removed and the reaction mixture heated up to 90°C. After completition, the polymerization was stopped and 2 mL of DCM were added. The polymers were purified by precipitation in diethyl ether once and centrifuged (10 min, 4500 rpm, 0°C). The supernatant was decanted and the colorless polymers dried *in vacuo*.

Yields:

$P(EEP)_{32}$ (entry 11.1): 0.6235 g (5,000 g/mol; 95%)

$P(GEP)_{39}$ (entry 11.2): 1.1266 g (9,400 g/mol; 97%)

$P(HEEP)_{36}$ (entry 11.3): 0.5007 g (9,400 g/mol, 92%)

$P(EEP)_{15}$-co-$(GEP)_{15}$ (entry 11.4): 0.4632 g (6,000 g/mol; 89%)

$P(EEP)_{15}$-co-$(HEEP)_{15}$ (entry 11.5): 0.6246 g (6,300 g/mol; 94%)

$P(EEP)_{18}$-co-$(HEEP)_{19}$ (entry 11.6): 0.7500 g (7,700 g/mol; 98%)

$P(HEEP)_{18}$-co-$(GEP)_{15}$ (entry 11.7): 0.4602 g (8,300 g/mol; 91%)

$P(HEEP)_{22}$-co-$(GEP)_{19}$ (entry 11.8): 0.6609 g (10,300 g/mol; 97%)

$P(HEEP)_{24}$-co-$(GEP)_{7}$ (entry 11.9): 0.5851 g (8,000 g/mol; 94%)

$P(HEEP)_{30}$-co-$(GEP)_{10}$ (entry 11.10): 0.6374 g (10,200 g/mol; 99%)

$P(HEEP)_{9}$-co-$(GEP)_{23}$ (entry 11.11): 0.5774 g (7,900 g/mol; 100%)

$P(HEEP)_{10}$-co-$(GEP)_{27}$ (entry 11.12): 0.7590 g (9,100 g/mol; 98%)

$P(EEP)_{11}$-co-$(HEEP)_{34}$ (entry 11.13): 0.6802 g (10,600 g/mol; 91%)

$P(EEP)_{11}$-co-$(HEEP)_{32}$ (entry 11.14): 0.5702 g (10,300 g/mol; 87%)

$P(EEP)_{20}$-co-$(HEEP)_{9}$ (entry 11.15): 0.5349 g (5,500 g/mol; 91%)

$P(EEP)_{27}$-co-$(HEEP)_{10}$ (entry 11.16): 0.4983 g (6,800 g/mol; 85%)

$P(EEP)_{26}$-co-$(GEP)_{10}$ (entry 11.17): 0.5332 g (6,400 g/mol; 97%)

$P(EEP)_{27}$-co-$(GEP)_{9}$ (entry 11.18): 0.6400 g (6,400 g/mol; 98%)

$P(EEP)_6$-co-$(GEP)_{23}$ (entry 11.19): 0.7633 g (6,500 g/mol; 94%)

$P(EEP)_{11}$-co-$(GEP)_{22}$ (entry 11.20): 0.4632 g (7,000 g/mol; 96%)

PEEP ^{1}H NMR (entry 11.1): (300 MHz, DMSO-d_6): 4.89 (t, 1H, P-O-CH_2-CH_2-O*H*), 4.18-4.15 (m, 62 H, O-C*H*$_2$-C*H*$_2$-O), 4.07 (m, 30H, O-C*H*$_2$-CH_3), 3.57 (m, 2H, P-O-CH_2-C*H*$_2$-OH), 1.25-1.27 (t, 132H, O-CH_2-C*H*$_3$), 0.82 (m, 3H, CH_2-CH_2-C*H*$_3$).

PHEEP ^{1}H NMR (entry 11.2): (300 MHz, DMSO-d_6): δ 7.17-7.44 (m ,75H, *Ar*), 4.76-4.80 (t, 1H, P-O-CH_2-CH_2-O*H*), 3.91-4.25 (m, 90 H, O-C*H*$_2$-C*H*$_2$-O), 3.65-3.84 (m, 2H, P-O-CH_2-C*H*$_2$-OH), 3.52-3.64 (m, 30H, Ar-C*H*$_2$-O), 0.82 (m, 3H, CH_2-CH_2-C*H*$_3$).

P(GEP) ^{1}H NMR (entry 11.3): (300 MHz, DMSO-d_6): δ 4.83 (t, 1H, O*H*), 4.13-4.29 (m, 100H, O-C*H*$_2$-C*H*$_2$-O), 3.91-4.10 (m, 40H, O-C*H*$_2$-CH), 3.54-3.64 (m, 2H, Ar-CH_2-O-C*H*$_2$-CH_2), 3.35-3.37 (m, 2H, P-O-CH_2-C*H*$_2$-OH), 1.33 (s, 60H, C*H*$_3$), 1.27 (s, 60H, C*H*$_3$), 0.82 (m, 3H, CH_2-CH_2-C*H*$_3$).

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