Synthesis and characterisation of polyphosphazenes with controlled drug release

Research stay at the Institute for Bioscience and Biotechnology Research, University of Maryland, USA in the group of Prof. Alexander K. Andrianov
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Marshall Plan Scholarship

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Contents

List of Abbreviations

COMU	1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DIEA	N,N-diisopropylethylamine
DLS	dynamic light scattering
DMF	dimethylformamide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimid
EPR	enhanced permeation and retention
FFF	field flow fractionation
Fmoc	fluorenylmethoxycarbonyl
GFLG	glycine-phenylalanine-leucine-glycine
Gly	glycine
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
HPMA	N-(2-hydroxypropyl)methacrylamide
Leu	leucine
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
PGA	polyglutamic acid
Phe	phenylalanine
PLGA	poly(lactide-*co*-glycolide)
PVP	polyvinylpyrrolidone
ROP	ring-opening polimerization
RES	reticuloendothelial system
TAEA	tris(2-aminoethyl)amine
TFA:	trifluoroacetic acid
THF	tetrahydrofuran
UV-Vis	ultraviolet–visible

1. Introduction

1.1. General introduction

During the last decade, polymer-based nanomedicines or polymer therapeutics have become a important tools for increasing the efficacy and positively influencing the bio-distribution of cancer therapeutics. Polymers are suitable to exploit the enhanced permeation and retention effect (EPR) and consequently to reduce side effects due to their adjustable hydrodynamic volume[1]. Moreover, polymeric drug carriers have been demonstrated to enhance plasma solubility and blood circulation time of small drug compounds. Poly(organo)phosphazenes are a versatile class of polymers with immense potential for application in nanomedicine[2]. Recent advances in polyphosphazene synthesis allow controlled molecular weights, narrow molecular weight distributions, controlled hydrodynamic volumes and high water solubility. Thus, their properties can easily be tuned to obtain polymer-drug conjugates with desired characteristics. The most remarkable properties of poly(organo)phosphazenes, which distinguish them from most other known polymers used as drug delivery systems, are their hydrolytically degradable backbones and their non-toxic degradation products, an essential feature in avoiding the deleterious effects associated with post-drug-release accumulation of high molecular weight macromolecules in the organism .This work describes the synthesis of novel polyphosphazene-based drug carriers with tailored characteristics, controlled degradability and drug release.

1.2. Outline

The first part of this work (chapter 1 and 2) gives a detailed overview over the theoretical background of the work presented in the main part (chapter 3 and 4), including polymers as nanomedicines in general, the chemistry and structure of polyphosphazenes and polyphosphazenes for biomedical applications.

The main part of this work consists of two different approaches to preapre biodegradable polyphosphazene-based drug delivery systems which are divided in two different chapters (3 and 4). Both chapters (listed below) consist of an abstract, introduction, materials and methods, results and conclusion. Most of the data was collected during the research stay at the Institute of Bioscience and Biotechnology Research (University of Maryland, USA) in the group of Prof. Alexander Andrianov. The polymers were previously synthesized at the Institute of Polymer Chemistry (Johannes Kepler University Linz, Austria) supervised by Prof. Ian Teasdale and Prof. Oliver Brüggemann.

- Chapter 3: *Biodegradable polyphosphazene based peptide-polymer hybrids*
- Chapter 4: *(Bio)degradable polyvinylpyrrolidone based hybrid polymers*

2. Theoretical background

2.1. Polymer based drug delivery systems

2.1.1. Definitions

Different terms have been created during the last decades to describe different polymer based systems for drug delivery applications. To give an overview, it might be helpful to start with some definitions:

a) Nanomedicine and nanomedicines

"Nanomedicine" is a relatively young field and relates to nano-sized materials as diagnostics and for prevention and treatment of diseases[3]. The term "nanomedicines" includes nanopharmaceuticals, nanoimaging agents and nanotheranostics[3] like liposomes, organic or inorganic nanoparticles, polymer therapeutics, micelles, nanocrystals or carbon nanotubes. Over the last two decades more than 40 nanomedicines haven been approved for human use and many others are in clinical trials[3].

b) Polymer therapeutics

The term polymer "polymer therapeutics", coined by Ruth Duncan, refers to a family of polymer based nanomedicines[3],[4]. It covers a variety of complex macromolecular systems with chemical bonds between bioactive molecules and water-soluble polymeric carrier with or without inherent activity[5]. A tripartite design comprising polymer, linker and bioactive molecule, has been typically described for these systems although the use of additional targeting moieties or imaging agents are also used[3]. Polymer therapeutics include different types of drug delivery systems: polymeric drugs[6],[7], polymer-drug conjugates[4], polymer-protein conjugates[4],[8], polymeric superstructures like micelles to which a drug is covalently bound[9] , dendrimers[10] and polyplexes[11],[1] (Figure 2-1).

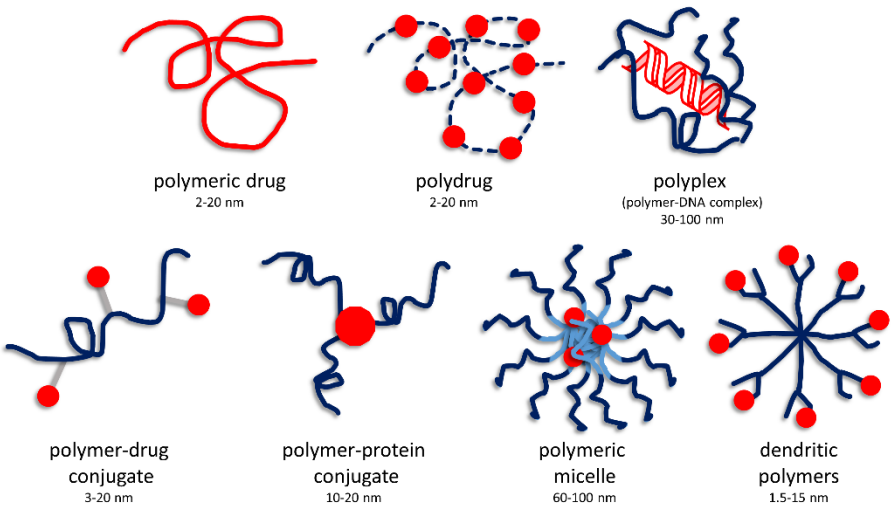


Figure 2-1: Scheme showing the family of polymer nanomedicines called polymer therapeutics.

c) Prodrug

"Prodrugs" are constructs including drugs, which remain inactive during delivery to the site of action and is activated by specific conditions present in the targeted site[12]. Targeted sites can be different organs, tissues or cells. Common chemical or metabolic processes like the cleavage of a bond between a polymer and a drug at specific pH values or by specific enzymes, are used to obtain prodrug reconversion[12]. If polymers are used to create an inactive form of a drug, the conjugate is sometimes called a "polymeric prodrug"[12].

d) Polymer-drug conjugates

In this work polyphosphazene based polymer-drug conjugates are presented. They can be termed nanomedicines, polymer therapeutics and prodrugs as well. Typical, polymer-drug conjugates consist as a minimum of three essential parts; the polymer backbone, a stimuli responsive linker and the therapeutic agent[3]. Ringsdorf described in 1975 a model (Figure 2-2) where a number of drug molecules are bound to a polymer backbone through linker molecules with a breaking point, to ensure controlled release of the drug at the target site, and targeting moieties like peptides, sugars moieties or antibodies to target disease-related receptors or antigens[13]. In addition, solubilizing groups and further therapeutic or imaging agents can be bound to the polymeric backbone.

Polymers for the preparation of polymer-drug conjugates should ideally be nontoxic, water-soluble and degraded or eliminated from the organism to avoid post-drug release accumulation in the body. Moreover, suitable functional groups at the polymer backbone are required for attachment of the functional side groups or drugs. Commonly used polymer backbones are, for instance, polyphosphazenes[14], polyethylene glycol (PEG), *N*-(2-hydroxypropyl)methacrylamide (HPMA), polyglutamic acid (PGA), dextran, chitosan[12].

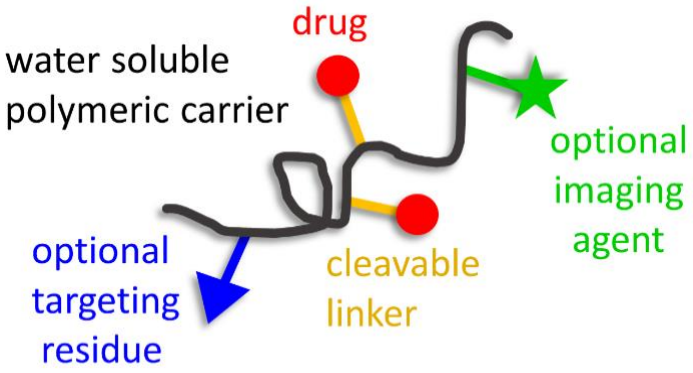


Figure 2-2: Scheme of polymer-drug conjugate containing targeting residue, cleavable linker and optional imaging agent.

2.1.2. Polymer therapeutics for cancer therapy

Conventional cancer chemotherapy – the standard treatment of cancer – in which high toxic, low-molecular-weight agents are indiscriminately delivered to tumors, as well as healthy tissue and organs, lead to severe, undesired side effects like nausea, bone-marrow toxicity, hair loss, increased susceptibility to illness or cardiotoxicity[15]. Effective drug delivery via intravenous injection involves the avoidance of elimination of the drug from the bloodstream by the kidneys, liver or other organs, the movement of the drug into the effected tissue/cell to enhance therapeutic efficacy and the prevention of side effects. Macromolecules are able to meet these requirements due to their high molecular weight and hydrodynamic volume, compared to usual low-molecular weight drugs. It is a combination of enhanced plasma circulation times and exploitation of the enhanced permeation and retention (EPR) effect that leads to passive tumor targeting and consequently a higher therapeutic efficacy of the drug, if macromolecules are used. Moreover they enable protection of drugs from deactivation, preservation of drug

activity during their way to the targeted site, active tumor targeting and improve of water solubility of low soluble or insoluble drugs[12]. In addition the combination with several other active components become possible[12]. These characteristics of low molecular weight drugs and high molecular weight drugs/prodrugs are compared in Table 2-1.

Table 2-1: Characteristics of low molecular weight drugs compared to those of high molecular weight drugs/produrgs.

low molecular weight drug	high molecular weight drug/prodrug
accumulation in tumor and healthy tissue	decreased accumulation in healthy tissue increased accumulation in tumor tissue passive targeting to tumor
short retention times in tumor tissue fast renal clearance → short plasma circulation times	enhanced retention times in tumor tissue enhanced plasma circulation times
only hydrophilic drugs	enhanced solubility of hydrophobic drugs
degradation or inactivation of drug during plasma circulation	protection of drug against degradation or inactivation
combination therapy difficult	possibility of combination therapy
cellular uptake by diffusion	cellular uptake by endocytosis
→low therapeutic efficacy and severe side effects	**→Improved therapeutic efficacy and pharmacokinetic profile of drug and avoidance of side effects**

a) Plasma circulation time - Bioavailability

The difference in size of polymers and classical small molecule drugs lead to a significantly different behavior *in vivo*. The hydrodynamic volume of polymers is in the same size range as the pores and openings present in the vasculature and elimination system of the body and has a significant effect on the transport of the polymer trough the body and on the removal from the body[15]. Usual drugs are eliminated from the body by renal clearance. The renal pore size, responsible for renal clearance, is 4-14 nm in diameter[16]. The threshold for renal clearance of macromolecules corresponds roughly with their molecular weights ranging from 30 to 50 kDa, depending on the macromolecular chemistry, shape, conformation and flexibility[4]. Drugs or macromolecules with sizes below 4-14 nm readily permeate the pores and are removed from the body by urine[16]. The liver and components of the reticuloendothelial system (RES) can also remove macromolecules from the bloodstream. Particles or aggregates

above 200 nm in diameter are readily bound to opsonin proteins and cleared by the liver and spleen[15],[17]. Consequently, synthesis of macromolecules with controlled molecular weights and narrow molecular weight distribution is of significant importance for drug delivery applications. With careful choice of molecular weight and hydrodynamic volume of the macromolecular drug or drug carrier, clearance from the bloodstream can be avoided and the plasma circulation time can be increased.

b) Passive Targeting - Enhanced permeation an retention effect

As already mentioned the EPR effect is one of the reasons why macromolecules are of high interest for drug delivery applications. This important effect is based on the abnormal characteristics of tumor vessels and the lack of effective tumor lymphatic drainage[18]. When tumor tissue is growing, new blood vessels or neovasculature is necessary to supply nutrients and oxygen[19]. Usually, these newly formed vessels own irregular and incomplete architectures[19],[20]. The endothelial cells of tumors are not as tightly packed as in healthy tissue leading to higher permeability to macromolecules. Consequently, macromolecular drug delivery systems are able to enter the tumor tissue but not the healthy tissue, whereas small molecular drugs distribute indiscriminately in the whole body (Figure 2-3).

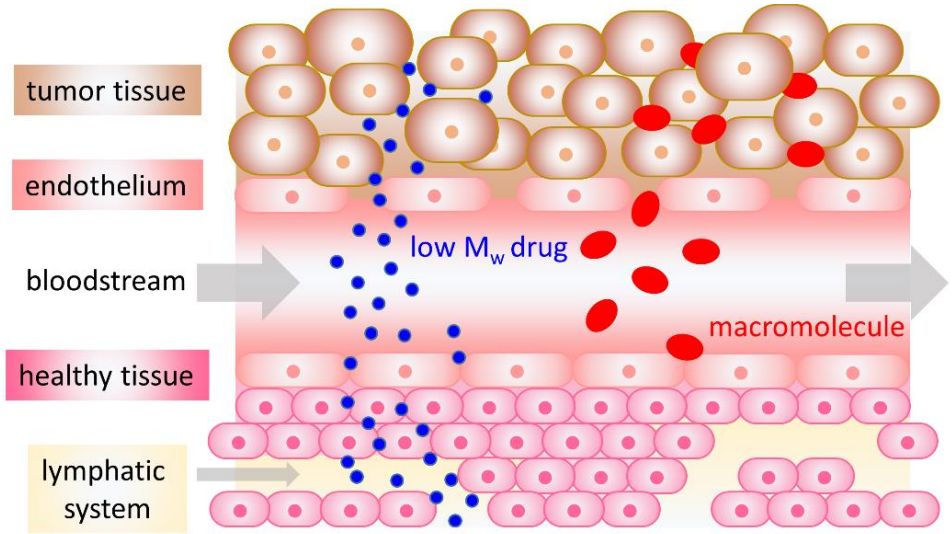


Figure 2-3: Scheme of EPR effect of low-molecular-weight drugs (blue) and high-molecular-weight drug or prodrug (red). The fast growing tumor tissue is uneven and large gaps exist between its endothelial cells that almost all molecules can path through.

The gap size in tumor vasculature is dynamic and varies between different types of tumor as well as between vessels in the same tumor, but particles in a size range of 20 to 100 nm are reported to be the optimum for prolonged circulation times and high accumulation in the tumor tissue[21]. The same is reported for molecular weights in the range of 20-200 kDa to exploit the EPR effect. Moreover the lymphatic system of rapidly growing tumors is defective or nonexistent, thus there is no lymphatic elimination of polymers and drugs from the tumor tissue[22]. Due to their size it is moreover difficult for low-molecular-weight drugs, to keep the drug concentration in the tumor tissue higher than in the blood, whereas macromolecular drugs are retained for a prolonged time at higher concentrations in the tumor tissue (Figure 2-4)[20].

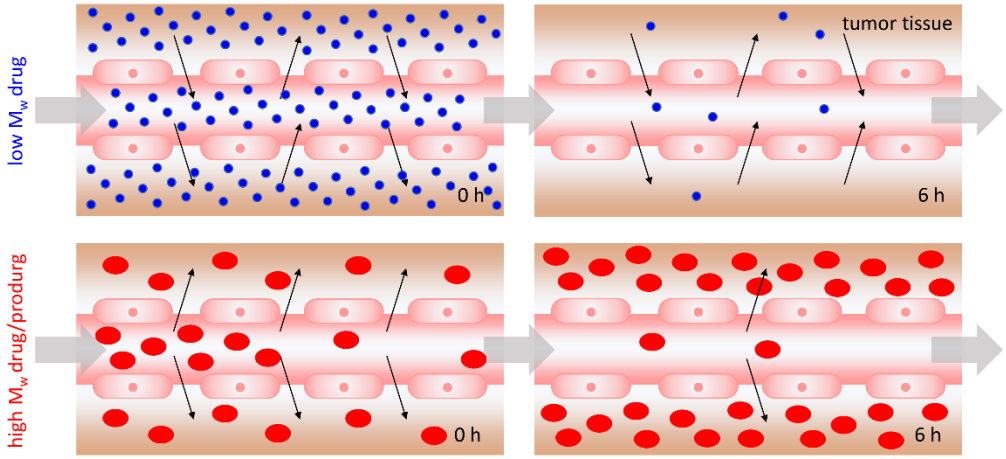


Figure 2-4: Scheme of EPR effect of low-molecular-weight drugs (blue) and high-molecular-weight drug or prodrug (red). The fast growing tumor tissue is uneven and large gaps exist between its endothelial cells that almost all molecules can path through. Compared to high-molecular weight drugs, it is more difficult for low-molecular-weight drugs to keep the drug concentration in the tumor tissue higher than in the blood for long periods of time. Macromolecules show enhanced tumor uptake and are retained for an extended time at high concentrations in the tumor tissue[20].

c) Active Targeting

The passive targeting associated with the EPR effect can be significantly improved by active mechanisms like receptor-ligand interactions[10, 21d]. This type of targeting is only possible if targeting moieties are added to the macromolecular drug delivery system and when specific molecular receptors like transferrin receptor or folate receptor are present in malignant tumor cells[10]. Usually, a targeting moiety is focused on specific receptors or antigens overexpressed in the plasma membrane or intracellular membrane in tumor cells. Examples for targeting moieties are peptides, carbohydrates

or other substrates which selectively bind to cell surface receptors[23], monoclonal or polyclonal antibodies which interact with antigens on cell surface[24], or moieties with high affinity to compounds in the extracellular matrix of the target site[25]. Thus, with the use of such targeting moieties, polymer–drug conjugates can be selectively transported into tumor tissues.

d) Cellular uptake and controlled intercellular drug release

In contrast to low molecular weight drugs, macromolecules are taken up by the cell via receptor-mediated endocytosis, adsorptive endocytosis or fluid-phase endocytosis[26]. During endocytosis the pH value decreases from the physiological value of pH 7.2–7.4 in the extracellular space to pH 6.5–5.0 in the endosomes and to around pH 4.0 in primary and secondary lysosomes[1]. Moreover, a great number of enzymes are present in the lysosomal compartment, for instance, phosphatases, nucleases, proteases, esterases, and lipases. In addition, tumor tissue contains at least 4-fold higher reducing glutathione levels relative to normal tissues[27]. Polymer-drug conjugates should be sufficiently stable in the blood stream prior to the drug being released at the target site and in an ideal case, the drug should be released from the conjugate initiated by biochemical or physiological conditions unique for the targeted site[1]. To obtain controlled drug release the different conditions present in tumor tissue and tumor cells, mentioned above, can be exploited. In the case of polymer-drug conjugates, drugs are conjugated via special linkers which are sensitive to the described changes in tumor environment. Based on the acidic environment in tumor tissues and tumor cells, pH-sensitive linkers, such as hydrazone, cis-acotinyl or acetal, were designed to obtain drug release in the tumor cells[28]. The high redox potential can be exploited by using redox potential sensitive disulfide bridges to conjugate therapeutic agents and moreover, the highly upregulated cathepsin B in lysosomes of malignant tumor cells can been exploited as drug-release trigger by incorporation of lysosomally cleavable oligopeptide linkers like Gly-Phe-Leu-Gly (GFLG) between drug and polymeric backbone[29].

2.2. Structure and synthesis of polyphosphazenes

Polyphosphazenes are a group of polymers with a flexible inorganic backbone based on the repeat unit consisting of phosphorus and nitrogen, which are connected by alternating single and double bonds (Figure 2-5)[30]. Many unique characteristics of polyphosphazenes, like high flexibility, high thermal stability[31], ionic conductivity[32],[32b] and hydrolytic degradability[33] are based on this inorganic backbone. A variety of organic, organometallic or inorganic side groups can be attached to the backbone[34]. The most common substituents are of organic character and lead to poly(organo)phosphazenes. The nature of the side groups has a decisive effect on the properties of the resulting polymer. Thus the characteristics of polyphosphazenes can be specifically adjusted by selective choice and combination of a large number of different side chains with regard to their desired applications[35].

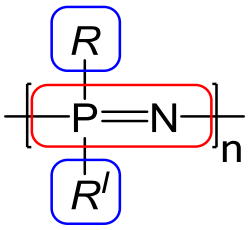


Figure 2-5: General structure of poly(organo)phosphazenes with their unique inorganic backbone (red) and organic side groups (blue).

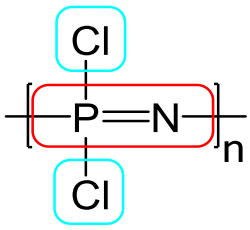


Figure 2-6: Structure of poly(dichlorophosphazene).

2.2.1. Poly(dichloro)phosphazene

Poly(dichloro)phosphazene $(NPCl_2)_n$ is, due to its role as fundamental macromolecular precursor, of decisive significance for the synthesis of poly(organo)phosphazenes. The repeat unit of poly(dichloro)phosphazene contains two highly labile chlorine atoms, which leads to a high synthetic flexibility by allowing the straightforward substitution of a huge number of organic nucleophiles and hence the achievement of a wide variety of properties (Figure 2-6)[36],[37]. Commonly two methods are used to obtain poly(dichloro)phosphazenes:

a) Ring-opening polymerization (ROP) of hexachlorocyclotriphosphazene

The traditional and most common route to prepare high molecular weight poly(dichloro)phosphazenes, is the thermally induced ring-opening polimerization (ROP) of hexachlorocyclotriphosphazene at high temperatures, leading to polymers with broad molecular weight distribution (Figure 2-7)[36],[37].

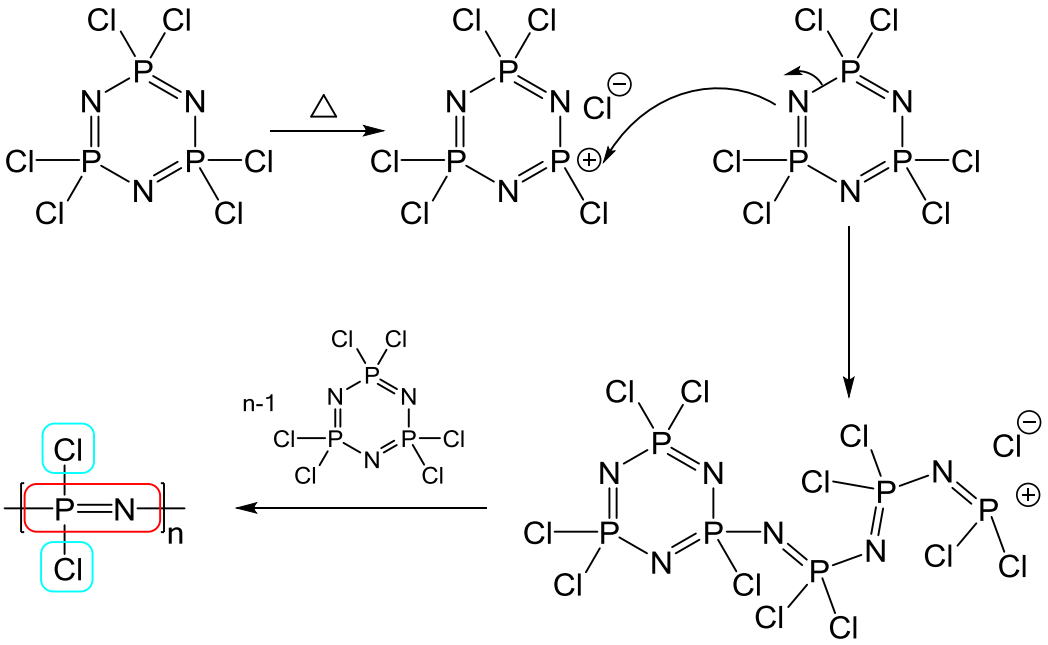


Figure 2-7: Mechanism for ring-opening polymerization of hexachlorocyclotriphosphazene.

The ROP procedure requires a high purity of the monomer and high temperatures for reproducible results[38]. Furthermore a drawback of ROP is the tendency to produce branching and crosslinked substances at higher conversions (Figure 2-8)[36]. It is assumed that this is due to moisture and the resulting formation of hydroxyphosphazenes, but there is growing evidence that this is a polymerization-based phenomenon, because no effort to reach higher drying or purification, prevents branching or crosslinking [36]. Moreover, ROP leads to polymers with broad polydispersities (M_w/M_n >2) due to its uncontrolled initiation mechanism with continuous formation of new chain. Polydispersities in this range are tolerable for a lot of medical applications but furthermore there are applications in which exact molecular weights are a requirement. Moreover, this polymerization technique allows no end-group control and thus there is not yet a possibility to obtain higher polymer architectures.

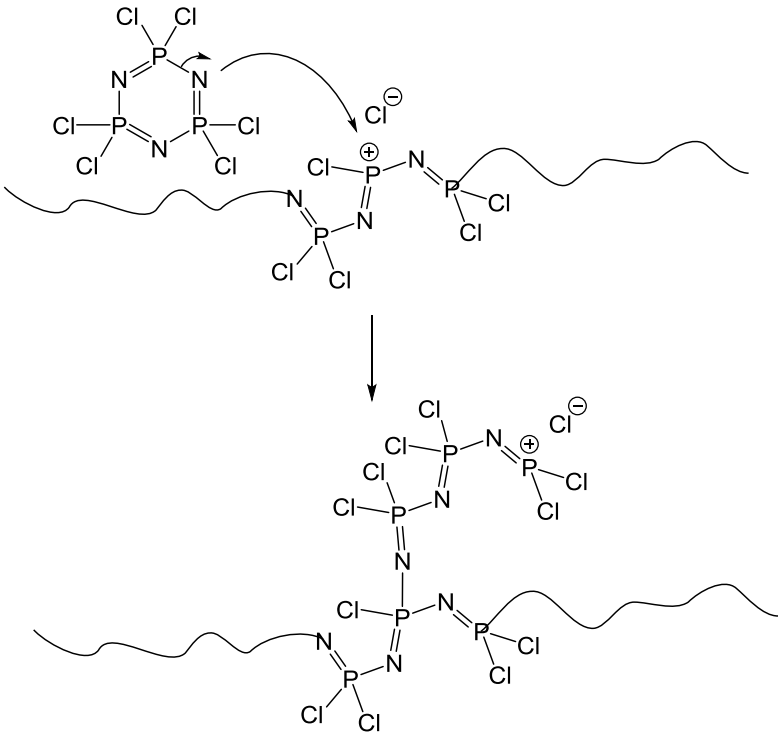


Figure 2-8: Supposed reason for branching and crosslinking during the synthesis of $[NPCl_2]_n$ during ROP.

b) Living cationic chain growth polycondensation of trichlorophosphoranimine

The second method to obtain poly(dichloro)phosphazene is the living cationic chain growth polycondensation of N-(trimethylsilyl)-trichlorophosphoranimine ($Cl_3PNSi(CH_3)_3$) at room temperature, which was developed by Manners and Allcock (Figure 2-9)[39],[40]. The poly(organo)phosphazenes synthesized for this thesis were obtained via this route. This method leads to polyphosphazenes with controlled molecular weights and narrow molecular weight distributions due to its controlled initiation and chain growth mechanism and thus opens the door to advanced macromolecular structures and architectures which are of great interest for many medical applications[41].

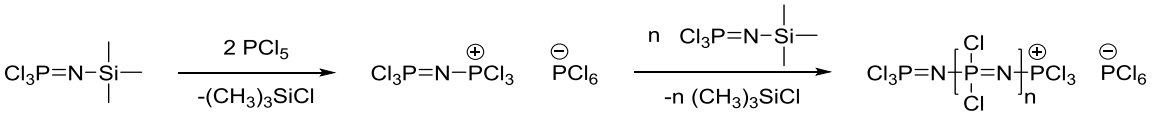


Figure 2-9: Living cationic chain growth polycondensation of $Cl_3PNSi(CH3)_3$ initiated by PCl_5.

Trichlorophosphoranimine ($Cl_3PNSi(CH_3)_3$) is initiated with phosphorus pentachloride (PCl_5) and the initiating species with the PCl_3^+ cationic end group and PCl_6^- as counter ion is formed. It is supposed that two PCl_5 molecules are needed to form this initiation species. The chain grows via reactions with further monomer molecules occurs in a living, cationic, chain growth polycondensation mechanism with the elimination of trimethylchlorosilane ($(CH_3)_3SiCl$) until monomer conversion is complete[42]. The molecular weight can be controlled by the ratio of PCl_5 to $Cl_3PNSi(CH_3)_3$[40, 43].

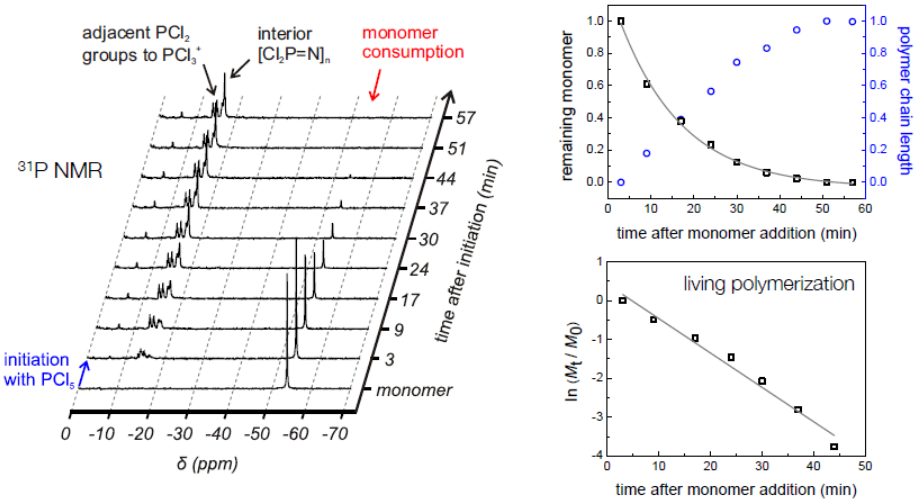
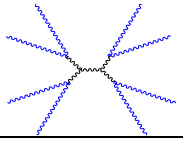
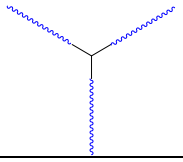
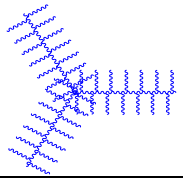
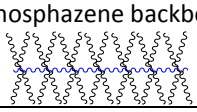
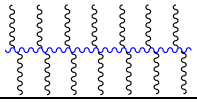
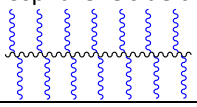
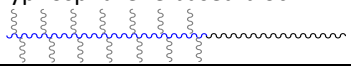
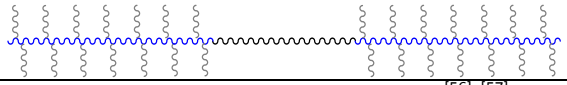
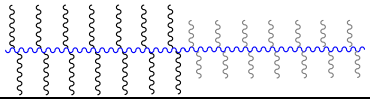
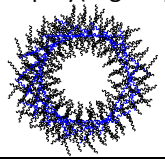
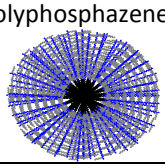


Figure 2-10: Monomer consumption and linear chain growth (right) of PCl_5 initiated polymerisation of trichlorophosphoranimine in CH_2Cl_2 followed by 31P nuclear magnetic resonance spectroscopy (left)[44].

Analysis of poly(organo)phosphazenes after macromolecular substitution of the chlorine atoms of poly(dichloro)phosphazene which were synthesized by this method, shows linear increase in chain length with progressing conversion, which is representative for a living polymerization (Figure 2-10) and the polydispersity index of the obtained polymers is generally low (M_w/M_n = 1.01-1.4)[39]. Thus, in contrast to the ring-opening route polymers with controlled molecular weight and narrow molecular weight distribution are obtained. Moreover, branching of $(NPCl_2)_n$ is generally not obtained with this polymerization pathway[43c]. Due to the chain ends remain active, controlled termination reactions or the addition of other phosphoranimines become possible[45]. This allows the synthesis of block and graft copolymers and a variety of polymer architectures like star or brush-type structures. Following Table 2-2 gives an overview over the variety of polymer architectures formed by or in combination with poly(organo)phosphazenes.

Table 2-2: Architectures and superstructures formed by or in combination with polyphosphazenes (blue).

architecture	role of polyphosphazene and scheme
dendrimer	polyphosphazene side arm[46] 
triarmed star	polyphosphazene arms[47] 
branched star	polyphosphazene backbone and side arms[48] 
brush, grafting-to	polyphosphazene backbone[49] 
brush, grafting-from	polyphosphazene backbone[50] 
brush	polyphosphazene side chain[51] 
diblock-copolymers	one polyphosphazene based block[52],[53],[54],[55] 
triblock-copolymer	two polyphosphazene based blocks[54] 
diblock-copolymers	two polyphosphazene based blocks[56], [57] 
polymersomes	random substituted poly(organo)phosphazenes[58] 
micelles	amphiphilic polyphosphazene diblock[59],[53], 

The mechanism of the living chain growth polycondensation is still a matter of on ongoing investigation[43c],[60], which include, for example, the influence of monomer to initiator ratio on the obtained molecular weight[43a] the forming and influence of the counter ion[60], the delocalization of the cationic propagating site and the resulting bidirectional chain growth[43a],[43c]. As the two chain ends may react at different rates, broader molecular weight distributions could be obtained[43c]. The influence of different solvents and initiators have also been observed, but PCl_5 in dichloromethane seems to be the best combination with regard to solubility of the initiator and reaction rates[43a].

Moreover, monomers like trialkoxyphosphoranimines[61] or initiators like $[R_3P{=}N{=}PCl_3][PCl_6]$ in CH_2Cl_2 can be used to ensure mono-directional growth[62]. Alternative approaches to synthesize polyphosphazenes are, for example, the direct synthesis starting from other phosphoranimines including organophosphoranimines[63], N-silylphosphoranimines[64] and N-phosphorylphosphoranimines[65], or the chain growth polymerization of poly-bis(trifluoroethoxy)phosphazene starting from $(F_3CH_2CO)_3P{=}N\text{-}SiMe_3$ induced by an anionic catalyst[66].

c) Phosphine-initiated living polymerization of trichlorophosphoranimine

Chlorinated triphenylphosphines with or without different functional groups like a double bond or a protected carboxylic acid can also be used as initiating species to polymerize N-(trimethylsilyl)-trichlorophosphoranimine to yield mono-end-functionalized polyphosphazenes (Figure 2-11)[67]. The functional end groups of the resulting polymers enable further modification reactions by, for example, esterification or thiol-ene chemistry and the resulting polyphosphazenes represent suitable building blocks for the synthesis of controlled molecular architectures such as block copolymers, branched or star-shaped polyphosphazenes.

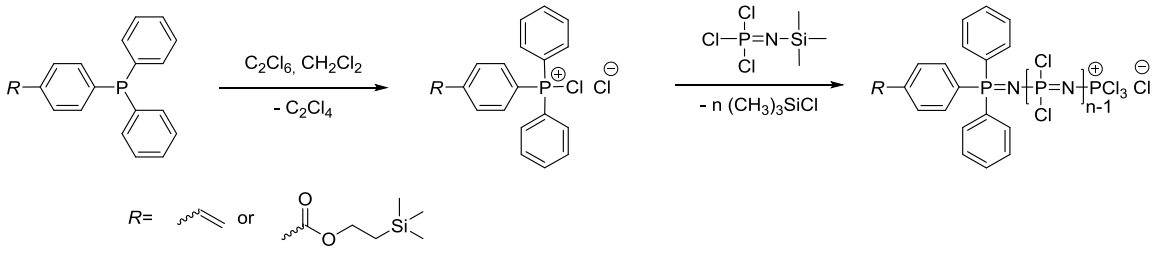


Figure 2-11: Synthesis of mono-end group functionalized poly(dichloro)phosphazenes in a one-pot reaction using a functional phosphine as initiating species.

Moreover, the phosphine mediated polyphosphazenes synthesis opens the door to new brushed, star and dendrimer-like structures. Both, hexachlorocyclotriphosphazene or polyphosphazenes substituted with, for example, (diphenylphosphino)-1-propylamine can act as macroinitiator after chlorination with C_2Cl_6 for the polymerisation of trichlorophosphoranimine leading to star- or brush-shaped polymers (Figure 2-12).

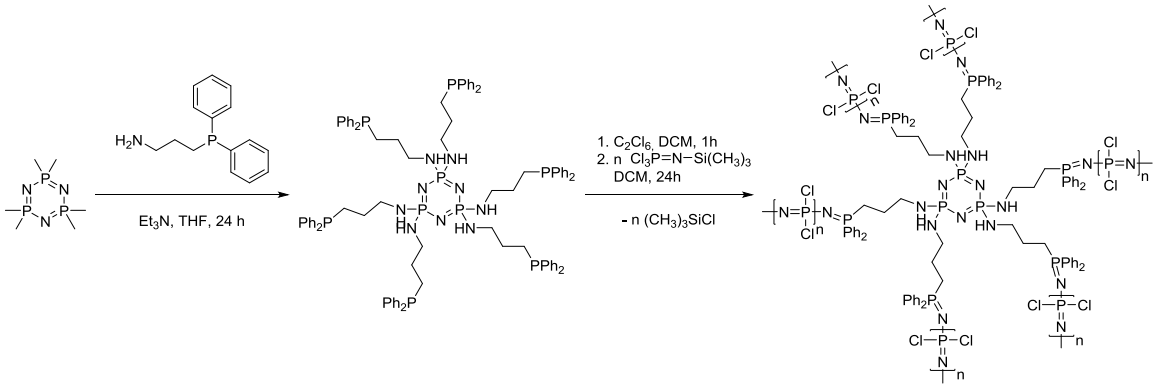


Figure 2-12: Synthesis of star-shaped poly(dichloro)phosphazene using hexachlorocyclotriphosphazene substituted with 3-(diphenylphosphino)-1-propylamine as macro-initiator for the polymerization of trichlorophosphoranimine.

2.2.2. Macromolecular substitution

Poly(dichloro)phosphazene is known to be hydrolytically very unstable but its highly labile chlorine atoms can be readily replaced by organic nucleophiles via macromolecular substitution to obtain the respective poly(organo)phosphazene and to give better hydrolytically stability (Figure 2-13).

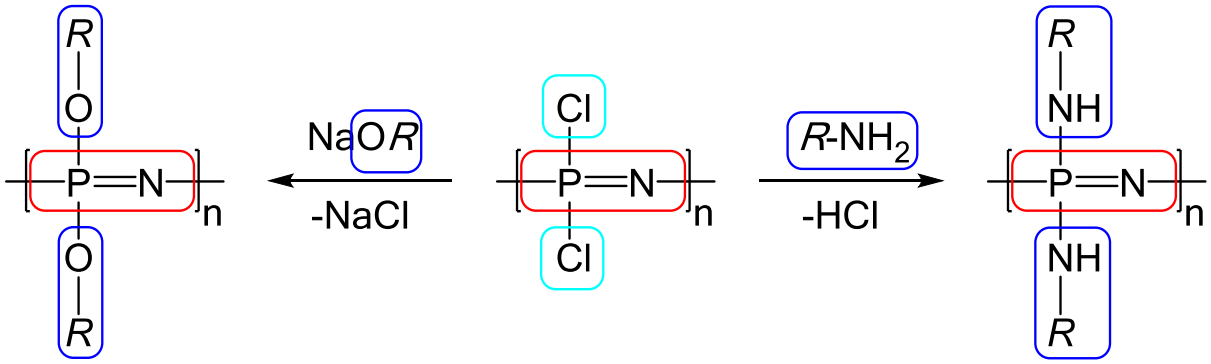


Figure 2-13: Most common routes to replace the chlorine atoms if poly(dichloro)phosphazenes by macromolecular substitution with organic nucleophiles.

This procedure is the most important step during poly(organo)phosphazene synthesis in respect to the resulting polymer properties, because during this step the properties can be tuned by considered choice from a wide variety of substituents. Thus a library of polymers can be prepared originating from simple poly(dichlorophosphazenes). Due to this fact a wide range of different poly(organophosphazenes) with diverse characteristics and various applications could be prepared in the past[68].

During macromolecular substitution multiple parallel substitutions on a single poly(dichloro)phosphazene molecule take place and it has to be guaranteed, that al chlorine atoms are substituted during this step, because remaining P-Cl groups could lead to structural irregularities, uncontrolled crosslinking, inconsistent polymer functionality and uncontrolled, accelerated degradation rates[69]. The undesired and uncontrolled nature of the resulting polymer can be easily avoided by adding an excess of the nucleophile and long reaction times. Thus these methods leads to polymers with suitable reproducibility and easy adjustable properties, which makes them very attractive for drug delivery applications, where this factors are important basic requirements[68c].

2.2.3. Degradation of polyphosphazenes

The most essential feature of poly(organo)phosphazenes, which distinguish them from many other polymers proposed for biomedical applications, is the inherent degradability

of the polyphosphazene backbone at clinically relevant degradation rates. They are known to degrade to a nontoxic mixture of the organic side groups, phosphates and ammonia, resulting from the backbone[70],[71],[33]. Phosphates can be easily metabolized and ammonia easily excreted by the organism. Only the organic side group has to be chosen carefully in respect of biocompatibility, molecular weight and toxicity. The side chains should be of molecular weight below the renal clearance limit, to avoid long-term accumulation. Moreover, the pH of the resulting mixture is in the neutral range, in contrast to polyesters forming acidic degradation mixtures during hydrolytic degradation, which can be problematic for biomedical applications. However, investigations of polyphosphazene-polyester blends indicated that the phosphate and ammonia buffer, resulting from polyphosphazene backbone degradation, was able to neutralize the acidic polyester degradation products[72],[73].

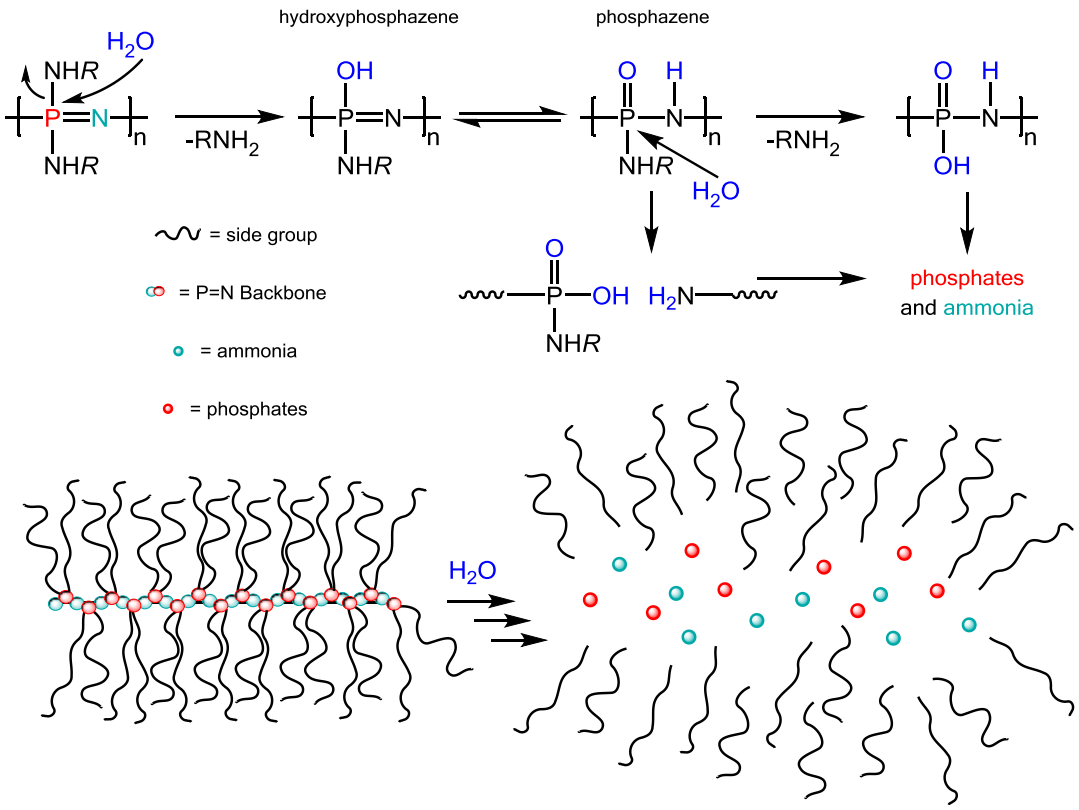


Figure 2-14: Proposed mechanism for hydrolytic degradation of poly(organo)phosphazenes. First the side groups are cleaved from the polyphosphazene backbone and highly water sensitive intermediates are formed, which degrade further to phosphates and ammonia.

The assumed degradation mechanism of poly(organo)phosphazenes is shown in Figure 2-14. In the presence of water, side groups are first hydrolyzed from the backbone leading to the formation of high hydrolytically sensitive hydroxyphosphazenes and phosphazenes[33],[74]. These intermediates undergo further degradation to low molecular weight oligophosphazene fragments followed by complete hydrolytic degradation to phosphates and ammonia. Backbone degradation can be followed by different methods including gel permeation chromatography[75], field flow fractionation, ^{31}P-NMR Spectroscopy[70] and phosphate determination using UV-Vis spectroscopy[70] and dynamic light scattering.

The stability or degradation rate is highly dependent on the nature of the organic side group. Consequently the rate of hydrolysis can be easily adjusted by careful choice and combination of side groups attached to the polyphosphazene backbone[76]. Thus, a broad range of polymers with different degradation rates are accessible. If highly hydrophobic side groups like CF_3CH_2O- are used, the resulting polymers are stable for many years as long as they are not exposed to strong bases, whereas polymers with remaining P-Cl moiety are known to be most susceptible to hydrolysis. The P-Cl moiety is extremely labile and in addition it leads to HCl as a degradation by-product, which is known to catalyze hydrolysis[69].

To tailor degradation rate of polyphosphazenes often a combination of hydrophilic and hydrophobic moieties is used as substituents. It is known that steric bulky residues in direct vicinity of the backbone can help to protect the backbone from hydrolytic attack. One example route is the combination of imidazole and 4-methylphenol[77]. In this case the hydrophilic imidazole groups sensitize the polymer towards hydrolysis whereas the bulky, hydrophobic phenol shields the backbone from hydrolytic attack. Similarly, the degradation rate can be tailored with the use of different amino acid esters. Different studies show that substitution with amino acid esters with bulky side groups at the α-C position, hinder hydrolytic attack of the backbone. For example, polyphosphazenes substituted with glycine ethyl ester are reported to have 3 month half-life whereas 6 month are obtained with alanine ethyl ester and about 1 year if valine ethyl ester is used[33].

Furthermore it is known that not only the hydrophilicity or hydrophobicity of the side groups control the hydrolytic sensitivity and degradability of polyphosphazenes, but the type of linkage to the phosphorus. As example, polyphosphazenes with ethyl esters of threonine or serine connected via the N-terminus to the backbone are reported to degrade faster than their more hydrophilic equivalent connected via the O-terminus[78]. Thus, nitrogen linkages are suggested to lead to higher hydrolytic lability. However, hydrolytic sensitive polyphosphazenes with side groups connected by oxygen atoms are also known. Consequently a number of concomitant mechanisms with influence on the polyphosphazene degradation rates and it is also feasible that other side group functionalities influence the degradation behavior. Exemplarily, an intramolecular carboxylic acid-catalysed mechanism is known for amino acid ester substituted polyphosphazenes, which takes place as soon as the pendent ethyl ester is hydrolysed[79],[80].

Moreover, the degradation rate of polyphosphazenes can be influenced by external parameters like temperature and pH. It is known that hydrolytic breakdown can be accelerated in acidic environments[81],[70] and in addition enzymatic induced degradation of polyphosphazenes is also possible, if enzymatic vulnerable peptide sequences are attached to the polyphosphazene backbone (Figure 2-15).

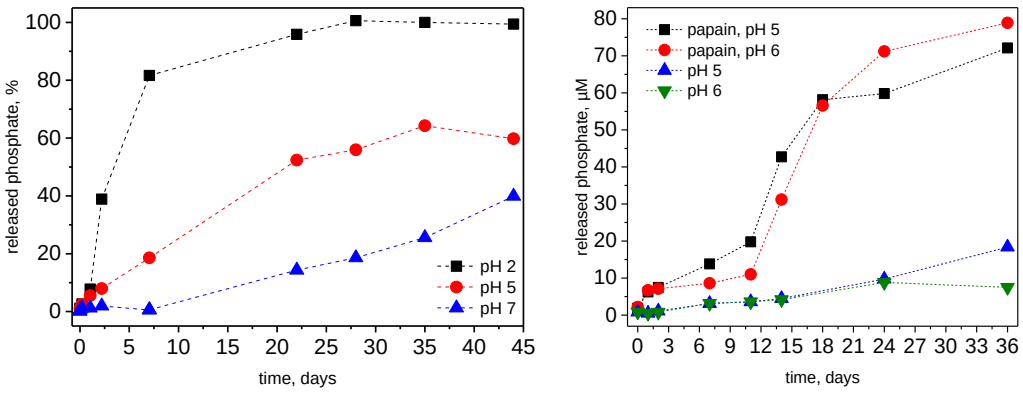


Figure 2-15: Degradation rates of two different poly(organo)phosphazenes und different conditions. Degradation rate at different pH values (left) and enzymatic (papain) driven degradation (right).

2.3. Polyphosphazenes for medical applications

Polyphosphazenes offer a unique combination of hydrolytic degradability, nontoxic degradation products, narrow polydispersity, controlled molecular weight and multifunctionality leading to their immense potential for biomedical applications. As shown previously, the desired physicochemical and biological properties of the polyphosphazene can be tuned via careful choice of the side-substituents attached to the backbone. The incorporation of side groups that show biocompatibility and promote degradability, is of significant importance for the development of polyphosphazene-based materials for medical applications[68b, 82]. Polyphosphazene research in the biomedical field focused on vaccine delivery, drug delivery, injectable hydrogels and degradable scaffolds for tissue engineering[2, 14, 83]. This work focuses on polyphosphazene-based polyphosphazene-drug conjugates for cancer therapy (especially cancer immunotherapy).

2.3.1. Polyphosphazene-drug conjugates

Due to its unique degradable backbone, polyphosphazenes have been intensively studied for their use in polymer-drug conjugates to deliver and release several drugs to the affected tissue. Firstly, for conjugates with anti-cancer drugs like Pt(II) or doxorubicin, the drugs were bound without any degradable linker directly to the polyphosphazene backbone to be released during backbone degradation[84]. However, for drug release by degradation of the backbone, slow release profiles are obtained. Consequently new strategies to control and trigger drug release had to be found. To this end the chemotherapeutic drugs epirubicin or doxorubicin were bound to the polyphosphazene backbone via a hydrazine linker and a rapid, pH stimulated drug release was obtained at pH values found in endo- and lysosomal compartment of cells[85]. Moreover, the polyphosphazene multifunctionality was used to add a targeting moiety to the delivery system, to direct the drug to the site of action[85]. In addition biocompatible, plasma-soluble groups (polyalkylene oxide) were added to enhance solubility and to exploit the “stealth-effect” to minimize immunogenicity. In vitro tests

showed excellent cytotoxicity whilst no toxicity of the free polyphosphazene was observed. Similar polyphosphazene-based systems with the photoactive drug hypericin covalently attached to the polyphosphazene backbone have also been prepared with narrow polydispersity via the living polymerization method. These polymers significantly enhanced the solubility of the drug and maintained photo-toxicity of the free drug. Thus, these polymers represent promising candidates for polymer assisted photodynamic therapy[86].

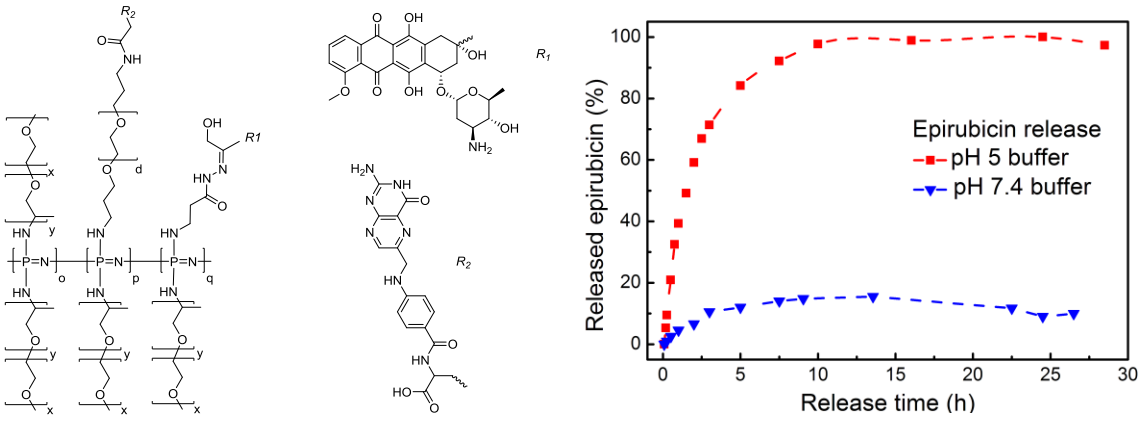


Figure 2-16: Water soluble polyphosphazene loaded with epirubicin via a pH labil hydrazone bond and the targeting ligand folic acid (left) and release rate of epirubicin from the conjugateat pH 5 and pH 7.4 (right)[85].

2.3.2. Polyphosphazenes in immunology

Water-soluble, polyelectrolyte poly(organo)phosphazenes with free carboxylic acid side groups, like polydi(sodiumcarboxylatophenoxy)phosphazene (PCPP) and poly-di(sodiumcarboxylatoethylphenoxy)phosphazene (PCEP) belong to the most well-investigated synthetic polymer immunoadjuvants[87]. Their use in complexes together with protein antigens leads to activation and antigen presentation of DCs. These high molecular weight polyelectrolytes show both adjuvant activity and improvement of the immune response of vaccines. Thus, these formulations lead to an increased antibody response in comparison to the vaccine antigen alone[88].For immunization, protein antigens can either be mixed with the water-soluble PCPP to form a complexes which can be injected directly or the antigen can be encapsulated through ionic gelation of PCPP with divalent calcium ions to obtain physical crosslinked hydrogel microspheres[89].

Moreover, these poly(organo)phosphazene based formulations for immunotherapies underwent clinical studies to a large extent, leading to the introduction in the vaccine industry[83].

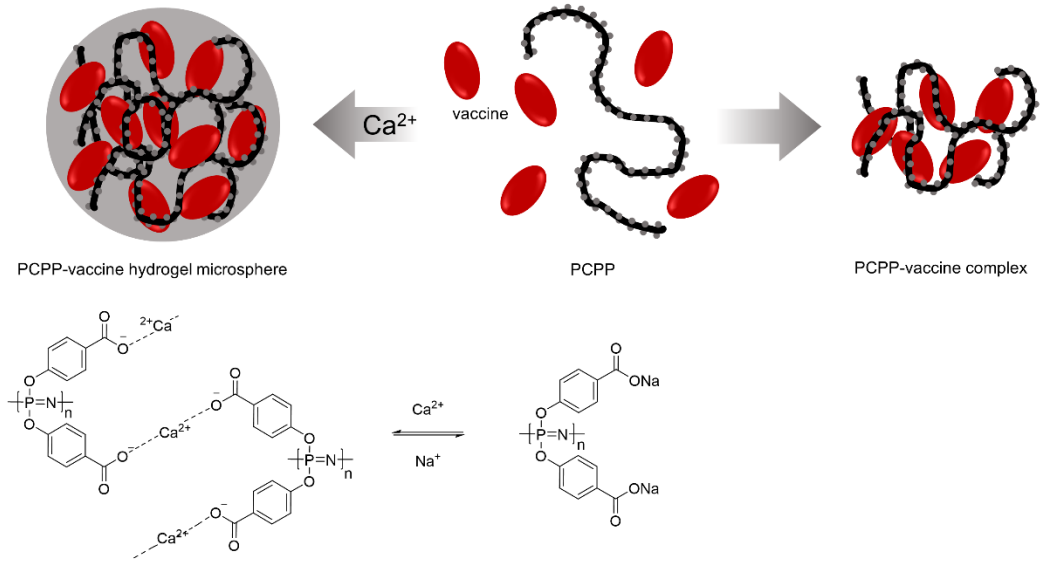


Figure 2-17: Scheme of water-soluble polydi(sodiumcarboxylatophenoxy)phosphazene (PCPP) and its complexation with protein antigens (vaccines) and ionic crosslinking with divalent calcium ions resulting in vaccine-encapsulation and hydrogel formation.

2.3.3. Injectable polyphosphazene-based hydrogels

Injectable hydrogels as drug depots are also of great interest for cancer therapy especially for cancer immunotherapy. Due to their degradability and biocompatibility poly(organo)phosphazene-based hydrogels have received increasing attention during the last decades[2, 83]. For example, thermosensitive poly(organo)phosphazene with functional groups that forms a hydrogel via phase transition at temperatures above the lower critical solution temperature (LCST) are reported[90]. These polymers are obtained *via* co-substitution of hydrophilic PEG oligomers and hydrophobic amino acid esters like isoleucine ethyl ester, which leads to thermosensitive hydrogels. Gelation appears due to hydrophobic interactions between the hydrophobic amino acid ester groups in aqueous solution. The amount of amino acid ester side groups can be used to tune the amphiphilicity, LCST and degradability of the resulting polymer. Another group employed noncovalent interactions between polyethylene oxide and α-cyclodextrin

(αCD) or adamantine and β-cyclodextrin to prepare polyphosphazene hydrogels[91].However, the poor mechanical properties of these physical cross-linked hydrogels, limits their practical application. Thus, chemical cross-linking groups where introduced into polyphosphazene hydrogel to prevent it from dissolving in water and improve the mechanical characteristics of the resulting hydrogels[92]. Moreover nanoparticles[93], carbon fibers[94], graphene[95], and clays like montmorillonite[96] can be added as crosslinked sites to hydrogels to enhance their mechanical strength. However, these additives and the chemical crosslinking are not biodegradable. Thus, the application of such hydrogels in medical applications is not useful. Consequently, a new strategy to improve mechanical strength, to avoid dissolving of the prepared hydrogels in water and to ensure biodegradability should be found. One could be chemical crosslinking via stimuli sensitive linkers or the combination of different kinds of physical cross-linking.

3. Biodegradable polyphosphazene based peptide-polymer hybrids

3.1. Abstract

A novel series of peptide based hybrid polymers designed to undergo enzymatic degradation is presented, via macrosubstitution of a polyphosphazene backbone with the tetrapeptide Gly-Phe-Leu-Gly. Further co-substitution of the hybrid polymers with hydrophilic polyalkylene oxide Jeffamine M-1000 leads to water soluble and biodegradable hybrid polymers. Detailed degradation studies, via ^{31}P-NMR spectroscopy, dynamic light scattering and field flow fractionation show the polymers degrade via a combination of enzymatic, as well as hydrolytic pathways. The peptide sequence was chosen due to its known property to undergo lysosomal degradation; hence, these degradable, water soluble polymers could be of significant interest for the use as polymer therapeutics. In this context we investigated conjugation of the immune response modifier imiquimod to the polymers via the tetrapeptide and report the self-assembly behavior of the conjugate, as well as its enzymatically triggered drug release behavior.

3.2. Introduction

Polypeptides have gained importance for biomedical applications during the last decades due to their unique physical, chemical and biological properties[5, 97]. For instance, poly(glutamic acid), poly(lysine) or poly(aspartate) have been heavily-investigated as polymer therapeutics, meeting most of the requirements including biocompatibility, biodegradability, high drug loading capacity and non-toxicity[5]. Moreover, peptide-polymer conjugates make up an interesting new class of polymeric materials combining the advantages of both peptides and synthetic polymers to generate hybrid materials with novel properties which cannot be realized with one of the components alone[97-98]. They take advantage of the flexibility of polymer synthesis

and diverse peptide functionality and properties. Furthermore, responsiveness of peptides to external stimuli can be exploited to create smart materials that change structure, size or other properties, when desired. A further important aspect of polypeptides is their inherent capacity to adopt stable conformations and self-assemble into highly organized nanoscale structures[98b]. Potential applications of peptide-polymer conjugates are in both biological and non-biological applications, but a vast majority of the work to date has focused on biomedical applications such as drug delivery[99], imaging[100], tissue engineering[101] or vaccines[102]. Peptide functionalization or grafting of synthetic polymers improves for example, degradation profiles[103], drug loading and release[99b, 104], cell adhesion[105] or controlled self-assembly to superstructures[106].

Herein, we present a hybrid approach combining inorganic polyphosphazenes decorated with multiple oligopeptides. Poly(organo)phosphazenes are a versatile class of polymers with immense potential for application in nanomedicine[2, 14, 107]. Recent advances in polyphosphazene synthesis allow controlled molecular weights, narrow molecular weight distributions[43a, 67, 108], controlled hydrodynamic volumes and high water solubility[109]. Furthermore, their properties can easily be tuned via facile post-polymerization modification to obtain polymer-drug conjugates with tailored characteristics. The most remarkable properties of poly(organo)phosphazenes, which distinguish them from many other known high molecular weight polymers, e.g. poly(ethylene glycol) PEG[110] and N-(2-hydroxypropyl)methacrylamide (HPMA)[111], used as drug delivery systems, is that their high synthetic flexibility and loading capacity is combined with an inherently hydrolytically degradable backbone (to non-toxic degradation products)[112], an essential feature in avoiding the deleterious effects associated with post-drug-release accumulation of high molecular weight macromolecules in the organism[21d]. Thus, the development of fully biodegradable polymeric drug delivery systems with molecular weights above the renal clearance limit and non-toxic degradation products with molecular weights under the renal clearance limit are of significant importance for drug delivery applications via blood vessels[21d, 113]. In addition, controlled intracellular drug release is also required and can be obtained by incorporating stimuli sensitive linkers in between the polyphosphazene backbone and drug [114].

Herein, a series of hybrid polymers consisting of the enzymatic degradable tetrapeptide and the unique biodegradable backbone of poly(organo)phosphazenes in combination with hydrophilic, oligomers (Jeffamine M-1000) to insure water solubility and controlled hydrodynamic volumes are presented. Many different, selectively degradable peptide sequences are described in the literature[115], but this initial study focuses on the Gly-Phe-Leu-Gly (GFLG) sequence, with it being well-reported to be susceptible to cathepsin B catalyzed hydrolysis in the intracellular lysosomal compartment [104b, 115a] due to its hydrophobic amino acid residues in P_2 and P_3 positions enabling an energetically favorable interaction with the active site of the lysosomal enzyme[104a, 116]. GLFG has been extensively studied as linker or spacer in combination with synthetic polymers like HPMA[99b, 103, 115a] or PEG[99c] for drug and gene delivery applications to obtain lysosomal drug release or degradable drug carriers. In this context, we also investigated the covalent linkage of drugs to the degradable carriers, leading to both enzymatic controlled drug release and simultaneous initiation of the degradation of the polyphosphazene backbone, due to the peptide is bound directly to the polyphosphazene backbone. The immune response modifier imiquimod (R837) is used as exemplary drug, due to the fact that the carbonic acid chain end of the peptide offers the possibility to attach different other drugs like doxorubicin[99c], epirubicin[117], adriamycin[104a] and cyclopamine[29], as well. The synthesis, characterization and self-assembly of the hybrid polymers are presented, as well as detailed degradation studies of the polymers and the drug conjugate.

3.3. Materials and Methods

All solvents were dried using standard laboratory procedures. Synthesis of polymers was carried out either in a glove box (MBRAUN) under argon or under nitrogen using standard Schlenk line techniques. The polyetheramine copolymer (PEO-PPO-NH_2) with an ethylene oxide / propylene oxide ratio of 19/3 and a M_n of 1000 g mol-1, sold under the trade name Jeffamine M-1000, was donated by Huntsman Performance Products and used as received. PCl_5 was purified by sublimation and stored under argon. Triethylamine was dried over molecular sieves and distilled prior to use. Fmoc- amino

acids, COMU and DIEA were purchased from Novabiochem. TAEA was purchased from Acros Organics, solvents were from VWR-Chemicals and used without further purification. Other chemicals were purchased from Sigma Aldrich or TCI chemicals.

^{1}H NMR spectroscopy was recorded on a Bruker 300 or 400 MHz spectrometer and referenced to the signal of internal $CDCl_3$. ^{31}P NMR spectra were recorded in decoupled mode on the same spectrometers at 121 MHz or 162 MHz. Gel permeation chromatography (GPC) was measured with a Viscothek GPCmax instrument equipped with a PFG column from PSS (Mainz, Germany; 300 mm x 8 mm, 5 µm particle size). The samples were eluted with DMF containing 10 mM LiBr at a flow rate of 0.75 mL/min at 60 °C. The molecular weights were estimated using a conventional calibration of the refractive index detector versus linear polystyrene standards. A Malvern ZetaSizer Nano-ZS analyzer (Malvern Instruments, UK) was used for dynamic light scattering (DLS) measurements. The 4 mW HeNe laser was set at λ=633 nm with the detector angle at 173° for backscattering measurements. The measurements were carried out in aqueous solution (1 mg/ mL) and all samples were filtered through a Millipore Millex-GV 0.22 µm PVDF filter and measured in a disposable polystyrene ultra-micro cuvette at 25 °C. UV-Vis spectra were carried out on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer. Field flow fractionation measurements were carried out on a Postnova AF2000 Ambient Temperature Asymmetric Flow FFF equipped with an UV detector. A 1290 Infinity UHPLC from Agilent Technologies (Agilent, Vienna, Austria) equipped with a reversed-phase C18 silica-based chromatographic column (Rapid Resolution HD Eclipse Plus C18; 2.1 mm x 50 mm, particle size 1.8 µm) was used for kinetic studies of the drug release. The samples were eluted at a flow rate of 0.3 mL min^{-1} at room temperature with a mobile phase composition of 20 % acetonitrile in water (v/v) containing 0.1% formic acid (v/v) in isocratic mode. UV detection was carried out at 254 nm. The amount of the released drug was estimated using a calibration curve for the free drug.

3.3.1. Tetra peptide synthesis (Gly-Phe-Leu-Gly-OtBu)

First peptide coupling: In a 100 ml round bottom flask 1.167 g (3.3 mmol) of Fmoc-Leu-OH were suspended in 30 ml EtOAc. Subsequently, 1.15 ml (6.6 mmol) DIEA and 1,413 g

(3.3 mmol) COMU were added and the mixture was stirred until a deep orange color was obtained. After the addition of 504 mg (3 mmol) of H-Gly-OtBu · HCl the reaction was stirred for 1 h and the solvent was removed in vacuo.

General procedure for Fmoc deprotection using TAEA: The crude residue from the precedent coupling was dissolved in 20 ml dichloromethane, 11.25 ml (75 mmol) of TAEA were added and the reaction was stirred for 30 min. The mixture was transferred to a separatory funnel containing 100 ml EtOAc and 50 ml brine, the funnel was shaken and the layers were separated. The organic phase was washed two times with 50 ml brine and two times with 50 ml pH 5.5 phosphate buffer, dried over anhydrous $MgSO_4$, and evaporated.

General procedures for COMU coupling: In a 50 ml round bottom flask 3.3 mmol of Fmoc- amino acid were suspended in 30 ml EtOAc followed by the addition of 1.15 ml (6.6 mmol) DIEA and 1.413 g (3.3 mmol) COMU. The resulting mixture was stirred for 2 min, added directly to the residue from the precedent deprotection and the resulting mixture was stirred for 1 h. After completion of the reaction, the solvent was removed on a rotary evaporator.

Modified work-up after final coupling step: After completion of the peptide coupling, the reaction solution was diluted with 100 ml EtOAc and washed with 1M HCl (2x 50 ml), saturated $NaHCO_3$ (2x 50 ml) and brine (2x 50 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated. The resulting residue was dissolved in CH_2Cl_2 and purified via DCVC[118]: SiO_2, cyclohexane/EtOAc 50:0 to 0:50 in 5 % increments. The product was further eluted by applying additional fractions of EtOAc if necessary. The protected tetrapeptide could be obtained as 1.4 g (2.09 mmol) of a colourless foam in 70 % yield. ^{1}H-NMR (300 MHz, DMSO-d_6, δ): 8.14-8.09 (m, 2H, -CO-N<u>H</u>-), 8.01 (d, 1H, J^3(H,H) = 8.3 Hz, -CO-N<u>H</u>-), 7.88 (d, 2H, J^3(H,H) = 7.7 Hz, Fluorenyl C^5<u>H</u> and C^4<u>H</u>), 7.69 (d, 2H, J^3(H,H) = 7.3 Hz, Fluorenyl C^1<u>H</u> and C^8<u>H</u>), 7.41 (t, 2H, J^3(H,H) = 7.3 Hz, Fluorenyl C^3<u>H</u> and C^6<u>H</u>), 7.32 (t, 2H, J^3(H,H) = 7.7 Hz, Fluorenyl C^2<u>H</u> and C^7<u>H</u>), 7.22-7.14 (m, 5H, Phe Ar-<u>H</u>), 4.56-4.51 (m, 1H, FmocNH-CH_2-), 4.35-4.18 (m, 4H, Fluorenyl C^9<u>H</u> and Fmoc –CH_2-O- and Leu –CH_2-C<u>H</u>-NH-), 3.72-3.45 (m, 4H), 3.02 (dd, 1H, J^3(H,H) = 13.8 Hz, J^4(H,H) = 4.4 Hz, Phe –C<u>H</u>(H)-Ph), 2.80-2.73 (m, 1H, Phe –CH(<u>H</u>)-Ph),

1.62-1.56 (m, 1H, Leu –C<u>H</u>(CH_3)$_2$), 1.48 (t, 2H, J^3(H,H) = 7.3 Hz), Leu –CH-C<u>H_2</u>-CH(CH_3)$_2$), 1.39 (s, 9H, -COOC(C<u>H_3</u>)$_3$), 0.88-0.82 (m, 6H, Leu –CH(C<u>H_3</u>)$_2$) ppm.

Removal of the terminal Fmoc-group[119]: The purified tetrapeptide (1 g) was suspended in 19 ml MeCN and 1 ml DBU and 3.7 ml 1-dodecanethiol were added. The mixture was stirred for 1 h at room temperature and afterwards 15 ml of n-heptane was added. The layers were separated and the MeCN phase was washed four times with 10 ml n-heptane, the solvent was evaporated and the residue dried under high vacuum. The crude product was directly used in the next step.

3.3.2. Synthesis of $Cl_3PNSiMe_3$

N-(trimethylsilyl)-trichlorophosphoranimine was synthesized similar to literature procedures[120]. 26 g LiN($SiMe_3$)$_2$ (155 mmol) were dissolved in 500 mL anhydrous diethylether under nitrogen at 0 °C and stirred for 30 min. 13.59 mL PCl_3 (155 mmol) were then added dropwise at 0 °C. The solution was allowed to warm to room temperature and stirred for 1 hour. After cooling to 0 °C again, 12.56 mL SO_2Cl_2 (155 mmol) were added and the mixture was stirred for another hour at 0 °C. Afterwards, the reaction was filtered and the solvent removed under vacuum. The product was purified by vacuum distillation at 40-50 °C and 5 mbar to yield chlorophosphoranimine as colorless liquid. The product was stored under inert argon atmosphere at –35 °C. Yield: 14 g (40%), ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.18 (s, 9H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): - 54.3 ppm.

3.3.3. Polymerisation procedure

The polymer was synthesized via the living cationic polymerization of trichlorophosphoranimine[43a] with PCl_5. In the following, the procedure used for the synthesis of polymer **2** is described. Polymer **3** and **4** were synthesized accordingly, with varied ratio of peptide to Jeffamine M-1000. For polymer **5**, H-Gly-Jeffamine M-1000 was used as hydrophilic sidechain and H-Gly-Phe-Leu-Gly-imiquimod instead of H-Gly-Phe-Leu-Gly-OtBu. In case of polymer **1**, no hydrophilic sidechain was used. The chlorine

atoms were only substituted by H-Gly-Phe-Leu-Gly-OtBu. In the glove box, initiator PCl_5 (13.0 mg, 0.0624 mmol) and monomer $Cl_3PNSiMe_3$ (0.350 g, 1.56 mmol) were dissolved in CH_2Cl_2 (5 mL) at room temperature. The solution was stirred for 12 h and the solvent removed under vacuum. The resulting polydichlorophosphazene (0.350 g, 1.56 mmol) was then dissolved in anhydrous THF in the glovebox. 1 equivalent of H-Gly-Phe-Leu-Gly-OtBu (0.700 g, 1.56 mmol) and NEt_3 (0.16 g, 1.56 mmol) were then added to the polymer solution and allowed to react for 24 hours. An excess of Jeffamine M-1000 (1.5 eq, 2.34 g, 2.34 mmol) was then added to the reaction mixture and allowed to react for further 24 hours. The solvent was then removed under vacuum and the resulting polymer was purified by dialysis (12 kDa cut-off) for one week against EtOH. The solvent was removed and the polymer was dried under vacuum to give a waxy solid.

Polymer **1**: Yield: 50%, ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.86 (br, 6H), 1.45 (br, 9H) 2.26 (b, 1H), 3.63 (s, 82H), 7.18 (br 5H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): 0.67 ppm.

Polymer **2**: Yield: 0.5 g (22 %), ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.85 (br, 6H), 1.12 (br, 3H), 1.24 (br, 2H), 1.44 (br, 9H) 3.37 (s, 2H), 3.63 (m, 29H), 7.21 (br 5H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): -0.03 ppm.

Polymer **3**: Yield: 38 %, ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.88 (br, 4H), 1.13 (br, 3H), 1.26 (br, 3H), 1.45 (br, 9H) 3.38 (s, 2H), 3.66 (m, 39H), 7.20 (br 5H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): 0.45 ppm.

Polymer **4**: Yield: 38 %, ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.89 (br, 4H), 1.13 (br, 3H), 1.26 (br, 3H), 1.45 (br, 9H) 3.38 (s, 4H), 3.66 (m, 46H), 7.23 (br 5H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): -0.35

Polymer **5**: Yield: 30 %, ^{1}H NMR (300 MHz, $CDCl_3$, δ): 0.86 (br, 1H), 1.13 (br, 3H), 1.25 (br, 2H) 3.38 (s, 2H), 3.65 (s, 23,78H) ppm, ^{31}P NMR (121 MHz, $CDCl_3$, δ): 0.07 ppm.

3.3.4. Gly-Phe-Leu-Gly-imiquimod

Firstly, Fmoc-Gly-Phe-Leu-Gly was stirred overnight in TFA to remove the tert-butyl protective group. TFA was removed under vacuum and CH_2Cl_2 was added to the product and evaporated three times to obtain a white powder. 161 mg imiquimod (0.67 mmol)

were dissolved in DMF containing 0.2 ml trimethylamine (1.34 mmol) by heating to 50 °C in DMF for 20 min. To the reaction mixture 0.57 g EDCI (3 mmol) and 413 mg Fmco-Gly-Phe-Leu-Gly-OH (0.67 mmol) were added and left overnight. The reaction progression was indicated by TLC (EtOAc:cyclohexane, 1:1). The reaction was then diluted in EtOAc and washed two times with $NaHCO_3$, two times with saturated NaCl and dried over $MgSO_4$. The solvent was removed and the product was dried under vacuum. Yield: 0.38 g (68 %).

3.3.5. Gly-Jeffamine M-1000

Gly-Jeffamine was synthesized similar to literature[112b]. The BOC-protected amino acid BOC-Gly-OH (1.3 g, 7.5 mmol), N-hydroxysuccinimide (0.86 g, 7.5 mmol) and N,N´-dimethylaminopyridine (0.09 g, 7.5 mmol) were dissolved in 80 mL CH_2Cl_2 and cooled to 0 °C. Separately, 1.55 g N,N´-dicyclohexylcarbodiimide (7.50 mmol) were dissolved in 15 mL CH_2Cl_2 and transferred in to the cooled reaction mixture. The mixture was stirred overnight at room temperature. The formed precipitate was removed by filtration and the filtrate was added to a solution of 7.5 g Jeffamine M-1000 (7.5 mmol) in CH_2Cl_2 and stirred for two days at room temperature. The reaction was extracted two times with 10 % ammonium chloride, two times with 5 % $NaHCO_3$, two times with saturated NaCl and dried over $MgSO_4$. The solvent was removed and the product dried under vacuum to yield a white wax-like product. Yield 7.2 g (88 %), ^{1}H NMR (300 MHz, $CDCl_3$, δ): 1.39 (s, 9H), 3.31 (s, 3H), 3.58 (m, 82H) ppm.

3.3.6. Hydrolytic degradation study– Field flow fractionation

Polymer 2 (5 mg/ml) was incubated in pH 2, pH 5 and pH 7.4 citrate/phosphate buffer at 37 °C during the time of analysis. Aliquots (50 µl) of the samples were taken after certain time intervals and measured *via* field flow fractionation.

3.3.7. Hydrolytic degradation study – Dynamic light scattering

Polymer 5 (1 mg/ml) was incubated in pH 2, pH 5 and pH 7.4 citrate/phosphate buffer at 37 °C during the time of analysis in disposable polystyrene ultra-micro cuvettes and measured after different times at 25 °C.

3.3.8. Enzymatic and hydrolytic degradation study– NMR

For ^{31}P NMR degradation studies, 10 mg papain (13 units/mg) were activated with 0.01 M L-cysteine in 0.9 mL citrate buffer (0.1 M, pH 6) and added to 10 mg polymer. Separately, 10 mg polymer were dissolved in 0.9 mL of the same solution but without papain. Moreover, 10 mg papain were mixed with 0.9 ml buffer containing 0.01 M of the inhibitor cystamine. Although phosphate buffer is reported to be the best choice for papain[121], citrate buffer had to be used to avoid buffer related signals in the ^{31}P NMR spectra. Immediately when the polymer was dissolved, 0.1 M D_2O was added to the solutions and the sample incubated at 37 °C. The changes of the phosphorus signals were monitored over a time period of 28 days. Between each measurement the samples were stored at 37 °C.

3.3.9. Degradation - Phosphate determination

The polymers (1.3 mg/ml) were incubated in pH 5 and pH 6 sodium acetate buffer containing 0.01 M L-cysteine and 0.057 mM Papain (3 units/mg), at 37 °C during the time of analysis. Hydrolytic degradation was measured under the same conditions but without papain. Aliquots (0.4 ml) of the samples were taken after certain time intervals and tested for the presence of inorganic phosphate using 1 ml of a reagent solution containing 5 mL of 0.4 % ammonium molybdate, 5 mL of 0.7 % ascorbic acid, 12.5 mL of 0.2 M sulfuric acid, and 2.5 mL of 0.018 % potassium antimonyl tartrate[71]. UV–Vis analysis of the mixtures was performed at 885 nm after 15 min incubation. The concentration of phosphate was calculated using a calibration curve measured with potassium dihydrogen phosphate and compared to the theoretical phosphate amount that can be released from the polymer backbone. Although phosphate buffer is reported

to be the best choice for papain[121], acetate buffer had to be used to avoid buffer related signals in the UV-Vis measurements.

3.3.10. Drug release – HPLC measurements

The release of imiquimod from the polymer 5 was analyzed by HPLC. 10mg Papain (3 units/mg) dissolved in 1ml 0.1 M citrate buffer containing 0.01M L-cysteine were stirred for 5 minutes to activate the enzyme and subsequently added to 10 mg of polymer 5. The amount of released drug was then investigated by HPLC measurements after certain periods of times and the samples were stored at 37 °C between each measurement. UV detection was carried out at 254 nm and the amount of the released drug was estimated using a calibration curve for the free drug measured under the same conditions. For comparison the drug release was also studied without papain to investigate the hydrolytic drug release. In this case, polymer **5** was dissolved in 1 ml 0.1 M citrate buffer containing 0.01 M L-cysteine and stored and measured like the papain containing sample.

3.4. Results

3.4.1. Synthesis

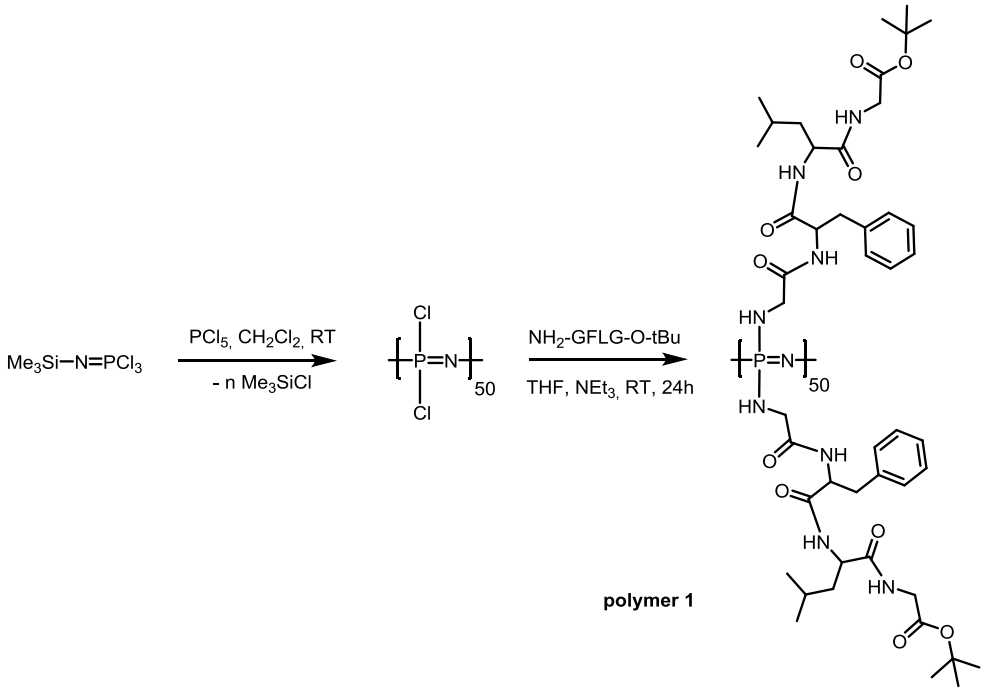


Figure 3-1. Living polymerisation of poly(dichlorophosphazene) and macromolecular substitution of the chlorine atoms by Gly-Phe-Leu-Gly-OtBu.

Poly(dichloro)phosphazene $[Cl_2P=N]_n$ with approximately 50 repeat units was synthesized via the room temperature, living cationic polymerization of trichlorophosphoranimine[43a, 109]. This reaction was followed by the complete macromolecular substitution of the chlorine atoms with separately prepared H-Gly-Phe-Leu-Gly-OtBu to obtain peptide based poly(organo)phosphazene (Figure 3-1, polymer **1**). ^{31}P NMR, ^{1}H NMR spectroscopy and GPC were used to confirm successful preparation of the hybrid polymer (Table 3-1 and Figure 3-2 and Figure 3-3). With two tetrapeptide groups per repeat unit, the resulting hybrid polymer consists mostly of peptide (95 wt%), and thus, the chemical and solution characteristics are peptide dominated for the hybrid polymer. Due to the hydrophobicity of the peptide sequence, the resulting polymer had limited aqueous solubility, thus, a further series of polymers were prepared using Jeffamine M-1000 as a co-substituent in various ratios (polymers **2-4**, Figure 3-4A) to obtain hybrid polymers with excellent water solubility.

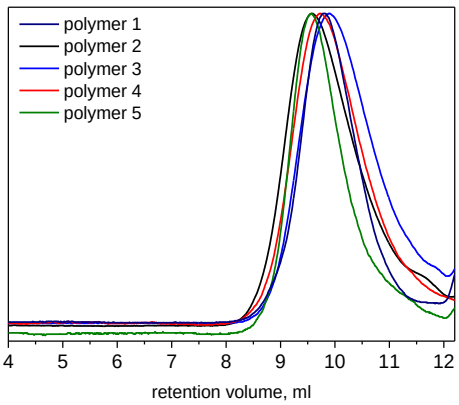


Figure 3-2: GPC chromatographs of polymer 1-5. All polymers elute at similar retention volumes indicating similar molecular weights.

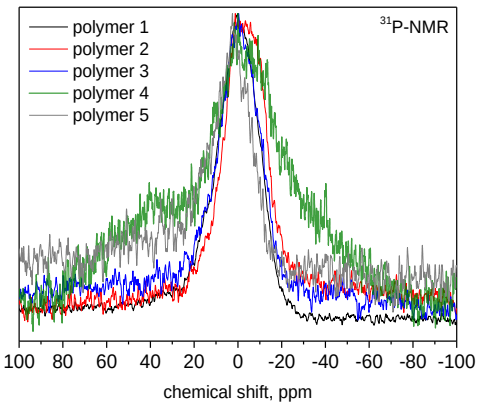


Figure 3-3: ^{31}P NMR spectrum of polymer 1-5. A broad peak at about 0 ppm indicates complete substitution of the chlorine atoms at polyphosphazene backbone and the absence of degradation products.

The relative ratio of the tetrapeptide and the Jeffamine side chains was calculated by ^{1}H-NMR spectroscopy, through integration of the OCH_3 Jeffamine end-group protons (s, 3H) at 3.37 ppm versus the tert-butyl-group (s, 9H) of the protected tetrapeptide sidechain at 1.44 ppm (Table 3-1). Furthermore, all polymers were characterized with ^{31}P NMR spectroscopy to confirm the absence of P-Cl units (within NMR limits) and thus, complete substitution of all chlorine atoms in the polyphosphazene backbone (Figure 3-3).

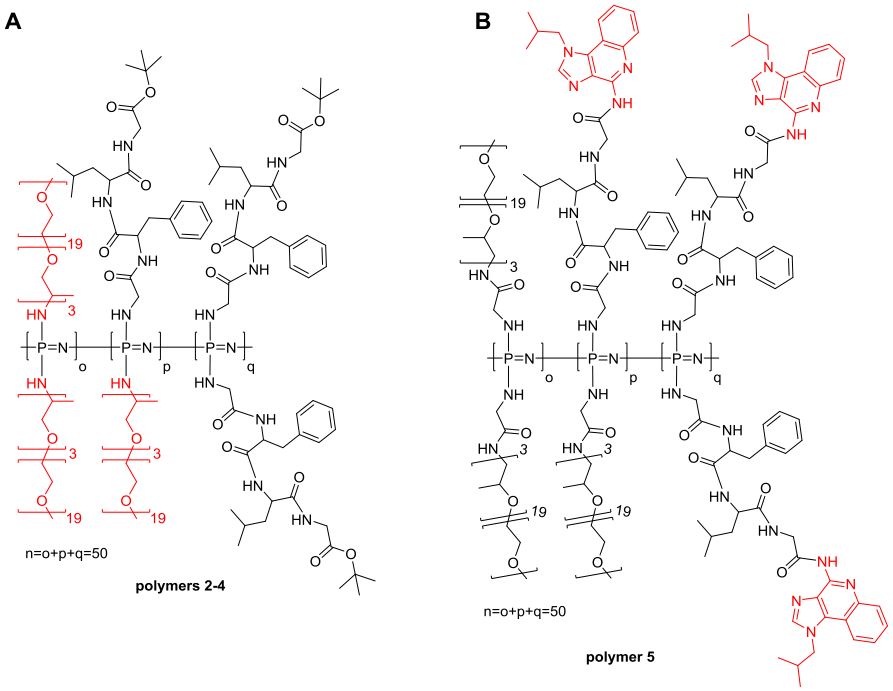


Figure 3-4. A: Poly(organo)phosphazenes 2-4 with the tetrapeptide linker GFLG and Jeffamine M-1000 coupled to the polyphosphazene backbone. B: Poly(organo)phosphazene 5 loaded with imiquimod via the tetrapeptide linker GFLG and Jeffamine M-1000 coupled via a glycine spacer to the polyphosphazene backbone. The combinations of the two different side chains are statistically distributed.

Table 3-1. Structural data for polymers 1-5.

polymer	GFLG-OtBu, %[a]	Jeffamine M-1000, %[a]	Imiquimod loading, wt%[b]	M_w/M_n (GPC)[c]	M_n (GPC), kDa[c]	D_h (DLS), nm[d]
1	100	0	0	1.3	6.84	-[f]
2	84	16	0	1.9	5.13	20,1 ± 1.6
3	57	43	0	1.9	4.05	14.2 ± 0.58
4	47	53	0	1.6	5.58	14.9 ± 0.58
5	-[e]	-[e]	2.4	1.6	6.20	201.6± 17.32

[a] percentage of total substituents calculated from ^{1}H-NMR measurements.
[b] weight percent of total conjugate calculated from UV-Vis measurements
[c] measured by GPC analysis in DMF and calibrated against linear polystyrene standards.
[d] peak mean from DLS size distribution by intensity
[e] calculation not possible due to overlapping NMR-signals of side chains and imiquimod
[f] not soluble in water

A common tactic in polymer therapeutics involves the coupling of amino-functionalized drugs to the C-terminus of the Gly-Phe-Leu-Gly sequence, facilitating intracellular lysosomal drug release[29, 99c, 104a, 117]. Thus, in this work we coupled the immune response modifier imiquimod to Fmoc-Gly-Phe-Leu-Gly-OH via its aromatic NH_2. After Fmoc-deprotection, the resulting conjugate H-Gly-Phe-Leu-Gly-imiquimod was added as co-substituent alongside H-Gly-Jeffamine M-1000 to –[Cl_2P=N]–, to obtain polymer **5** (Figure 3-4B). The amount of imiquimod attached to polymer 5 via the tetrapeptide linker was calculated using the UV-Vis absorbance at 246 nm (Figure 3-5) and gave a drug loading of 2.4 wt % of the conjugate (Table 3-1).

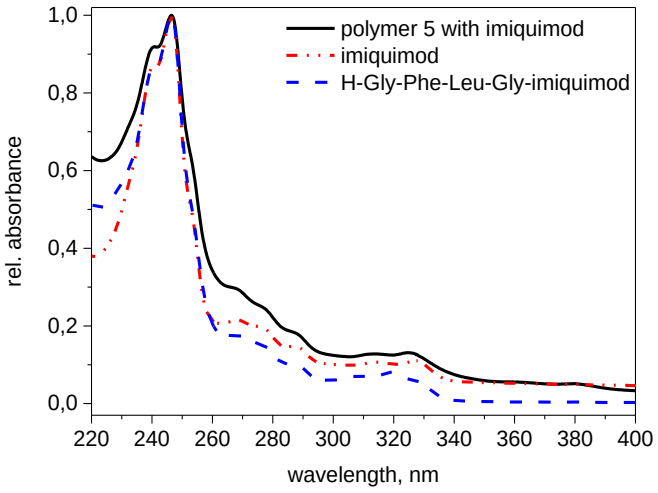


Figure 3-5: UV-Vis spectra in acetonitrile of polymer 5 loaded with 2.4 wt% imiquimod (black), imiquimod (red) and Gly-Phe-Leu-Gly-imiquimod (blue).

3.4.2. Self-assembly

The hydrodynamic volumes of the hybrid polymer series was then investigated by dynamic light scattering (DLS, Figure 3-7) to determine the hydrodynamic diameter in aqueous solutions, a factor known to have considerable impact on plasma circulation time, cellular uptake and biodistribution of the polymers. Figure 3-7A shows the size distribution by intensity and Figure 3-7B by volume of polymers **2-5**. The intensity distribution of polymers **2-4** show a bimodal distribution, hinting at some self-assembly of the polymers. Such behavior has been reported previously for similar amphiphilic co-substituted polyphosphazenes[58, 79, 122]. Micellar-like superstructures with the hydrophobic side groups agglomerated in the core can be formed despite them being essentially random copolymers, presumably due to the high flexibility of the backbone, allowing for folding and agglomeration of the hydrophobic sections (Figure 3-6).

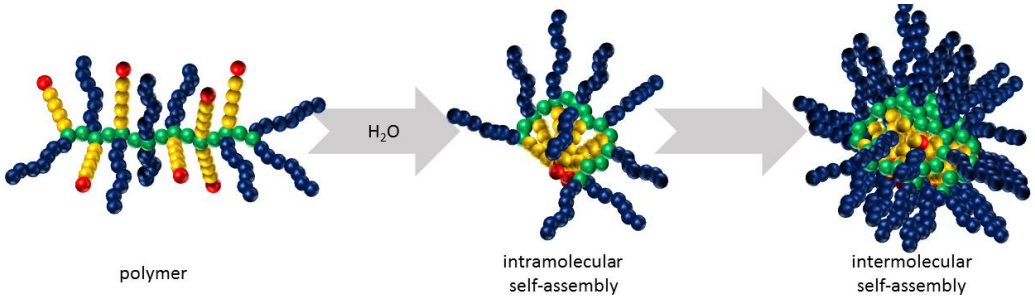


Figure 3-6. Intra- and intermolecular self-assembly of poly(organo)phosphazenes with amphiphilic character.

Interestingly, the distribution was observed to be monomodal in the region of 200 nm for polymer **5**, loaded with the hydrophobic drug**,** suggesting this exists mostly in its agglomerated form for 1 wt. % solutions. Since larger particles show higher intensity, a closer inspection of the size distribution by volume was also carried out (Figure 3-7B). In summary, the DLS investigations show that although all polymers tend to form aggregates due to their amphiphilic character resulting from the combination of hydrophilic Jeffamine sidechains with the hydrophobic tetrapeptide, only in case of polymer **5** are the formed aggregates the dominating species present in the sample as a result of the increased hydrophobicity imparted by the hydrophobic drug.

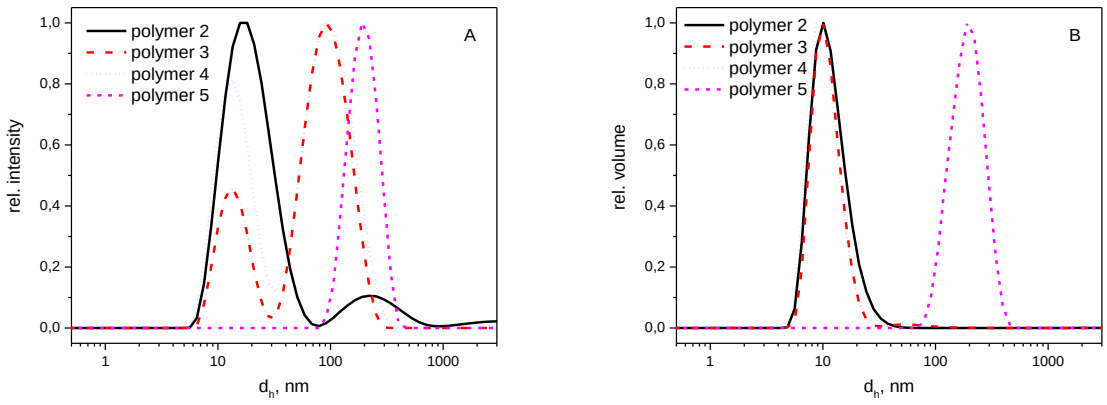


Figure 3-7. Intensity size distribution (A) and volume size distribution (B) obtained for polymers 2-5 in water (1 mg/ml) measured on a Nano ZS instrument.

3.4.3. Hydrolytic degradation

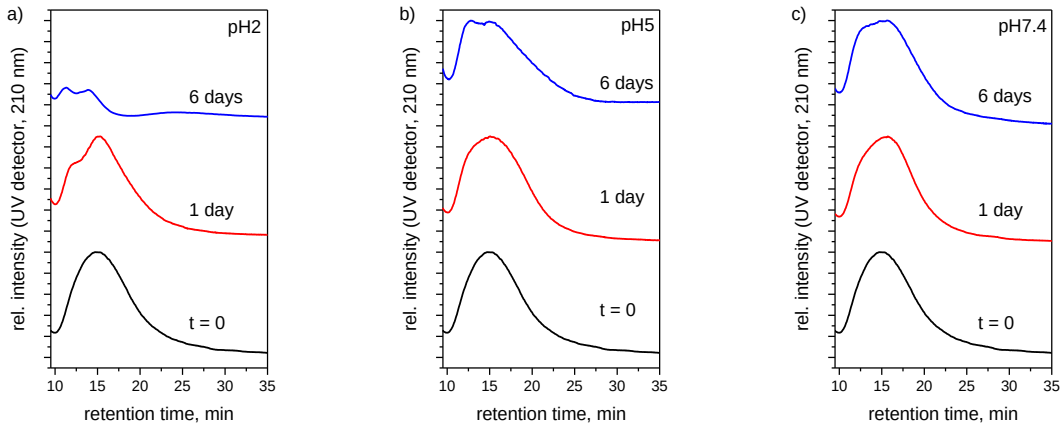


Figure 3-8. Normalized FFF elugramm showing the degradation of polymer 2 at 37 °C, in an aqueous solution at pH 2 (a), 5 (b) and 7.4 (c). Broadening and decrease in intensity and a shift to earlier retention time of the polymer peak are observed.

Degradation studies of polymer **2** measured by field flow fractionation at 37 °C, pH 2, 5 and 7.4 showed that the polymers are stable over a short period of time in an aqueous environment but degrade significantly to small molecules under simulated physiological conditions (pH 5 and 7.4) with a broadening and a shift to earlier retention times of the polymer peak being observed (Figure 3-8). A more rapid degradation occurred under enhanced (pH 2) conditions (Figure 3-8a), with the entire polymer being observed to degrade within two weeks (Figure S4), in accordance with previous reports into amino acid substituted poly(organo)phosphazenes[112b].

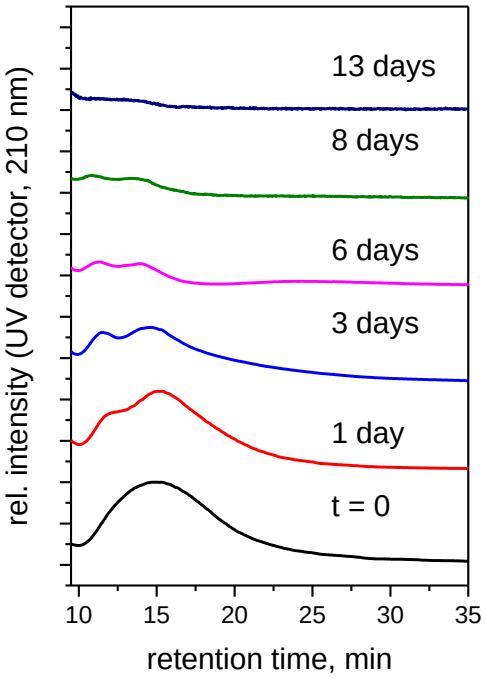


Figure 3-9: Normalized FFF elugram showing the degradation of polymer 2 at 37 °C in an aqueous solution at pH2. Broadening and decrease in intensity and a shift to earlier retention time of the polymer peak are observed.

Moreover, the hydrolytic degradation of polymer **5** was followed by DLS under enhanced conditions (pH 2) to show the behavior of the formed aggregates during degradation (Figure 3-10). Interestingly, an initial increase in the aggregate size was observed, before complete degradation of the polyphosphazene backbone. After 17 days only small molecules, namely the M-1000 sidechains, could be detected (Figure 3-10). The temporary increase in aggregate size is supposed to be caused by small molecule, hydrophobic amino acid and drug residues being released from the polymer. Incorporation of the released hydrophobic residues in the hydrophobic core of the self-assembled structures and further intermolecular aggregation is facilitated to shield the non-soluble parts from the aqueous phase[123]. Further backbone degradation subsequently leads to collapse of the superstructures and then the complete polymer chains.

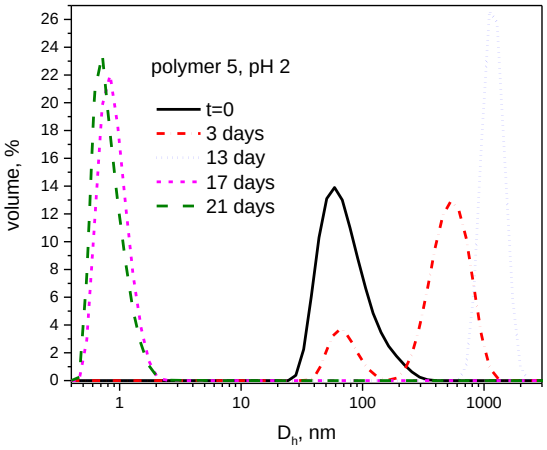


Figure 3-10: Volume size distribution obtained for polymer 5 in citrate/phosphate buffer pH 2 (1 mg/ml) measured on a Nano ZS instrument after certain times.

3.4.4. Enzymatic degradation

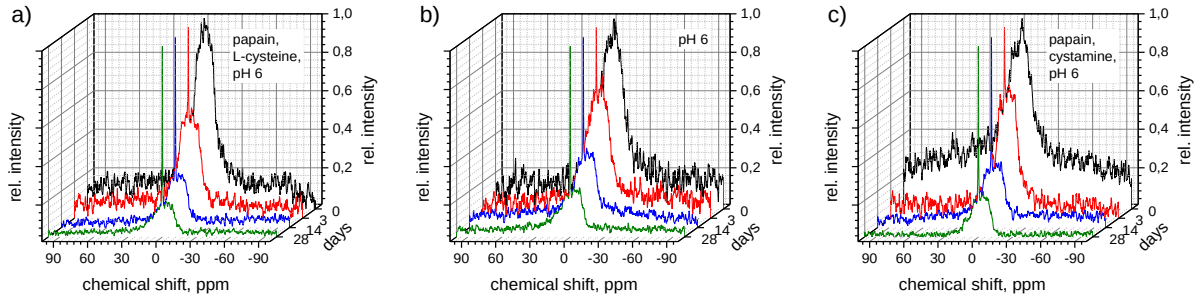


Figure 3-11. Enzymatic degradation of polymer 2 followed by ^{31}P NMR spectroscopy over 28 days in citrate buffer (pH6) containing L-cysteine and papain (a), hydrolytic degradation of polymer 2 in the same buffer system without papain (b) , with papain and cystamine as inhibitor (c). All samples were stored at 37°C.

Further to the hydrolytic degradation, the degradation of the hybrid polymers in the presence of the enzyme papain was also investigated. To this end, a photometric determination of the phosphate backbone degradation product was carried out, as well as ^{31}P NMR spectroscopy, due to interference of the macromolecular enzyme in DLS and FFF studies. Exemplarily ^{31}P NMR spectra are shown for polymer **2** (Figure 3-11). Since cathepsin B has phosphate incorporated in its structure, it was not deemed suitable for such studies and thus papain was used as model enzyme in combination with the activator L-cysteine. After one day, a sharp peak at around 0 ppm appeared in all ^{31}P NMR spectra and increased over time, whereas the broad signal associated with the polymer decreased. A clear decrease in the degradation rate was observed upon the

addition of the papain inhibitor cystamine (Figure 3-11c), suggesting the presence of a combination of both hydrolytic and enzymatic degradation mechanisms. Measurements without papain show a clear, but slower hydrolytic degradation under the same conditions (Figure 3-11b).

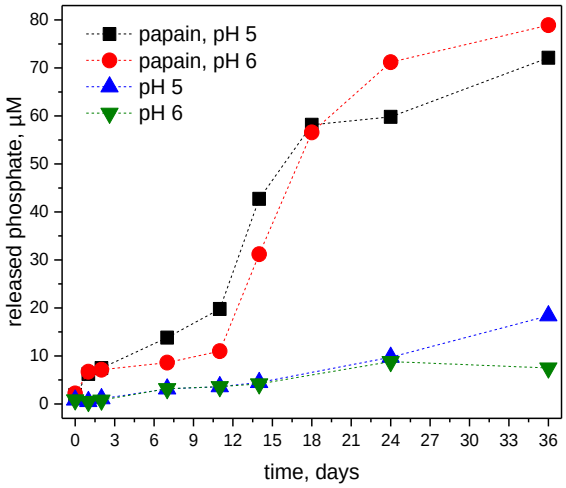


Figure 3-12. Phosphate determination of polymer 2 quantitatively determined by UV–Vis analysis to show the degradation profile of the polymer in aqueous conditions at pH 5 (▲), pH 6 (▼) and with papain at pH 5 (■) and pH 6 (●) at 37°C.

Furthermore formation of phosphate could also be tracked by photometric molybdate assay[112b]. The release of phosphate from polymer **2** was investigated at 37°C, pH 5 (lysosomal pH) and pH 6, the reported ideal pH for papain activity. Considerably higher degradation rates were observed for the polymer incubated with the L-cysteine activated papain than in its absence, suggesting a significant contribution from enzymatic degradation of the polymer (Figure 3-12).

3.4.5. Degradation mechanism

As previously discussed the hybrid polymers show a hydrolytic labile behavior, due to glycine bound directly to the polyphosphazene backbone, which could be indicated by the ^{31}P NMR spectra as well as photometric phosphate determination. The degradation rate was clearly accelerated by the addition of the enzyme papain, which has similar specificity as the lysosomal enzyme cathepsin B[113, 116], suggesting the involvement of an enzymatic degradation route. Papain has the preference for phenylalanine in position P2

(Schechter and Berger nomenclature) of the substrate[29]. Consequently, a cleavage between leucine and glycine-OtBu or glycine-imiquimod is expected (Figure 3-13). Despite the peptide preferential cleavage site not being directly at the polyphosphazene backbone, the degradation rate was nevertheless observed to be significantly accelerated upon the addition of papain. This observation is explained with an acid-catalysed mechanism (Figure 3-13), whereby after enzymatic cleavage of a peptide bond, free carboxyl groups are formed which would be expected to promote the backbone degradation. This carboxylic acid-catalysed degradation mechanism has already been proposed for poly(organo)phosphazenes substituted with amino acid esters[79, 122a]. Moreover, it is to be expected that the entire peptide subsequently disintegrates into its corresponding amino acids in presence of enzymes like papain[29, 104b], thus leading directly to the hydroxyphosphazene degradation intermediate. Thus, it is proposed that under lysosomal conditions, two different peptide cleavage mechanisms may occur, both of which would promote backbone degradation.

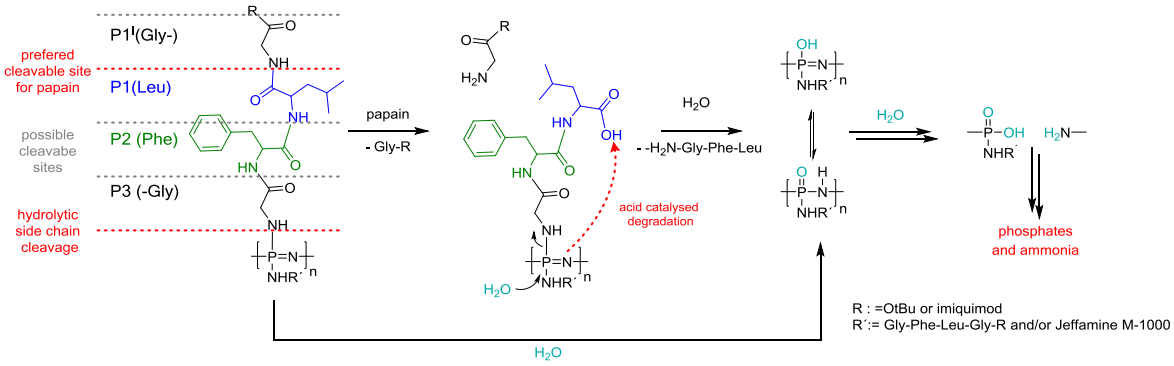


Figure 3-13. Preferential cleavable site of papain and proposed hydrolytic and enzyme initiated degradation mechanism of GFLG-peptide based poly(organo)phosphazenes.

3.4.6. Drug release

The release of imiquimod from the polymer **5** was analysed by HPLC and the amount of the released drug was estimated using a calibration curve for the free drug. The samples were stored at 37°C between each measurement and the investigations were carried out in citrate buffer at pH 6 and with papain in the same buffer system. Within a period of 14 days, 100 % release from the polymer-drug conjugate could be observed for the sample exposed to papain and only 65 % for the conjugate stored at pH 6 without

papain (Figure 3-14). This observation, suggests that, as expected hydrolytic and enzymatic drug release take place simultaneously. According to the supposed mechanism Gly-imiquimod is preferentially released by papain but according to other comparable published data from authors using similar GFLG based macromolecular drug delivery systems, Gly-drug degrades eventually to glycine and free drug[29, 104b]. It is assumed that the bond between glycine and drug is also a secondary cleavage site for papain. If used, as proposed, in polymer therapeutics in vivo, the lysosomal cathepsin B would be expected to preferentially cleave the Gly-imiquimod bond [29, 104b].

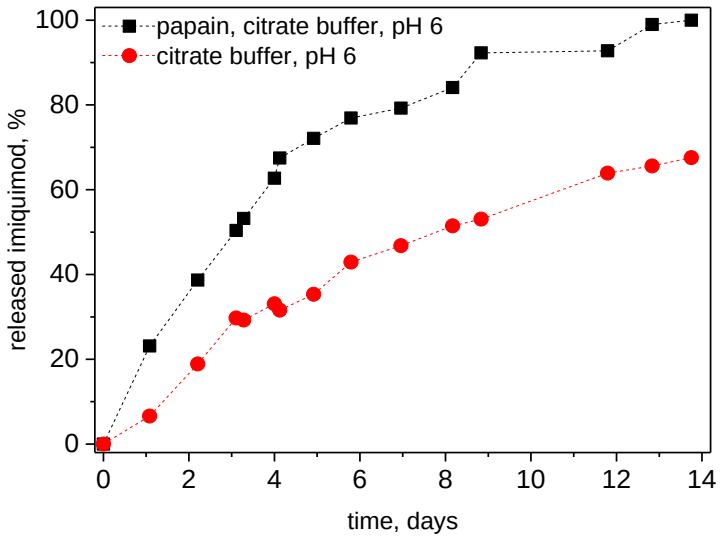


Figure 3-14. Hydrolytic release of imiquimod from polymer 5 at 37°C in acidic environment (citrate buffer, pH 6) (●), and enzymatic release of imiquimod from polymer 5 at 37°C in the same buffer with L-cysteine activated papain (■). The amount of the released drug was estimated using a calibration curve for the free drug.

3.5. Conclusions

A series of novel peptide hybrid polymers are reported via the grafting of the tetrapeptide sequence Gly-Phe-Leu-Gly onto a polyphosphazene backbone with successful synthesis being confirmed by GPC, ^{31}P and ^{1}H NMR spectroscopy. The polymers showed excellent solubility in water upon co-substitution with hydrophilic side chain Jeffamine M-1000. Degradation studies showed the polymers degraded via both enzymatic, as well as hydrolytic pathways, with the degradation rates significantly enhanced in the presence of papain. The degradation products include phosphate and ammonia, as well as the respective amino acids and Jeffamine M-1000 oligomers. A

suggested application of such materials is polymer therapeutics due to their showing sufficient stability under physiological conditions to be used as drug carriers delivering drugs via bloodstream, whilst subsequently disintegrating into low-molecular weight, non-toxic degradation products capable of undergoing renal clearance. This, combined with the observed spontaneous self-assembly upon conjugation of the hydrophobic drug imiquimod, make the presented degradable polymers potentially interesting for such drug delivery applications.

4. (Bio)degradable polyvinylpyrrolidone based hybrid polymers

4.1. Abstract

Polyvinylpyrrolidone (PVP) grafted onto a degradable polyphosphazene backbone gives hybrid polymers with precisely controlled molecular weights, multifunctionality and tunable degradability. The hybrid PVP polymers have a >97% organic proportion and hence mimic the many useful solution properties of PVP but with the added characteristics of covalent binding sites and degradability.

4.2. Introduction

Polyvinylpyrrolidone (PVP) can be regarded as a genuine all-rounder in polymer chemistry[124], with its numerous useful properties, such as water solubility, non-covalent bonding power, as well as film forming and adhesive properties. Due to these properties, aligned with its toxicological benignity,[125] PVP is used extensively in biomedical applications and widely used in the pharmaceutical industry, with many commercial formulations produced, as well as hundreds of papers reporting preparation of drugs in various using PVP as an excipient in soluble or in cross-linked forms.[126]

In this regard, PVP belongs to one of a wide range of water soluble polymers which are used for a multitude of pharmaceutical applications.[127] Indeed, ever more applications are being investigated, for example exploiting the excellent aqueous solubility, extended plasma retention times,[128] and low tissue accumulation compared to other commonly used polymers.[128] They have been investigated, amongst others, for use as nanocarriers for drug-delivery,[129] as polymer drug-conjugates,[130] polymer-protein conjugates[131] and for the solubilisation of hydrophobic drugs.[132]

However, in particular in nanomedicine, the safety of using non-degradable polymers at molecular weights above the renal clearance limit is of significant concern. Although a high molecular weight is usually required for pharmacokinetic enhancement, biopersistent polymers with molecular weights below the renal clearance limit run the risk of lysosomal accumulation.[3, 21d, 111] Polydisperse non-bioerodble polymers, even when used with a molecular weight just below the renal threshold will inherently have some biopersistent polymer chains. PVP above molecular weights above approximately 10 000 g mol^{-1} are thus not recommended for repeated use in intravenous preparations.[126] In this work it was attempted to circumvent this limitation by coupling a large number of end-functionalized PVP oligomers onto a hydrolytically instable polyphosphazene backbone.

4.3. Materials and Methods

All synthetic procedures were carried out air-free either in a glovebox (MBRAUN) under argon or under nitrogen using standard Schlenk line techniques. Glassware used for polymerization steps was dried in an oven at 120°C prior to use. PCl_5 was purified by sublimation and stored in the glovebox under argon. NEt_3 was dried over molecular sieves and distilled prior to use. All other chemicals were purchased from Sigma Aldrich and used without further purification. $Cl_3P{=}N\text{-}SiMe_3$ was prepared using reported procedure.[49] S-2-cyano-2-propyl O-ethyl xanthate was synthesized using literature procedures.[131a] Instrumentation. 1H NMR spectra were measured on a Bruker Avance 300 spectrometer with CDCl3 or D2O as an internal reference. The 31P NMR experiments were conducted at 121 MHz, using 85 % phosphoric acid as an external standard. Gel permeation chromatography (GPC) was carried out on a Viscothek GPCmax instrument using a PFG column from PSS (Mainz, Germany) (300 mm x 8 mm, 5 µm particle size). The samples were eluted with DMF containing 10mM LiBr as the mobile phase at a flow rate of 0.75 mL min-1 at 60°C. The molecular weights were calculated relative to polystyrene standards from PSS using a multidetector calibration. UV-Vis spectra were carried out on a Perkin Elmer Lambda 25 UV/VIS spectrophotometer. ESI-TOF spectrometry was measured on a Bruker MaXis instrument

(ACN/MeOH 1% H2O). A Malvern ZetaSizer Nano-ZS analyser (Malvern Instruments, UK) was used for dynamic light scattering (DLS) measurements. The 4 mW HeNe laser was set at λ = 633 nm with the detector angle at 173° for backscattering measurements. The samples were dissolved in phosphate buffer (pH 7.4) to give a 1 mg/ mL concentration, filtered through a Millipore Millex-GV 0.22 µm PVDF filter and measured in a disposable polystyrene ultra-micro cuvette at 25 °C.

4.3.1. Phosphate determination.

The degradation behaviour of the polymer **5** was studied by inorganic phosphate determination monitored by UV-Vis spectroscopy[71]. The polymer was incubated in TRIS buffer (pH 7.4), sodium acetate buffer (pH 5), or acidified H_2O (pH 2, enhanced degradation conditions) in a concentration of 7 mg mL^{-1} at 37°C during the time of analysis. Aliquots of the degradation medium (0.4 mL) were taken in regular time intervals and mixed with a reagent solution (1 mL) consisting of ammonium molybdate, ascorbic acid, sulfuric acid and potassium antimonyl tartrate. UV-Vis analysis of the mixtures were performed after 15 minutes of incubation time at 885nm. The concentration of phosphate was calculated from a calibration curve using potassium dihydrogen phosphate and is given as the percentage compared to the theoretical phosphate amount that can be released from the polymer backbone. In case of polymer **6** the polymer was incubated in acetate buffer at pH 5 or in acetate buffer containing $5.7x10^{-6}$ mol L^{-1} papain, preactivated for 5 min in acetate buffer with cysteine (0.01 mol L^{-1}), in a concentration of 1.3 mg mL^{-1} at 37°C during the time of analysis. Papain was replenished every day or every three days.

4.3.2. Synthesis of ω-hydroxyl polyvinylpyrrolidone (PVP-OH) (1).

Azobisisobutyronitrile (AIBN) (0.075 g, 0.45 mmol), the CTA S-1-cyanoethyl *O*-ethyl xanthate (0.75 g, 3.96 mmol) and N-vinylpyrrolidone (NVP) (19,55 g, 175.9 mmol) are degassed with argon and heated with stirring in an oil bath at 60 ºC for 6 h. The reaction mixture was then cooled to room temperature and precipitated into diethylether. The

filtered white powder was dried under vacuum. The polymer was then dissolved in deionized H_2O (5 ml) and the solution stirred at 40 ºC for 16 h. The solution was then co-evaporated with toluene under vacuum, dissolved in THF and precipitated into diethyl ether. The powder was filtered and dried under vacuum to give a white, hygroscopic powder.

Polymer **1a:** Yield 7.6 g (39 %). ESI-TOF m/z: 676.4; ^{1}H NMR (300 MHz, D_2O, δ): 3.6 (br, 1H), 3.3 (br, 2H), 2.4 (br, 1H), 2.3 (br, 2H), 2.0 (br, 2.8H) 1.7 (br, 2H), 1.27 (s, 0.7H) ppm.

Polymer **1b:** ^{1}H NMR (300 MHz, D_2O, δ): 3.6 (br, 1H), 3.3 (br, 2H), 2.4 (br, 0.6H), 2.3 (br, 1.4H), 2.0 (br, 2H) 1.7 (br, 2H), 1.32 (s, 0.2H) ppm.

4.3.3. Polydichlorophosphazene (2).

In a glovebox, the monomer $Cl_3P{=}N\text{-}SiMe_3$ (0.45 g, 2.01 mmol) and the initiator PCl_5 (0.02 g, 0.08 mmol) were dissolved in CH_2Cl_2 and stirred at room temperature. After 12 hours, the solvent was removed under vacuum. The obtained poly(dichlorophosphazene) was used for macromolecular substitution without further purification. Yield quantitative. ^{31}P NMR (121 MHz, $CDCl_3$, δ): -18 ppm.

4.3.4. Polyphosphazene-PVP hybrid polymer (3).

A suspension of sodium hydride (60% in mineral oil) (0.2 g, 5.0 mmol) in THF was prepared and PVP-OH (1) (3.5 g, 5.2 mmol) was added. The reaction mixture was stirred for 1 hour at room temperature before poly(dichlorophosphazene) (2) dissolved in THF (5 mL) was added and the reaction was stirred for further 24 hours at room temperature. The reaction mixture was filtered and the solvent was removed under vacuum and purified by dialysis against H_2O (24 hours) and then ethanol (48 hours). Yield 2.8 g (85 %). ^{1}H NMR (300 MHz, D_2O, δ): 3.6 (br, 1H), 3.3 (br, 2H), 2.4 (br, 1H), 2.3 (br, 2H), 2.0 (br, 2.8H) 1.7 (br, 2H), 1.27 (s, 0.7H) ppm; ^{31}P NMR (121 MHz, D_2O, δ): -7.5 ppm; SEC: M_n = 90 8001 g mol^{-1}, M_w = 102 600 g mol^{-1}, M_w / M_n = 1.2.

4.3.5. Glycin polyvinylpyrrolidone (H-Gly-PVP) (4).

PVP-OH (2g, 0.5 mmol), Fmoc-Gly-OH (163.5 mg, 0.55 mmol) and N,N' -dimethylaminopyridine (6.12 mg, 0.05 mmol) were dissolved in 15 mL CH_2Cl_2 and cooled to 0 C. In a second flask, 123.8 mg N,N' -dicylcohexylcarbodiimide (0.6 mmol) was dissolved in 5 mL CH_2Cl_2 and transferred to the reaction mixture at 0̊ C. The mixture was allowed to warm to room temperature and stirred for 24 h. The formed precipitate was then removed by filtration and the product precipitated in diethyl ether to obtain a white powder. Yield 1.5 g (70 %), ^{1}H NMR (300 MHz D_2O, δ): 6.5-8.0 (br, 0.4H), 3.6 (br, 1.4H), 3.3 (br, 1.9H), 2.4 (br, 0.6H), 2.3 (br, 1.4H), 2.0 (br, 2H) 1.7 (br, 2H), 1.32 (s, 0.2H) ppm

For Fmoc deprotection the product (1.5 g) was then dissolved in 19 ml MeCN with 1 ml DBU and 0.83 ml 1-dodecanethiol were added. The mixture was stirred for 1 h at room temperature. Afterwards the solvent was reduced and the product precipitated from diethyl ether to obtain a white powder.

4.3.6. Polyphosphazene-Gly-PVP hybrid polymer (5).

1.1 g H-Gly-PVP (0.28 mmol) were dissolved 3 ml THF and 0.4 ml Et_3N and added to a solution of poly(dichlorophosphazene) (25.0 mg, 0.11 mmol) dissolved in 2 ml THF and stirred for 24 hours at room temperature. The reaction mixture was filtered, the solvent evaporated and the product further purified by dialysis against H_2O (24 hours) and Ethanol (72 hours). Yield 595 mg (66.5 %) ^{1}H NMR (300 MHz D_2O, δ): 3.6 (br, 1H), 3.3 (br, 2H), 2.4 (br, 0.6H), 2.3 (br, 1.4H), 2.0 (br, 2H) 1.7 (br, 2H), 1.32 (s, 0.2H) ppm; ^{31}P NMR (121 MHz, D_2O, δ): -0.4 ppm.

4.3.7. Gly-Phe-Leu-Gly-OtBu peptide synthesis.

First peptide coupling: In a 100 ml round bottom flask 1.167 g (3.3 mmol) of Fmoc-Leu-OH were suspended in 30 ml EtOAc. Subsequently, 1.15 ml (6.6 mmol) DIEA and 1,413 g (3.3 mmol) COMU were added and the mixture was stirred until a deep orange color was

obtained. After the addition of 504 mg (3 mmol) of H-Gly-OtBu · HCl the reaction was stirred for 1 h and the solvent was removed in vacuo.

General procedure for Fmoc deprotection using TAEA: The crude residue from the precedent coupling was dissolved in 20 ml dichloromethane, 11.25 ml (75 mmol) of TAEA were added and the reaction was stirred for 30 min. The mixture was transferred to a separatory funnel containing 100 ml EtOAc and 50 ml brine, the funnel was shaken and the layers were separated. The organic phase was washed two times with 50 ml brine and two times with 50 ml pH 5.5 phosphate buffer, dried over anhydrous $MgSO_4$, and evaporated.

General procedures for COMU coupling: In a 50 ml round bottom flask 3.3 mmol of Fmoc- amino acid were suspended in 30 ml EtOAc followed by the addition of 1.15 ml (6.6 mmol) DIEA and 1.413 g (3.3 mmol) COMU. The resulting mixture was stirred for 2 min, added directly to the residue from the precedent deprotection and the resulting mixture was stirred for 1 h. After completion of the reaction, the solvent was removed on a rotary evaporator.

Modified work-up after final coupling step: After completion of the peptide coupling, the reaction solution was diluted with 100 ml EtOAc and washed with 1M HCl (2x 50 ml), saturated $NaHCO_3$ (2x 50 ml) and brine (2x 50 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated. The resulting residue was dissolved in CH_2Cl_2 and purified via DCVC[118]: SiO_2, cyclohexane/EtOAc 50:0 to 0:50 in 5 % increments. The product was further eluted by applying additional fractions of EtOAc if necessary. The protected tetrapeptide could be obtained as 1.4 g (2.09 mmol) of a colourless foam in 70 % yield. ^{1}H-NMR (300 MHz, DMSO-d_6, δ): 8.14-8.09 (m, 2H, -CO-N<u>H</u>-), 8.01 (d, 1H, J^3(H,H) = 8.3 Hz, -CO-N<u>H</u>-), 7.88 (d, 2H, J^3(H,H) = 7.7 Hz, Fluorenyl C^5<u>H</u> and C^4<u>H</u>), 7.69 (d, 2H, J^3(H,H) = 7.3 Hz, Fluorenyl C^1<u>H</u> and C^8<u>H</u>), 7.41 (t, 2H, J^3(H,H) = 7.3 Hz, Fluorenyl C^3<u>H</u> and C^6<u>H</u>), 7.32 (t, 2H, J^3(H,H) = 7.7 Hz, Fluorenyl C^2<u>H</u> and C^7<u>H</u>), 7.22-7.14 (m, 5H, Phe Ar-<u>H</u>), 4.56-4.51 (m, 1H, FmocNH-CH_2-), 4.35-4.18 (m, 4H, Fluorenyl C^9<u>H</u> and Fmoc –CH_2-O- and Leu –CH_2-C<u>H</u>-NH-), 3.72-3.45 (m, 4H), 3.02 (dd, 1H, J^3(H,H) = 13.8 Hz, J^4(H,H) = 4.4 Hz, Phe –C<u>H</u>(H)-Ph), 2.80-2.73 (m, 1H, Phe –CH(<u>H</u>)-Ph), 1.62-1.56 (m, 1H, Leu –C<u>H</u>(CH_3)$_2$), 1.48 (t, 2H, J^3(H,H) = 7.3 Hz), Leu –CH-C<u>H$_2$</u>-CH(CH_3)$_2$), 1.39 (s, 9H, -COOC(C<u>H$_3$</u>)$_3$), 0.88-0.82 (m, 6H, Leu –CH(C<u>H$_3$</u>)$_2$) ppm.

Removal of the terminal Fmoc-group[119]: The purified tetrapeptide (1 g) was suspended in 19 ml MeCN and 1 ml DBU and 3.7 ml 1-dodecanethiol were added. The mixture was stirred for 1 h at room temperature and afterwards 15 ml of n-heptane was added. The layers were separated and the MeCN phase was washed four times with 10 ml n-heptane, the solvent was evaporated and the residue dried under high vacuum. The crude product was directly used in the next step.

4.3.8. Polyphosphazene-Gly-PVP/GFLG-OtBu hydrid polymer (6).

Polydichlorophosphazene (0.028 g, 0.125 mmol) was dissolved in anhydrous THF in the glovebox and 1 equivalent of polymer 4 (0.534 g, 0.125 mmol) and NEt3 (0.013 g, 0.125 mmol) were then added to the polymer solution and allowed to react for 24 hours. An excess of H-Gly-Phe-Leu-Gly-OtBu (1.5 eq, 0,126 g, 0.187 mmol) was then added to the reaction mixture and allowed to react for a further 24 hours. The solvent was then removed under vacuum and resulting polymer was purified by dialysis (12 kDa cut-off) for one week against EtOH and afterwards the solvent was reduced and the product precipitated from diethyl ether to obtain a white powder. Yield 320 mg (51.4 %) ^{1}H NMR (300 MHz D_2O, δ): 7.3 (br, 0.01H) 3.6 (br, 1.2H), 3.3 (br, 2H), 2.4 (br, 0.7H), 2.3 (br, 1.5H), 2.0 (br, 2H) 1.7 (br, 2H), 1.4 (s, 0.07H), 1.32 (s, 0.2H), 0.8 (br, 0.02H) ppm; ^{31}P NMR (121 MHz, D_2O, δ): -0.4 ppm.

4.4. Results

In this work it was attempted to circumvent this limitation by coupling a large number of end-functionalized PVP oligomers onto a hydrolytically instable polyphosphazene backbone. To achieve this, PVP was first prepared via RAFT polymerization as reported by Pound et.al.[131a, 133] This preparation provides a simple and reproducible route to prepare ω-mono functionalized polyvinylpyrrolidone oligomers. For this work, PVP oligomers were prepared with molecular weights of approximately between 700 g mol^{-1} (Polymer **1a**) and 4000 g mol^{-1} (Polymer **1b**), as confirmed by ^{1}H-NMR spectroscopy and

ESI-TOF analysis. The xanthate end-groups could be readily converted to hydroxyl functionalities via hydrolysis (Polymer **1a** and **1b**).

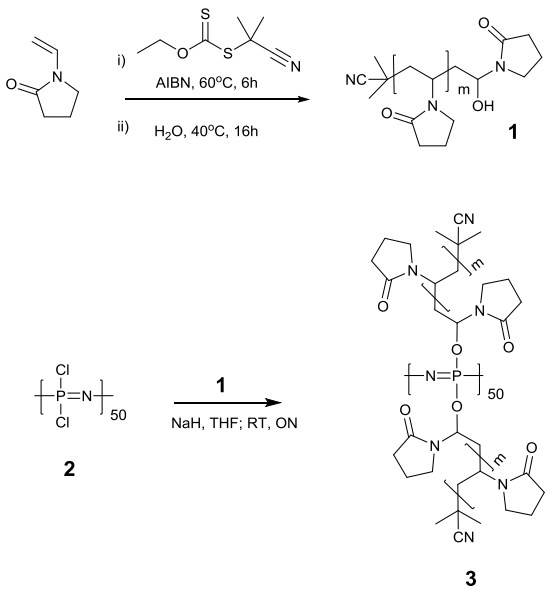


Figure 4-1: Coupling of ω-mono functionalized PVP (1) onto a polydichlorophosphazene backbone (2) to give the hybrid polymer (3).

The ω-hydroxyl functionalized vinylpyrrolidone oligomer (polymer **1a**) was then coupled to a dichloropolyphosphazene backbone (n=50) (Figure 4-1). An excess (2.5 equivalents per repeat unit) was added and complete substitution (within NMR limits) confirmed by 31P NMR spectroscopy. Through the use of controlled polymerisation techniques for the side group preparation, as well as a living cationic polymerisation for the polyphosphazene backbone, hybrid polymers with controlled molecular weights and relatively narrow polydispersities (Mw/Mn = 1.2-1.3) could be attained (Figure 4-2).

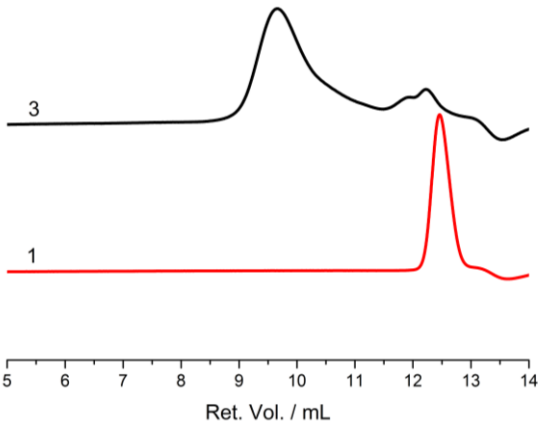


Figure 4-2: GPC Polymer 1a (PVP oligomer) and the grafted hybrid polymer 3.

It has been reported, that ethylpyrrolidone or propylpyrrolidone side groups can be attached to dichloropolyphosphazene to give degradable water soluble polymers with two pyrrolidone groups per repeat unit.[67, 81] Through the attachment of PVP oligomers, as presented here, however, the proportion of pyrrolidone units can be greatly increased and indeed easily varied. Indeed for the hybrid polymers prepared in this work, the amount of PN is extremely low (<3 wt. %) and hence the polymers can be expected to have chemical and biological properties dominated by that of PVP, rather than polyphosphazene. With an organic component of over 97% the solution state properties of the resulting polymer are expected to be dominated by those of PVP. The flexible polyphosphazene backbone and the highly branched, molecular brush type structure of the resulting hybrid polymers, with approximately 100 end-groups (two side chains per repeat unit), is clearly expected to have a positive influence on the solubility of the polymers.

Pristine PVP, despite offering excellent non-covalent binding properties, does not offer the opportunity of covalent loading of, for example drugs, targeting ligands, fluorescent markers as with multifunctional polymers used for polymer conjugates such as for example other commonly used functional polymers such as HPMA, or PGA.[134] Backbone functionalization is achievable, but with harsh conditions are required and the reaction is difficult to control.[130] However, through exploitation of a mixed substitution mechanism, it is a simple exercise to covalently link a relatively high loading of functional groups onto a polyphosphazene backbone, for example drug carriers[75], imaging agents,[135] photo-reactive moieties[136] or under charged groups to act as antibacterial[137] or vaccine adjuvants.[138] Such functionality thus significantly broadens the spectrum of PVP usage as a functional polymer. Recent techniques also allow the precise end group functionalization of the polyphosphazene[67].

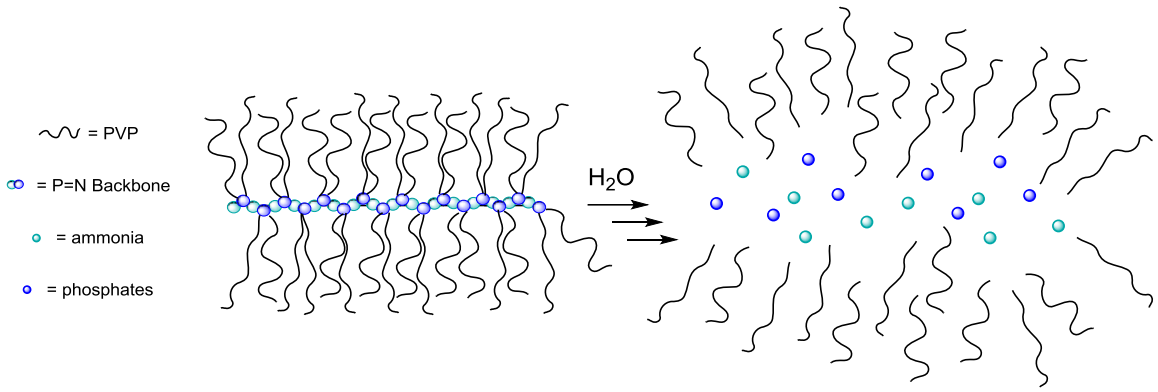


Figure 4-3: Schematic representation of the hydrolytic degradation process.

The degradation products of hybrid poly(organo)phosphazenes are well-studied[70, 85] and are known to consist of the organic component, in this case the intact PVP oligomers, plus phosphates and ammonia (Figure 4-3). As anticipated from previous studies[70, 75] the rate of degradation was observed to be significantly faster at lower pH values. The rate of degradation is relatively slow (data not shown) for some medical applications but could be readily accelerated and fine-tuned through the insertion of amino acid moieties between the organic component and the phosphorus backbone, as recently reported by Wilfert et. al.[70]. These previous studies focusing on polyalkylene oxide bound via different amino acids to the polyphosphazene backbone indicated glycine as hydrolytically very unstable spacer[70]. Therefore the hydroxyl functionalities of polymer 1b were reacted with Fmoc-Gly-OH using DMAP and DCC as coupling agent, to obtain Fmoc-Gly-PVP (Polymer **4**). Polymer **4**, was then coupled to the polydichlorophosphazene, to achieve PVP bound via a glycine spacer to the degradable backbone (polymer **5**, Figure 4-4).

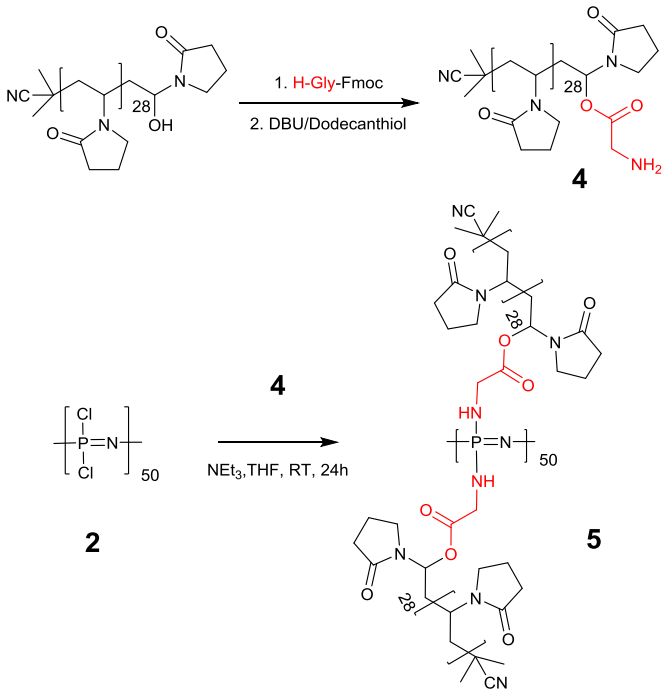


Figure 4-4: Coupling of glycine functionalized PVP (4) onto a polydichlorophosphazene backbone (2) to give the hybrid polymer (5).

The formation of phosphate, a final backbone degradation product, could be tracked by UV-Vis (Figure 4-5) and NMR spectroscopy (Figure 4-6). The release of phosphate from the synthesized polymer 5 was investigated under enhanced degradation conditions in water at pH 2, at lysosomal conditions at pH 5 and at pH 7.4. All samples were stored at 37°C between the measurements to simulate physiological conditions. Polymer **5** degraded at pH 7.4 with the slowest rate, with only 106 µM release of phosphate, after 44 days.

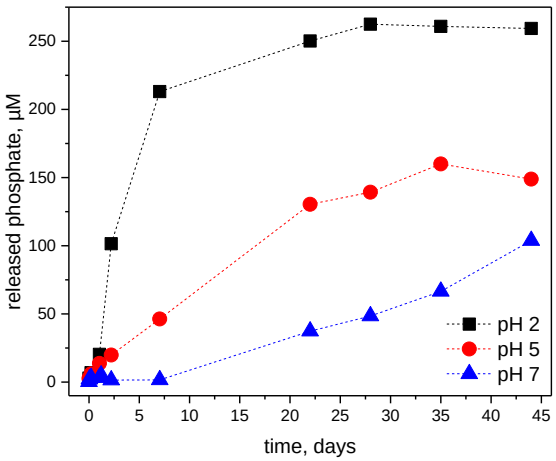


Figure 4-5: Phosphate determination of polymer 5 quantitatively determined by UV–Vis analysis to show the degradation profile of the polymer in aqueous conditions at pH 2 (■), pH 5 (●) and pH 7 (▲) at 37oC.

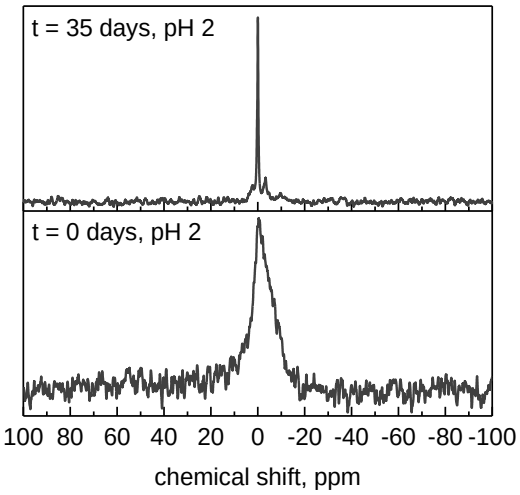


Figure 4-6: Degradation of polymer 5 followed with 31P-NMR to confirm that the polyphosphazene backbone degrades within 35 days and phosphates are released.

Considerably higher degradation rates were observed at acidic conditions with 64 % at pH 5 and total degradation of the backbone at pH 2 within 27 days. After 35 days, a sharp peak at around 0 ppm dominated the ^{31}P-NMR spectrum of the sample stored at pH 2, which is associated with the formation of inorganic phosphate. These results are comparable to published data and affirm the expected degradation mechanism of polyphosphazenes with the hydrolytic cleavage of the side groups from the polyphosphazene backbone, leading to very unstable hydroxyphosphazenes and phosphazanes[75].

It is also possible to insert a selective, biodegradation through co-substitution with an enzymatic vulnerable peptide sequence. The structure of the tetrapeptide Gly-Phe-Leu-Gly contains hydrophobic amino acid residues in P2 and P3 positions enabling an energetically favourable interaction with the active site of the lysosomal enzyme cathepsin B[104a, 116] or papain, an enzyme with similar specificity as the lysosomal enzyme cathepsin B[103a]. After enzymatic degradation, the resulting hydroxyphosphazene is extremely unstable and degrades rapidly (Figure 4-7). Moreover that the carbonic acid chain end of the peptide offers the possibility to attach different agents like targeting ligands or drugs (e.g. doxorubicin[99c], epirubicin[117], adriamycin[104a]

and cyclopamine[29]) covalently. An excess of tetrapeptide H-Gly-Phe-Leu-Gly-OtBu was used as second reagent, to substitute the remaining Cl atoms of a polyphosphazene (n=50) with polymer **1b** (Gly-PVP) side chains already bound to the backbone (Figure 4-7). The relative ratio of the Gly-Phe-Leu-Gly-Otbu and the Gly-PVP side chains was calculated by ^{1}H-NMR, through integration of the PVP end-group protons (s, 6H) at 1.32 ppm versus those from the tert-butyl-group (s, 9H) of the protected tetrapeptide sidechain at 1.4 ppm. The obtained ratio is 31 % tetrapeptide to 69 % Gly-PVP.

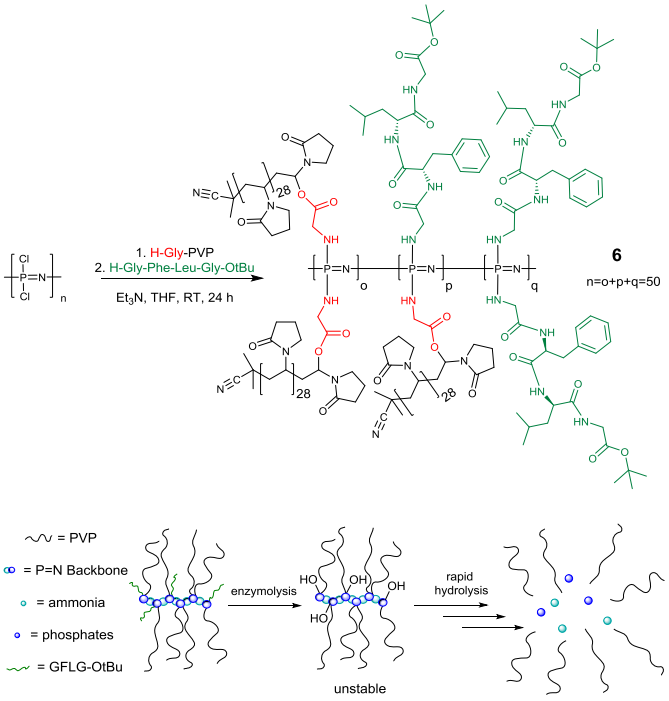


Figure 4-7: Coupling of the tetrapeptide Gly-Phe-Leu-Gly- OtBu and glycine functionalized PVP (4) onto a polydichlorophosphazene backbone (2) to give an enzymatically degradable hybrid polymer (6) and the expected degradation mechanism. The combinations of the two different side chains are expected to be statistically distributed.

The formation of phosphate from polymer **6** was again tracked by UV-Vis (Figure 4-8). The release of phosphate from polymer **6** was investigated in acetate buffer at pH 5, and under the influence of papain, pre-activated with L-cysteine in the same buffer. Papain was replenished every day or every three days. The samples were stored at 37 °C during the time of analysis. Polymer **6** degraded at pH 5 without papain with the slowest rate and with considerably higher degradation rates with papain. Moreover the degradation rate could be increased by adding fresh enzyme every day to the incubated polymer. Hence, the results show a significant influence of the enzyme on the degradation rate of polymer **6.**

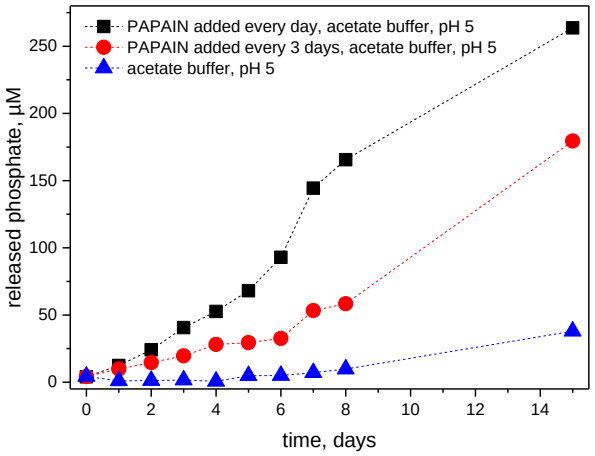


Figure 4-8: Phosphate determination of polymer 6 quantitatively determined by UV–Vis analysis to show the degradation profile of the polymer in aqueous conditions at pH 5 with papain added every day (■), papain added every three days (●) and at pH 5 without papain (▲) at 37oC.

4.5. Conclusion

Hybrid macromolecular constructs can be prepared with controlled molecular weights and narrow polydispersities. The hybrid polymers are shown to degrade to their small molecule components in hydrolytic and enzymatic environments. The degradation rate can be enhanced with lower pH values and with the addition of enzyme. The high organic component of these hybrid polymers renders them genuine degradable alternatives for solution state applications to the highly valuable, biopersistent polymer PVP. Furthermore, the multifunctional nature both along the backbone, as well as the end-groups opens the door to the functionalization a wide range of copolymers and/or co-functionalisation and/or cross-linking groups. All these characteristics make the presented degradable polymers potentially interesting for biomedical applications like drug delivery.

5. Summary and Outlook

In this work, polyorganophosphazenes with controlled degradability as well as controlled drug release are presented. The polymers were successfully synthesized by the living cationic polycondensation of trichlorophosphoranimine and macromolecular substitution with stimuli sensitive and water soluble side groups. The degradation behavior of these polymers was detailed studied using ^{31}P NMR, FFF and UV-Vis. Drug release was followed with HPLC.

The tetrapeptide Gly-Phe-Leu-Gly was used as stimuli sensitive linker and detailed degradation studies showed peptide containing polymers degraded via both enzymatic as well as hydrolytic pathways, with the degradation rates significantly enhanced in the presence of enzyme. Moreover, the immune response modifier imiquimod was coupled to the polyphosphazene backbone via the tetrapeptide linker, leading to enzymatic enhanced drug release.

Furthermore, biodegradable polyvinylpyrrolidone based hybrid polymers are shown. The degradation rate of these novel hybrid polymers could be significantly enhanced by incorporating a glycine-spacer between PVP and the polyphosphazene backbone or by using the tetrapeptide-linker as co-substituent.

A suggested application of the presented materials is nanomedicine. They show sufficient stability under physiological conditions to be used as drug carriers delivering drugs via bloodstream, whilst subsequently disintegrating into low-molecular weight, non-toxic degradation products capable of undergoing renal clearance. Moreover controlled drug release in the lysosomal compartment of cells can be obtained if drugs are coupled to the enzymatic degradable tetrapeptide linker Gly-Phe-Leu-Gly. In this work the immune response modifier imiquimod (R837) was used as exemplary drug, however the carbonic acid chain end of the peptide offers the possibility to attach different other drugs like epirubicin, doxorubicin, adriamycin or cyclopamine.

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