Charge modified, comb-like graft-polyesters for drug delivery and DNA vaccination: Synthesis and Characterization of Poly(vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide)s

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Chapter 1

Chapter 1: Introduction

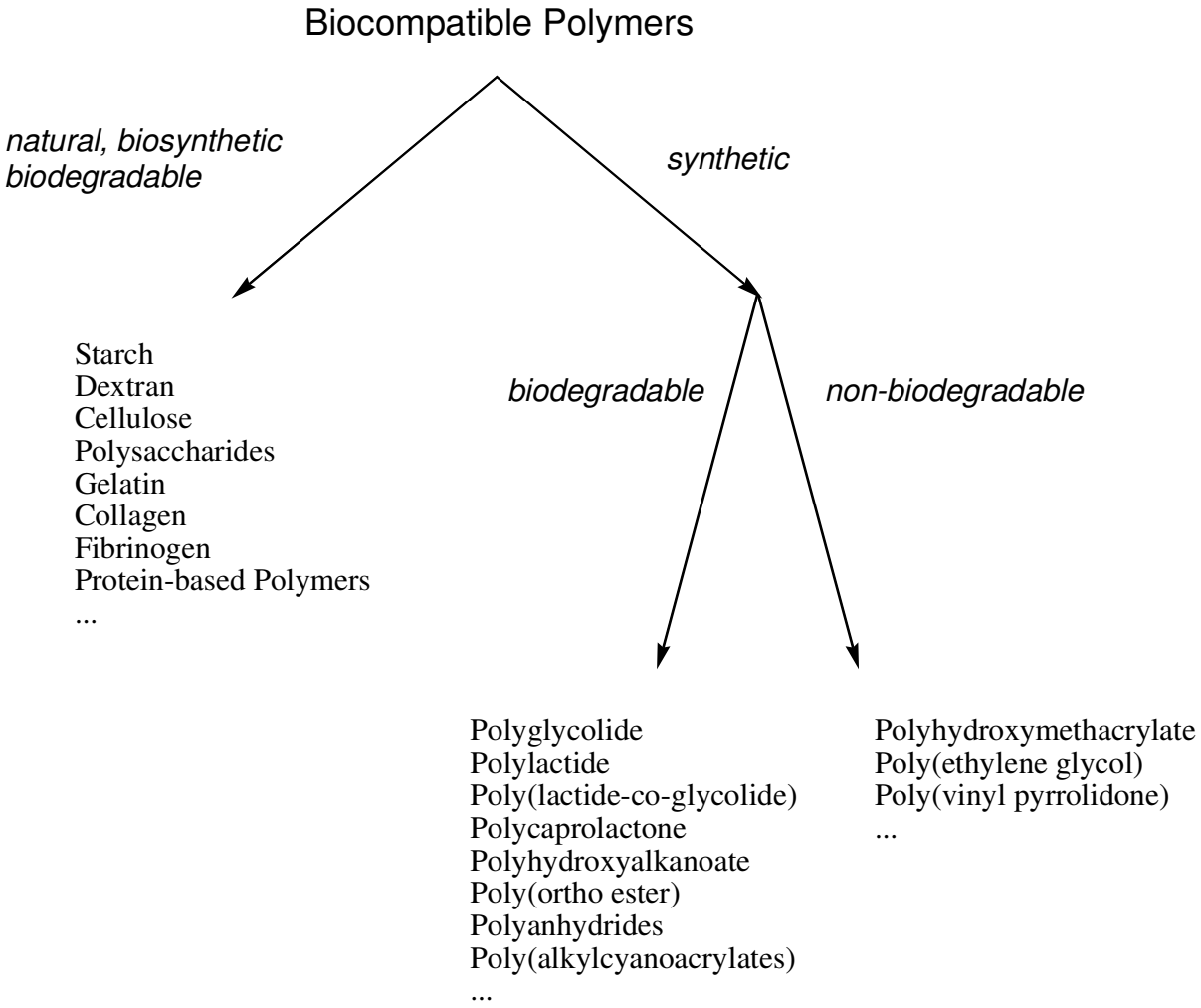
1.1 Introduction

Peptide and DNA-based drugs play a more and more important role in modern medicine and pharmacy. These hydrophilic, polymeric substances often work very effective and specific, however due to their instability they can not be delivered into the body in their bioactive form. The tertiary structure of large proteins like enzymes is the responsible for biological activity and is very sensitive against hydrophilic or lipophilic influences. For drugs based on RNA and DNA like antisense oligonucleotides, ribozymes and plasmids, the high molecular weight and distress of enzymatic degradation by DNases is one of the major problems for their the use as therapeutics.[1,2] These delivery problems could only be overcome by using of special carriers which are able to protect drugs against the loss of bioactivity. These carriers have a polymeric structure and can be understood as a protecting coat or scaffold which stabilizes the structure of proteins and/or RNA/DNA and protecting them against degradative attacks. The polymeric drug carriers should also promote the uptake of drugs and enhance their bioavailability. Drugs protected by the carriers can enter the body by different routes, such as the peroral,[3] nasal,[4] pulmonary[5] and parenteral[6,7] way. Mostly the parenteral route of application is preferred, since the injection of drug carrier systems leads to higher bioavailability and less degradation of the drugs than the oral, nasal and pulmonary application. Peroral applied drug carrier systems are exposed to digestion by enzymes and the acidic and basic milieu of the gastrointestinal tract. Using the nasal or pulmonary route the drug carrier systems are exposed to ciliated epithelium and surfactant which may reduce bioavailability and stability of drugs. Sometimes a damage of the epithelium could be caused by the carrier system.

Currently, there are different types of carrier systems under development. Beside liposomal approaches the use of biocompatible polymers in the form of micro- and nanoparticles as well as implants are promising vectors for drug delivery.[8,9] Biocompatibility is the ability of a material to perform with an appropriate host

response in a specific application.[10] Different types of biocompatible polymers are known. Beside natural and biosynthetic polymers like polysaccharides, gelatin and protein-based polymers synthetic biocompatible polymers are used. They can be subdivided into biodegradable polymers like polylactide and poly(ortho esters) and non-biodegradable like poly(hydroxymethacrylate) and poly(ethylene glycol) (Scheme 1).

Scheme 1: A selection of biocompatible polymers for drug delivery[11,12]



Polyesters synthesized from the dimers of lactic and glycolic acids (PLGA) are the most important polymers for drug delivery by now (Figure 1).[13] They will be degraded into the physiological products, lactic and glycolic acid.

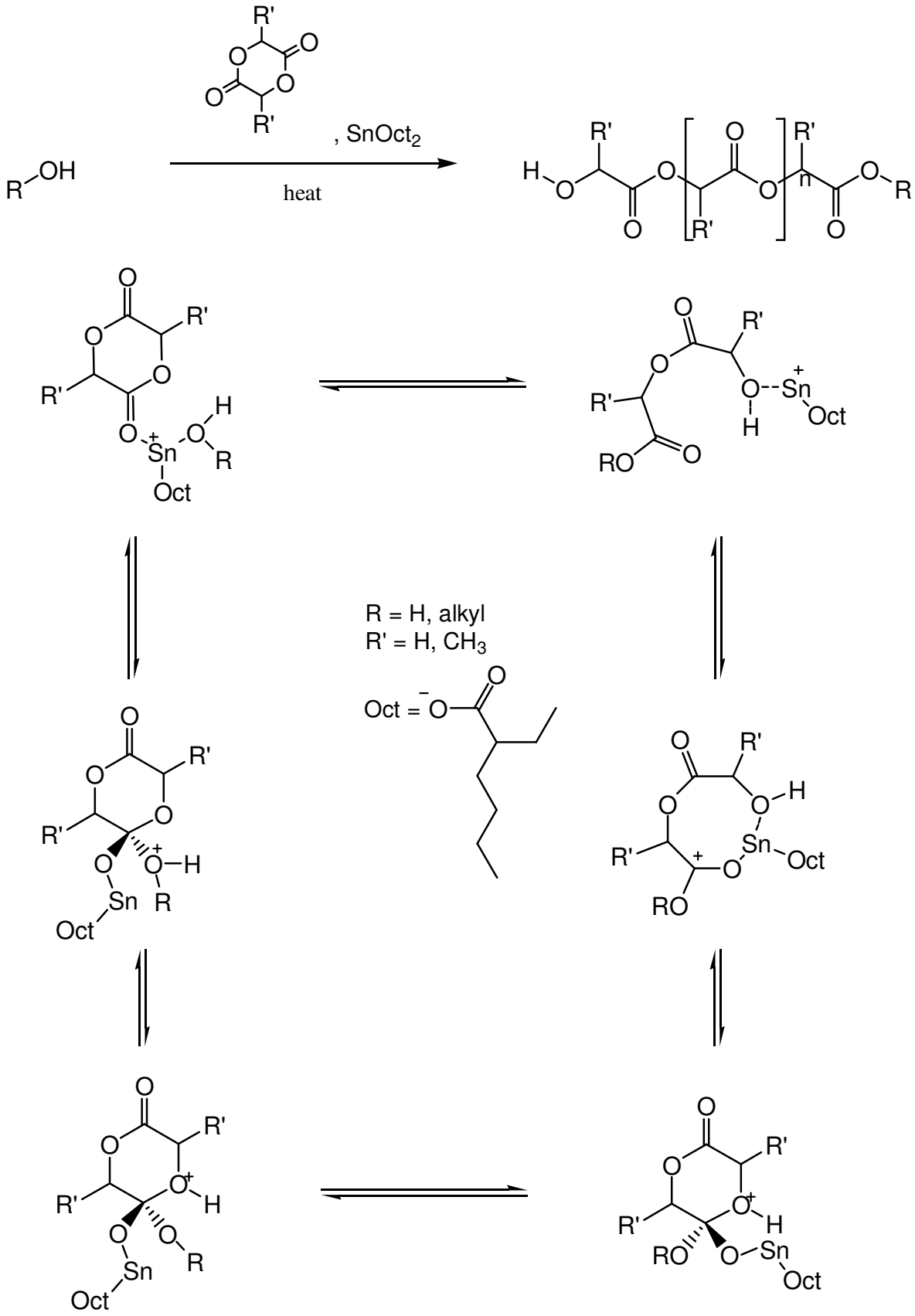


Figure 1: *Poly(lactide-co-glycolide) (PLGA): synthesis in bulk using tin(II) octoate as catalyst and mechanism of chain formation*

Biodegradation of polymers can be defined as a degradation of the polymeric structure to small molecules mediated at least partially by a biological system. [14] The mechanism of the degradation can be a hydrolytic or enzymatic cleavage of the polymer chain.[15,16] This cleavage is followed by an erosion process, a loss of material from the polymer bulk. [14] Bulk and surface erosion are known. In bulk erosion the degradation starts after water has entered the polymeric sample. Huge, water-insoluble degradation products are formed by random chain cleavage. At the beginning, this leads to a decrease of the molecular weight of the sample without mass loss (figure 2). In case of acidic degradation products the acidity within the specimen increases until the formed molecules are small enough to leave the system. This decrease of pH-value is very problematic for the bioactivity and stability of many protein- and RNA/DNA-based drugs. [15,17-20] In contrast, surface erosion takes place at the surface of the polymer sample. Immediately after cleavage of the polymer chain water-soluble degradation products are formed. The molecular weight of the sample remains constant. However, the mass of the system decreases immediately after the degradation has started (figure 2).[14,21]

Different degradation mechanisms lead to different release characteristics of the drug from the delivery device. The first release step is usually the so called burst release which is caused by detachment of surface absorbed drug. In case of surface erosion the burst release is followed by a release of the drug depending on the polymer degradation at the surface of the device. In contrast, during bulk erosion the burst is followed by a lag phase which means no drug release. In this phase water penetrates the device and the degradation begins. After Polymer degradation has advanced and pores are formed the drug release restarts from the whole device.[18,22-25]

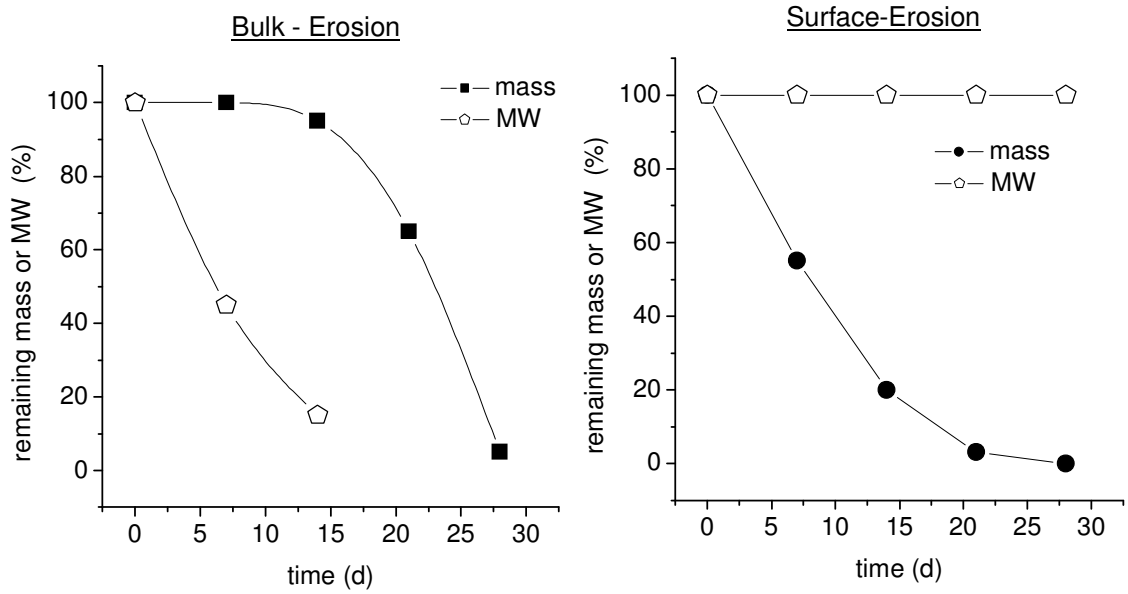


***Figure 2**: Comparison of bulk and surface erosion: Changes in mass and molecular weight (MW)*

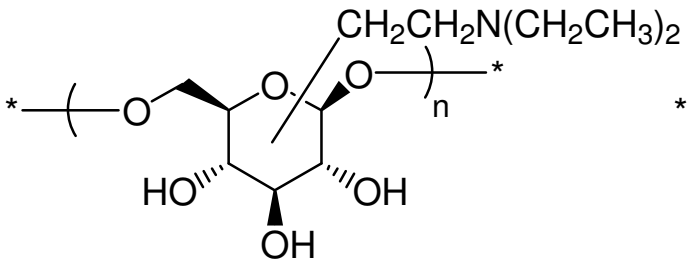
Linear PLGA polyesters which have been described for the preparation of nano- and microparticles or implants in many publications show bulk erosion with a typical release profile with a degradation lasting more than 20 days.[18,20,26-30] Drug delivery systems made of PLGA have a high initial burst release accompanied by low efficiency of drug encapsulation. Due to their neutral character only relative weak interactions between drug and polymer are possible. The resulting drug concentrations in the delivery systems are too low for a therapeutic application in patients. Furthermore, PLGA becomes more and more lipophilic with increasing chain length which destabilizes the structure of the incorporated hydrophilic and amphiphilic drugs leading to a loss in bioactivity. Furthermore, PLGA degradation follows a bulk erosion mechanism. This can affect stability and bioactivity of protein- and RNA/DNA-based drugs due to acidic pH values during degradation.[31]

To enter cells and to transport RNA/DNA into the nucleus the drug delivery device has to pass the negatively charged cell membrane and to enter the

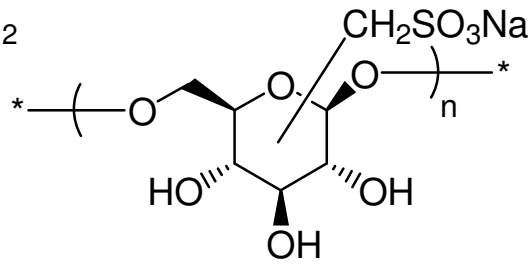
cytoplasm. The negative charges of DNA result in negatively charged drug delivery systems of PLGA and encapsulated DNA. These devices can hardly enter cells due to electrostatic forces of repulsion.

To overcome these disadvantages copolymers of PLGA and poly(ethylene glycol) (PEG) or dextran sulfate sodium (DSS) were synthesized (figure 3).

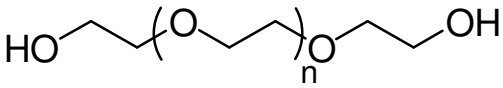
ABA (A = PLGA; B-block = PEG) have better degradation properties than linear PLGAs. However, they are still neutral and show relatively long degradation times. [18]



DEAE-Dextran



Dextransulfate sodium salt (DSS)

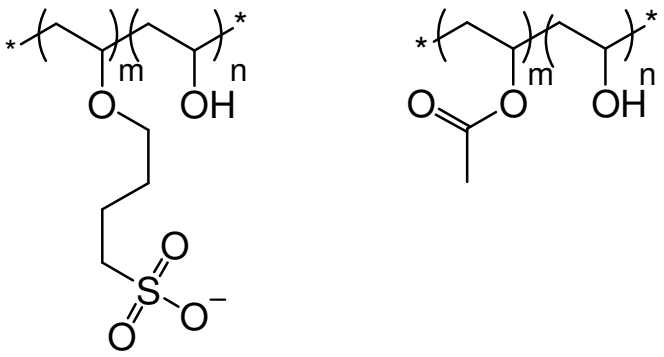


Poly(ethylene glycol) (PEG)
resp.
Poly(ethylene oxide) (PEO)

Figure 3: Different starting materials for the synthesis of PLGA-graft-polyesters and ABA-block-co-polymers

Using Comb-like, highly branched polyesters synthesized by grafting dextran-based backbones with lactide and glycolide a faster degradation was achieved. Unfortunately, the temperature stability of the backbone was too low leading to destruction of dextran during polymer synthesis. In addition, the backbone has bacterial origin which could lead to allergic reactions. Furthermore, the polymers show bulk erosion.[32-34]

Poly(vinyl alcohol) (PVA) or negatively charged PVAs have better temperature stability during bulk polymerization with lactide and glycolide (figure 4). However, because of their neutral respectively negative character these polyesters can not be used as RNA/DNA carrier systems. Furthermore, it could be supposed that due to low drug loading the use as drug delivery system for negatively charged protein-based drugs is inefficient. In addition, the degradation of these polyesters still lasts relatively long and can not be modified to get degradation times between 1 and 20 days.[35,36]



sulfobutylated PVA PVA (partly esterified with acetate)

Figure 4: Neutral and negatively charged PVAs as starting material for the polyester synthesis

1.2 Objects of this work

In this work a novel carrier agent or drug delivery and especially RNA/DNA delivery was synthesized. The relationship between polymer structure and polymer properties regarding behavior at different temperatures, degradation, solubility and transfection was investigated using different physico-chemical and biological characterization methods.

In this dissertation a new amphiphilic, biodegradable and completely excretable class of polyesters based on positively charged PVA backbones was developed. **Chapter 2** describes the synthesis and characterization of the polymer using physico-chemical methods. We postulated that due to basic characteristics of amino groups the integration of amines into PVA and grafting with lactide/glycolide leads to a faster degradation of the polyester. It was hoped that the degradation mechanism could be influenced by the comb-like design of these polymers, because water–soluble products should be formed early in the degradation process. It was expected that due to variation of hydrophilic and lipophilic compounds of the polyester the solubility of the resulting polymer could be modified. Furthermore, we postulated that insertion of short side chains and amine groups would increase the flexibility of chain segments. These insertions might also reduce the glass transition temperature due to sterical hindrance of hydrogen bonding and other interactions between the polymer chains. It was postulated that the highly branched character of the polymers could be proofed by static light scattering.

Using multidimensional NMR techniques covalent bonds and the structural geometry of organic substances could be investigated in detail. In **chapter 3** a study about the microstructure of amine- modified PVA backbones and its tacticity is described. It was postulated that the covalent bond between amine and PVA, the constitution and the configuration of the polymer chain could be studied in detail using multidimensional NMR techniques like COSY (Correlated Spectroscopy), HMBC (Heteronuclear Multiple Bond Correlation) and HMQC (Heteronuclear Multiple Quantum Correlation).

Positively charged polymers are well known as gene delivery devices.[37-39] **Chapter 4** describes a study about the degradation, particle formation and the transfection of the newly developed polymers. We assumed that using proton NMR a direct proof for the degradation of polymer side chains should be possible. It was supposed that a decrease in side chain length could be observed.

It was postulated that due to the positive charge of the inserted amine-groups particles of polyesters and DNA could be formed. Furthermore, we supposed that these particles have positive zeta-potentials, excellent abilities of cell uptake and high transfection efficiencies which could be modified by the degree of amine substitution in the backbone and particle composition.

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Chapter 2

Chapter 2: Fast degrading, high-molecular weight, brush-like branched, amine-modified poly(vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide)s as a platform for parenteral drug delivery systems: Synthesis, characterization and degradation behavior.

Submitted to Macromolecules (see appendix)

2.1 Summary

A new class of biodegradable branched polyesters, namely Poly[vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide), Poly[vinyl 2-(diethylamino)ethylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide) and Poly[vinyl 3-(dimethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide), abbreviated as (P[$VACB_{0\text{-}70}$-$VAc_{0\text{-}36}$-$VA_{15\text{-}195}$-$VPLGA_{75\text{-}240}$]), was designed for parenteral delivery of hydrophilic macromolecular drug substances such as proteins and DNA. To overcome protein/polymer compatibility and degradation problems of classic PLGA copolyesters a combined strategy of amine substitution (using 3-diethylaminopropylamine, 2-diethylaminoethylamine and 3-dimethylaminopropylamine) and the introduction of a hydrophilic poly(vinyl alcohol), PVA, backbone (P=300) using a polymer analogous reaction based on CDI activation and subsequent grafting with poly(lactide-co-glycolide) PLGA in bulk was evaluated. A series of 52 polyesters was synthesized in good yields and successfully characterized using ^{1}H NMR, ^{13}C NMR, DSC, TGA, TEM, WAXD and GPC-MALLS. The postulated structure of the branched polyesters was found to be in accordance with physiochemical characterization.

Depending on the PLGA side chain length and the degree of amine substitution, the solubility of the resulting polyesters could be designed to vary from water-soluble to water-insoluble. NMR measurements indicate an ionization of the inserted amino function within the polyesters. Glass transition temperatures (T_g) indicate miscibility of PVA and PLGA components. Nevertheless TEM suggests a microstructure of these polyesters possibly caused by a separation of hydrophilic and lipophilic segments. The high branching of the polyesters were demonstrated by multi angel laser light scattering.

Polymers with molecular weights above 100 000 g mol^{-1} show half-life degradation times smaller than one day. It could be shown that increasing degrees of amine substitution lead to smaller degradation times while elongation of side chains results in the opposite effect. A rapid bulk hydrolysis could be supposed. It could be shown that the synthesis of polyesters with a wide range of different properties is possible

without complicate reactions and in good yields. Elongation of the PLGA side chains lead to more and more lipophilic polymers. A higher degree of amine substitution results in an opposite effect. A structure-properties relationship of the solubility could be obtained. These polymers demonstrated a surprising degradation behavior and their potential as drug delivery system for hydrophilic drugs and for tailor made polymers for parenteral delivery. Further investigations should demonstrate these abilities within pharmaceutical studies.

2.2 Introduction.

Drug delivery of hydrophilic molecules such as proteins and DNA is generally considered as the Achilles heel for their therapeutic application.[1-3] These molecules are rapidly degraded by enzymes at the site of application and in the general circulation requiring frequent injections or infusions.[4-6] Their size and instability usually prevent uptake through epithelium of the gastro-intestinal tract and thus necessitates parenteral administration.[7,8] To overcome these application problems, carrier systems stabilizing hydrophilic macromolecular drug substances is a subject of intensive research efforts allowing efficient delivery to patients in a safe and convenient way.[9-20] In this context nano-scale carriers such as nano-particles and nano-complexes have found increasing attention since they allow intravenous application.[9,21,22] Controlled and sustained release of this class of drug candidates can be accomplished using microspheres and implants from biodegradable polymers.[1,14,20,23] Yet classic copolyesters of lactic and glycolic acid, PLGA, are not ideal for protein and DNA delivery since inactivation and uncontrolled release is a consequence of poor compatibility between lipophilic polymers and hydrophilic drug candidates[7,24-27].

In an attempt to overcome these problems associated with linear PLGA we hypothesized that modification of the polymer structure would allow modification of polymer solubility and degradation in a vast range suitable for drug delivery. A brush-

like branched structure was considered beneficial for accelerating degradation as fewer cleavage steps are necessary to generate water soluble degradation products.[28,29] As hydrophilic and water soluble backbone we selected poly(vinyl alcohol) (PVA) with a molecular weight of 15 000 g/mol which is considered biocompatible and can be eliminated from the body by renal excretion.[30-32]

To this backbone, amine groups were covalently coupled in a polymer-analogous reaction using carbonyl diimidazole, CDI, to introduce cationic charges under physiological conditions. This modification was thought to affect colloidal stability of carrier systems by imparting positive surface charges on one hand,[33] increasing protein or DNA loading of carriers by electrostatic interactions on the other hand.[34,35] Also acceleration of PLGA degradation by base catalysis could contribute to faster biodegradable delivery systems. Because of the lower cytotoxicity of secondary and tertiary amino-groups functions, diamines and PVA were coupled via the hydrolytically stable urethane bond.[36,37] Due to their partial protonation under physiological conditions, these amines will enhance the hydrophilic character of the polyester.

Finally poly(lactide-co-glycolide) (PLGA) was grafted onto the amine-modified PVA in a ring opening polymerization (ROMP) in bulk introducing lipophilic side chains. Due to this strategy we can design amphiphilic polymers with a broad range of solubility characteristics ranging from water soluble to organic soluble thus allowing the design of new carrier systems suitable for macromolecular drug candidates. We devised a relative simple strategy for synthesis to allow up-scaling and structure modification in a broad range.

Together with the amine substitution the PLGA side chains composed of a 1:1 ratio (n/n) of lactide and glycolide control the amphiphilic behavior of the branched polyester. Elongation of the side chain should move the polymer properties towards linear PLGA whereas short PLGA side chains should make them more hydrophilic. The basic amino functions will also enlarge the hydrophilic character of these polyesters. It should increase the loading capacity and degradation rate and should reduce the initial burst of a drug delivery system.[25,35]

Branched polyesters consisting of a non-polysaccharide backbone and PLGA side chains have not been studied to a great extend.[28,38,39] To establish structure-function-relationships a total of 55 different polymers was synthesized modifying amine-substitution and PLGA side chain length systematically. As known out of our former studies neutral and negatively charged PVA-g-PLGAs having chain length higher than ten showed potential for the formation of micro- and nanoparticles and they are water soluble and form complexes having chain length smaller than three.[12,29,39,40] Due to these former findings we decided to synthesize the polymers outlined in figure 1.

2.3 Experimental Section

Materials. 2-Diethylaminoethylamine (*purum*, >98%) , 3-diethylaminopropylamine (*purum*, >98%), 3-dimethylaminopropylamine (*purum*, >98%), poly(vinyl alcohol) (MW 15000 g mol^{-1}; degree of polymerization 300 (P=300); degree of hydrolysis 86-89%), carbonyl diimidazole (*purum*, ~97%), N-methyl pyrrolidone (NMP) (absolute), dimethylacetamide (DMAc)(for HPLC, 99.8% and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (*puriss.*, absolute, over molecular sieve) were purchased from Fluka GmbH (Germany) and used as received. D,L-lactide (S-grade) and glycolide (S and A-grade) (Boehringer Ingelheim, Germany) were used as received or if the melting point was too low[29] it was recrystallized twice from ethyl acetate. tin(II) 2-ethylhexanoate (Aldrich) ($Sn(oct)_2$) and lithium bromide (extra pure) (Merck) was used as received. Tetrahydrofuran (THF) (BASF, Germany) was dried over sodium and distilled under nitrogen before use. All other chemicals were used as received without further purification.

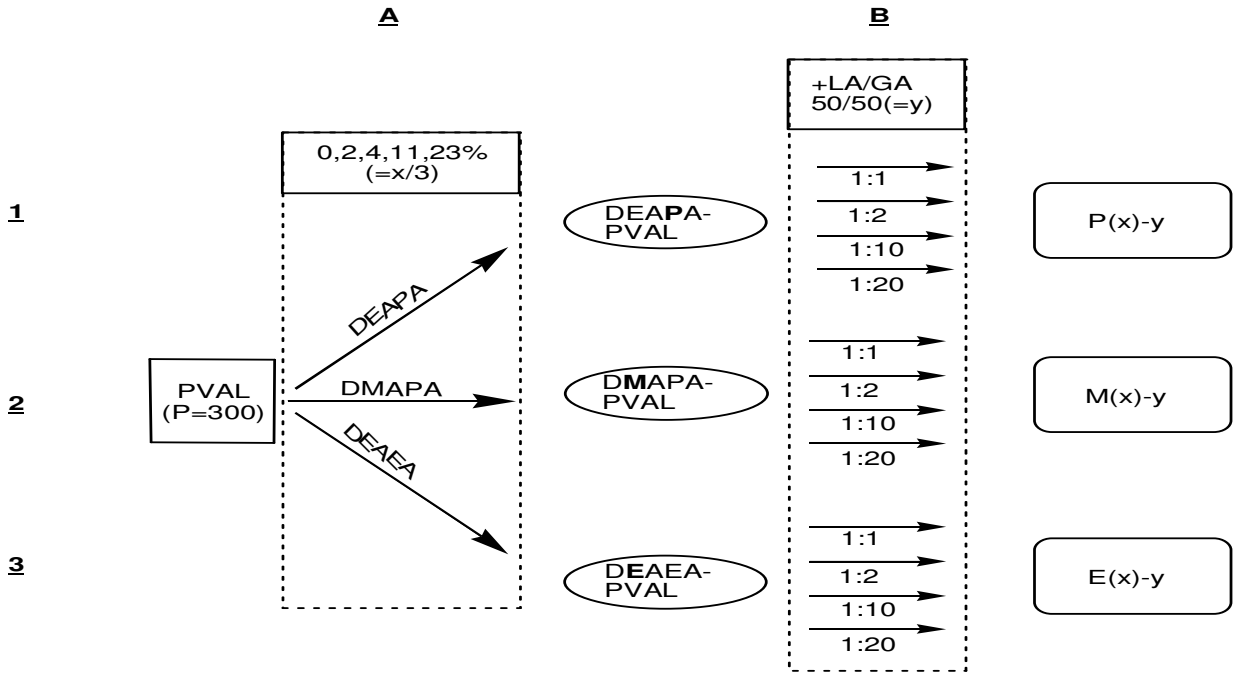


Figure 1*. Pattern demonstrating the composition and the corresponding nomenclature of the different polyesters, x symbolize the average amount of amine carrying monomers in a PVA having a degree of polymerization of 300, y is the theoretic chain length (1, 2, 10, 20)*

2.3.1 Synthesis

Synthesis of amine carbonylimidazoles (N-(3-(diethylamino)propyl)-1H-imidazole-1-carboxamide, N-(3-(dimethylamino)propyl)-1H-imidazole-1-carboxamide and N-(2-(diethylamino)ethyl)-1H-imidazole-1-carboxamide) (amine-CI): In a rigorously dried 100ml round-bottomed flask equipped with gas inlet, a septum cap and magnetic stirrer 90 ml dry THF were distilled under nitrogen gassing. Then 16.72 g carbonyl diimidazole (CDI) (0.10 mol) were dissolved and one of the three diamines (1:1 molar ratio) were injected using syringe so that the temperature in the flask did not exceed 55°C.[41]

After stirring for 16 hours at room temperature the resulting imidazole / amine-carbonylimidazole solution was isolated by distilling the THF off using a rotavapor. The resulting oily, slight yellow mixture was used without further purification after the amount of amine-CI was quantified by ^{1}H NMR spectroscopy. Yields: >90%

^{1}H-NMR:

DEAEA-CI: δ = 12.04 (broad, NH non-bonded imidazole), 8.27 ((Im-CO-N<u>H</u>-CH_2-CH_2-N(CH_2CH_3)$_2$, s), 8.21-8.19 (bonded imidazole [-N-C<u>H</u>=N-], m), 7.64-7.62 (non bonded imidazole [-N-C<u>H</u>=N-], m), 7.48-7.46 (bonded imidazole [-N-C<u>H</u>=CH-N=], m), 7.26 (chloroform), 7.05-7.03 (non bonded imidazole [-N-C<u>H</u>=C<u>H</u>-N=], m), 7.01-6.98 (bonded imidazole (-N-CH=C<u>H</u>-N=), m), 3.40 (Im-CO-NH-C<u>H</u>$_2$-CH_2-N(CH_2CH_3)$_2$, t, ^{3}J = 6.6 Hz), 2.61 (Im-CO-NH-CH_2-C<u>H</u>$_2$-N(CH_2CH_3)$_2$, t, ^{3}J=6.6 Hz), 2.51 (Im-CO-NH-CH_2-CH_2-N(C<u>H</u>$_2$$CH_3$)$_2$, q, ^{3}J = 7.1 Hz), 0.96 (Im-CO-NH-CH_2-CH_2-N(CH_2C<u>H</u>$_3$)$_2$, t, ^{3}J = 7.1 Hz)

DEAPA-CI: δ = 12.10 (broad, NH non-bonded imidazole), 9.58 (Im-CO-N<u>H</u>-CH_2-CH_2-CH_2-N(CH_2CH_3)$_2$, s), 8.13-8.08 (bonded imidazole [-N-C<u>H</u>=N-], m), 7.61-7.57 (non bonded imidazole [-N-C<u>H</u>=N-], m), 7.37-7.32 (bonded imidazole [-N-C<u>H</u>=CH-N=], m), 7.26 (chloroform), 7.02-6.97 (non bonded imidazole [-N-C<u>H</u>=C<u>H</u>-N=], m), 6.97-6.94 (bonded imidazole (-N-CH=C<u>H</u>-N=), m), 3.39 (Im-CO-NH-C<u>H</u>$_2$-CH_2-CH_2-N(CH_2CH_3)$_2$, t, ^{3}J = 6.3 Hz), 2.50 (Im-CO-NH-CH_2-CH_2-C<u>H</u>$_2$-N(CH_2CH_3)$_2$, t, ^{3}J=6.1 Hz), 2.46 (Im-CO-NH-CH_2-CH_2-CH_2-N(C<u>H</u>$_2$$CH_3$)$_2$, qua, ^{3}J = 7.2 Hz), 1.66 (Im-CO-NH-C<u>H</u>$_2$-CH_2-CH_2-N(CH_2CH_3)$_2$, qui, ^{3}J = 6.2 Hz), 0.93 (Im-CO-NH-CH_2-CH_2-CH_2-N(CH_2C<u>H</u>$_3$)$_2$, t, ^{3}J = 7.2 Hz)

DMAPA-CI: δ = 11.85 (broad, NH non-bonded imidazole), 9.56 (Im-CO-N<u>H</u>-CH_2-CH_2-CH_2-N(CH_3)$_2$, s), 8.12-8.08 (bonded imidazole [-N-C<u>H</u>=N-], m), 7.63-7.59 (non bonded imidazole [-N-C<u>H</u>=N-], m), 7.35-7.32 (bonded imidazole [-N-C<u>H</u>=CH-N=], m), 7.26 (chloroform), 7.04-7.01 (non bonded imidazole [-N-C<u>H</u>=C<u>H</u>-N=], m), 6.99-6.97 (bonded imidazole (-N-CH=C<u>H</u>-N=), m), 3.43-3.41 (Im-CO-NH-C<u>H</u>$_2$-CH_2-CH_2-N(CH_3)$_2$, m), 2.61 (Im-CO-NH-CH_2-CH_2-C<u>H</u>$_2$-N(CH_3)$_2$, t, ^{3}J=6.1 Hz), 2.21 (Im-CO-N<u>H</u>-CH_2-CH_2-CH_2-N(C<u>H</u>$_3$)$_2$, s), 1.70 (Im-CO-NH-CH_2-C<u>H</u>$_2$-CH_2-N(CH_3)$_2$, qui, ^{3}J = 6.2 Hz)

Synthesis of amine-modified poly(vinyl alcohol)s:

Poly(vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol), Poly(vinyl 3-(dimethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol) and Poly(vinyl 2-(diethylamino)ethylcarbamate-co-vinyl acetate-co-vinyl alcohol): For example: To synthesize P(6) a 250 ml round-bottomed flask with gas inlet and magnetic stirring bar was rigorously dried, filled with 10.00 g poly(vinyl alcohol) (PVA) (0.69 mmol) and 170 ml anhydrous NMP and heated to 80°C to dissolve PVA. After complete dissolution 1.00 g N-(3-(diethylamino)propyl)-1H-imidazole-1-carboxamide (4.46 mmol) was added and finally 0.06 g DMPU (10 mol% of the amine-CI) were injected. The mixture was heated to 80 °C under stirring for 4.5 days (table 1).

The resulting amine-modified poly(vinyl alcohol) was purified by ultrafiltration using an YM1 membrane (cut off 1000 g/mol, Millipore). During filtration the solvent was substituted by demineralized water. After filtration of 2.5 L water, the volume in the cell was reduced to 50 to 100 ml. The solution was frozen at -20°C and dried by lyophilisation (Edward Freeze Dryer Modulyo, standard conditions). The polymers were milled and stored until use at 40 °C in vacuum to minimize water uptake during storage. The polymers are obtained as slightly yellowish hygroscopic powders. Yields: ~ 83 %.

CHN: P(6): C 53.24 %, H 9.15 %, N 1.86 % (Theory: C 55.18 %, H 8.88 %, N 1.14 %); P(12): C 53.68 %; H 9.37 %; N 2.50 % (Theory: C 55.50 %, H 9.03 %, N 2.09 %); P(18): C 54.24 %; H 9.53 %; N 3.23 % (Theory: C 55.79%, H 9.17 %, N 2.97 %); P(33): C 55.18 %; H 9.97 %; N 5.66 % (Theory: C 56.47 %, H 9.46 %, N 4.96 %); P(68): C 56.62 %; H 9.93 %; N 7.67 % (Theory: C 57.63 %, H 9.67 %, N 7.98 %); M(69): C 54.31 %; H 9.58 %; N 8.30 % (Theory: C 55.24 %, H 9.21 %, N 8.63 %); E(70): C 55.75 %; H 9.59 %; N 7.90 % (Theory: C 56.51 %, H 9.40 %, N 8.29 %)

NMR: ^{1}H-NMR: Signals caused by PVA (and solvent): δ = 5.17-4.73 (-CH_2-C<u>H</u>($OCOCH_3$)-CH_2- and -CH_2-C<u>H</u>(OCONR)-CH_2-), 4.73-4.01 (hydroxyl groups), 3.97-3.30 (methine protons), 3.21 (broad, H_2O), 2.50 DMSO, 2.00-1.90 (acetate), 1.89 (NMP), 1.87-1.17 (methylene protons); Signals caused by amine substitution: DMAPA substitution: δ =7.05-6.75 (NH), 3.04-2.92 (-O-CO-NH-C<u>H_2</u>-CH_2-CH_2-$N(CH_3)_2$), 2.42-2.32 (-O-CO-NH-CH_2-CH_2-C<u>H_2</u>-$N(CH_3)_2$), 2.24 (-O-CO-NH-CH_2-CH_2-CH_2-$N(C\underline{H_3})_2$), 1.54-1.51 (-O-CO-NH-CH_2-<u>C</u>H_2-CH_2-$N(CH_3)_2$); DEAPA substitution: δ =7.07-6.74 (NH), 3.06-2.94 (-O-CO-NH-C<u>H_2</u>-CH_2-CH_2-$N(CH_2CH_3)_2$), 2.44 (-O-CO-NH-CH_2-CH_2-CH_2-$N(C\underline{H_2}CH_3)_2$), 2.39 (-O-CO-NH-$CH_2$-$CH_2$-C<u>$H_2$</u>-$N(CH_2CH_3)_2$), 1.52 (-O-CO-NH-$CH_2$-<u>C</u>$H_2$-$CH_2$-$N(CH_2CH_3)_2$), 0.95 (-O-CO-NH-$CH_2$-$CH_2$-$CH_2$-$N(CH_2C\underline{H_3})_2$); DEAEA substitution: δ = 6.83-6.47 (NH), 3.08-2.94 (-O-CO-NH-C<u>H_2</u>-CH_2-$N(CH_2CH_3)_2$), 2.49-2.43 (-O-CO-NH-CH_2-C<u>H_2</u>-$N(C\underline{H_2}CH_3)_2$), 0.94 (-O-CO-NH-$CH_2$-$CH_2$-$N(CH_2C\underline{H_3})_2$);

^{13}C-NMR: Signals caused by PVA: δ = 171.8 NMP, 170.3-169.2 (-O-<u>C</u>O-CH_3), , 70.0-63.0 (methine carbon), 45.7+45.4+45.0+44.5 (methylene carbon), 39.4 DMSO, 20.8 NMP, 21.0-20.4 (-O-CO-<u>C</u>H_3); Signals caused by amine substitution: DMAPA substitution: δ = 156.4 (urethane), 56.0 (PVA-CO-NH-CH_2-CH_2-<u>C</u>H_2-$N(CH_3)_2$), 44.3 ((PVA-CO-NH-CH_2-CH_2-CH_2-$N(\underline{C}H_3)_2$), 38.2 (PVA-CO-NH-<u>C</u>H_2-CH_2-CH_2-$N(CH_3)_2$), 26.6 (PVA-CO-NH-CH_2-<u>C</u>H_2-CH_2-$N(CH_3)_2$); DEAPA substitution: δ = 156.3 (urethane), 49.8 (PVA-CO-NH-CH_2-CH_2-<u>C</u>H_2-$N(CH_2CH_3)_2$), 46.1 ((PVA-CO-NH-CH_2-CH_2-CH_2-$N(\underline{C}H_2CH_3)_2$), 38.7 (PVA-CO-NH-<u>C</u>H_2-CH_2-CH_2-$N(CH_3)_2$), 26.8 (PVA-CO-NH-CH_2-<u>C</u>H_2-CH_2-$N(CH_2CH_3)_2$), 11.4 (PVA-CO-NH-CH_2-CH_2-CH_2-$N(CH_2\underline{C}H_3)_2$); DEAEA substitution: δ = 156.4 (urethane), 51.8 (PVA-CO-NH-CH_2-<u>C</u>H_2-$N(CH_2CH_3)_2$), 46.5 ((PVA-CO-NH-CH_2-CH_2-$N(\underline{C}H_2CH_3)_2$), 38.5 (PVA-CO-NH-<u>C</u>H_2-CH_2-$N(CH_2CH_3)_2$), 11.6 (PVA-CO-NH-CH_2-<u>C</u>H_2-CH_2-$N(CH_2\underline{C}H_3)_2$);

Table 1. Backbone polymers: synthesis and molar mass

Polymer	feeding [a]	M_n [b] g/mol	M_n [c] g/mol	M_w [c] g/mol	M_n/M_w [c]
PVAL	-	14730	11400	13960	1.2
P(6)	10.00/ 1.00/ 0.06/ *4.5*	15460	11690	14210	1.2
P(12)	10.00/ 2.01/ 0.11/ *4.5*	16100	12390	14690	1.2
P(18)	10.00/ 4.02/ 0.23/ *4*	16690	11920	13980	1.2
P(33)	10.00/ 10.16/ 0.57/ *4*	18410	16130	19770	1.2
P(68)	10.00/ 23.12/ 1.32/ *4*	23910	33590	67790	2.0
M(7)	12.00/ 1.20/ 0.07/ *4*	15320	9895	11710	1.2
M(13)	12.00/ 2.39/ 0.17/ *4*	15890	12070	14460	1.2
M(21)	12.00/ 4.81/ 0.31/ *4*	16750	11770	13690	1.2
M(32)	12.00/ 11.99/ 0.79/ *4*	17880	12170	14410	1.2
M(69)	11.00/ 23.09/ 1.51/ *4*	22420	15590	20060	1.3
E(6)	12.00/ 1.28/ 0.07/ *4*	15360	11050	12640	1.1
E(12)	12.00/ 5.13/ 0.16/ *4*	15980	11010	12900	1.2
E(20)	12.00/ 5.13/ 0.31/ *3*	16920	15430	17380	1.1
E(33)	12.00/ 12.85/ 0.79/ *3*	18370	14560	17150	1.2
E(70)	10.00/ 22.48/ 1.37/ *4*	23630	29580	32780	1.1

(a) Feeding (mass/grams): m(PVA) / m(Amine-CI) / m(DMPU) / *days of reaction*

(b) Calculated by 1H NMR depending on the degree of polymerization of PVA (P=300)

(c) Measured by GPC-MALLS

Grafting of poly(vinyl alcohol) with lactide and glycolide

(Synthesis of Poly(vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(lactide-co-glycolide), Poly(vinyl 3-(dimethylamino) propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(lactide-co-glycolide) and Poly(vinyl 2-(diethylamino)ethylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(lactide-co-glycolide):

In a glove box under anhydrous conditions modified poly(vinyl alcohol) was very carefully mixed with D,L-lactide and glycolide (lactide/glycolide = 1:1) in different molar ratios (1:1, 1:2, 1:10 and 1:20, this is the molar ratio between the average of free hydroxyl groups in the PVA (one) and acid molecules (lactic and glycolic acid)(1, 2, 10, 20)in the mixture). After that tin(II) 2-ethylhexanoate (1:1, 1:2: 12 % (w/w) 1:10,1:20 22 %(w/w) mass(PVA)), $SnOct_2$.(table 2) was added and mixed with the powder until a slight change in consistence appears. The mixture was transferred into a nitrogen filled, rigorously dried round bottomed flask with gas inlet. For 3 hours of bulk polymerization at 150 °C the filled flask was directly put into a preheated oil bath. After this time period the reaction was finished and the flask was immediately cooled down to 20 °C.[39]

Batches of polyesters with short side chains were solved in methanol, water, dimethylsulfoxide (DMSO) or 5% acetic acid and then precipitated in ether, isopropanol, THF or were ultra-filtrated like in the PVA in step two (yields ~59%). The polymer approaches for polyesters with long side chains were dissolved in acetone or DMSO and purified by precipitation into a mixture of isopropanol/water, isopropanol or pure water (yields ~80%). The isolated colorless to yellow polymers were dried at 20 °C in vacuum.

CHN: E(33)-1 C 47.84%, H 7.19 %, N 2.57 %; E(33)-2 C 48.11%, H 6.96 %, N 2.22; E(33)-10 C 46.29 %, H 5.19 %, N 1.07 %, E(33)-20 C 45.91 %, H 5.39 %, 1.10 %, P(33)-10 C 46.37 %, H 5.57 %, N 1.33 %, P(33)-20 C 46.22 %, H 5.01 %, N 1.00 %, M(32)-10 C 45.89 %, H 5.36 %, N 1.19 %;

^{1}H-NMR: *Signals caused by: PVA:* δ = 5.56-5.31, 4.60-4.30, 4.00 -3.37, 2.03-1.90 (acetate), 1.89-1.17; *PLGA:* δ = 5.35 (OH-end group), 5.30-5.02 (lactide central CH-groups), 4.96-4.75 (glycolide central CH_2-groups), 4.28-4.16 (lactide CH-end group), 4.15-4.04 (glycolide CH_2- end group), 1.54-1.42 (lactide central CH_3-groups), 1.33-1.27(lactide CH_3 end group); *Solvent*: δ =3.21 H2O, 2.50 DMSO; *DMAPA substitution (range):* δ = 7.18-6.86 (NH), 3.11-2.95, 2.95-2.81, 2.73-2.26; *DEAPA substitution (range):* δ = 7.18-6.69 (NH), 3.10-2.92, 2.92-2.45, 1.15-0.94 ; *DEAEA substitution (range):* δ = 7.10-6.66 (NH), 3.18-2.97, 2.79-2.52, 1.07-0.91

^{13}C-NMR: *Signals caused by: PVA:* δ = 69.8-67.0, 64.0-62.7, 46.4-43.8, 42.3-40.8, 20.6 (acetate) *PLGA:* δ = 173.7 (LA–CO-O- end group), 171.7 (GA–CO-O- end group), 169.4 (LA–CO-O- group next to PVA) and 166.9-166.8 (GA–CO-O- group next to PVA), 168.9-168.6 (LA–CO-O- central groups), 166.5-166.1 (GA–CO-O- central groups), 68.8-68.1 (LA-CH central groups), 67.6 (LA–CH group next to PVA), 65.9-65.2 (LA-CH end group), 59.6-59.0, 20.1 (LA-$\underline{C}H_3$ end group), 16.6-16.0 (LA-$\underline{C}H_3$ central groups); *Solvent:* δ =39.4 (DMSO); *DMAPA substitution:* δ = 54.8, 42.5, 37.3, 24.5; *DEAPA substitution:* δ =155.4 (visible with 60 000 scans), 49.2-49.0, 46.2-46.1, 37.7, 24.7-24.0, 9.7-9.4; *DEAEA substitution:* δ = 51.5,46.4, 38.2, 11.3

Table 2. *Synthesized polyesters: Molar mass, Tg, side chain length, amine substitution*

Polyester	feeding[a]	M_n[b] (kg mol^{-1})	M_n[c] (kg mol^{-1})	M_w[c] (kg mol^{-1})	M_w/M_n[c]	T_g[d] (°C)	Side chain[e]	Amine[f]
DEAPA(6)-PVA_{300}-g-PLGA(1)	2.00/1.18/0.95/0.24	49.0	55.5	104.3	1.88	43.9	(1.9)	2.1
P(6)-2	1.30/1.54/1.24/0.16	64.0	96.0	194.0	2.02	36.6	(3.1)	2.1
P(6)-10	0.39/2.37/1.91/0.09	207.6	211.3	281.1	1.33	30.6	11.2	2.1
P(6)-20	0.20/2.37/1.91/0.04	335.8	249.5	335.8	1.35	32.3	18.7	2.1
P(12)-1	2.00/1.09/0.88/0.25	50.8	77.7	171.2	2.20	44.3	(2.0)	4.0
P(12)-2	1.30/1.42/1.14/0.16	66.8	105.3	248.4	2.36	36.7	(2.9)	4.0
P(12)-10	0.40/2.19/1.76/0.09	201.4	195.8	262.6	1.34	30.8	10.8	4.0
P(12)-20	0.20/2.19/1.76/0.04	348.2	227.2	304.1	1.34	33.0	19.3	4.0
P(33)-1	2.00/0.84/0.68/0.24	46.1	1143.0	1498.0	1.31	44.2	(1.5)	10.9
P(33)-2	1.30/1.09/0.88/0.16	56.9	94.7	439.5	4.64	45.3	(2.2)	10.9
P(33)-10	0.50/2.10/1.69/0.11	181.2	194.6	366.9	1.89	27.7	9.4	10.9
P(33)-20	0.25/2.10/1.69/0.06	315.0	374.9	711.9	1.90	32.8	17.2	10.9
P(68)-1	3.00/0.83/0.67/0.20	n.d.	108.8	392.8	3.61	42.5	n.d.	22.7
P(68)-2	2.50/1.39/1.12/0.31	n.d.	397.8	467.7	1.16	31.4	n.d.	22.7
P(68)-10	0.75/2.09/1.69/0.16	134.5	282.1	798.5	2.83	11.5	7.4	22.7
P(68)-20	0.50/2.78/2.24/0.11	236.2	469.7	1203.0	2.56	26.1	14.1	22.7
DEAEA(6)-PVA_{300}-g-PLGA(1)	2.00/1.23/0.99/0.25	47.7	149.8	475.1	3.17	n.d.	(1.9)	2.0
E(6)-2	1.30/1.61/1.29/0.16	n.d.	170.4	638.9	3.75	n.d.	n.d.	2.0
E(6)-10	0.60/3.70/2.98/0.14	206.5	130.5	208.7	1.60	22.8	11.1	2.0
E(6)-20	0.30/3.70/2.98/0.11	341.6	233.6	325.3	1.39	25.3	19.1	2.0
DEAEA(12)-PVA_{300}-g-PLLGA(1)	2.00/1.18/0.95/0.24	n.d.	121.1	447.0	3.69	34.5	n.d.	3.9
E(12)-2_{LLG}	1.30/1.54/1.24/0.16	68.7	110.6	307.1	2.78	32.8	(3.1)	3.9
E(12)-10_{LLG}	0.60/3.55/2.86/0.13	225.6	143.2	240.0	1.68	29.9	12.3	3.9
E(12)-20_{LLG}	0.30/3.55/2.86/0.07	378.9	252.6	350.3	1.39	32.7	21.3	3.9
DEAEA(33)-PVA_{300}-g-PLGA(1)	2.00/1.00/0.81/0.24	n.d.	57.9	76.8	1.33	n.d.	n.d.	10.8
E(33)-2	1.30/1.30/1.05/0.16	50.6	123.4	496.2	4.02	33.0	(1.9)	10.8
E(33)-10	0.60/3.00/2.42/0.14	203.1	393.0	1199.0	3.05	24.0	11.1	10.8
E(33)-20	0.30/3.00/2.42/0.30	358.0	415.1	767.0	1.85	21.7	20.5	10.8
E(70)-1	2.00/0.67/0.54/0.19	51.0	49.5	86.9	1.76	n.d.	(1.9)	23.3
E(70)-2	1.30/0.87/0.70/0.16	57.7	56.7	119.3	2.12	n.d.	(2.4)	23.3
E(70)-10	0.60/2.00/1.61/0.16	165.2	651.3	2836.0	4.35	32.1	9.9	23.3
E(70)-20)	0.30/2.00/1.61/0.07	265.0	749.7	3299.0	4.40	20.9	17.0	23.3

Polyester	feeding[a]	M_n[b] (kg mol^{-1})	M_n[c] (kg mol^{-1})	M_w[c] (kg mol^{-1})	M_w/M_n[c]	T_g[d] (°C)	Side chain[e]	Amine[f]
DMAPA(7)-PVA_{300}-g-PLGA(1)	2.00/1.24/1.00/0.25	n.d.	195.2	394.9	2.02	n.d.	n.d.	2.3
M(7)-2	1.30/1.61/1.30/0.16	69.8	88.9	264.3	2.97	29.8	(3.1)	2.3
M(7)-10	0.60/3.72/3.00/0.13	227.6	177.0	247.6	1.40	27.8	12.4	2.3
M(7)-20	0.30/3.72/3.00/0.08	374.7	231.5	301.8	1.30	31.1	20.9	2.3
M(13)-1	2.00/1.19/0.96/0.25	54.5	65.8	160.4	2.44	39.1.	2.2	4.4
M(13)-2	1.30/1.55/1.25/0.16	67.0	68.6	138.2	2.01	29.5	3.0	4.4
M(13)-10	0.60/3.58/2.89/0.13	228.0	325.0	631.7	1.94	23.6	12.4	4.4
M(13)-20	0.30/3.58/2.89/0.07	386.3	340.2	589.4	1.73	25.7	21.6	4.4
M(32)-1	2.00/1.03/0.83/0.25	n.d.	63.2	87.4	1.38	n.d.	n.d.	10.8
M(32)-2	2.00/1.34/1.08/0.17	63.0	77.1	234.7	3.04	n.d.	(2.7)	10.8
M(32)-10	0.60/3.09/2.48/0.15	184.8	359.4	953.7	2.65	18.5	10.0	10.8
M(32)-20	0.30/3.09/2.49/0.09	333.2	770.7	1932.0	2.51	20.7	19.0	10.8
M(69)-1	2.00/0.72/0.58/0.17	n.d.	63.6	118.2	1.86	n.d.	n.d.	23.0
M(69)-2	1.30/0.93/0.75/0.18	n.d.	57.4	74.7	1.30	n.d.	n.d	23.0
M(69)-10	0.60/2.15/1.73/0.13	126.8	131.8	154.7	1.17	19.9	7.2	23.0
M(69)-20	0.30/2.15/1.73/0.09	303.2	647.7	2267.0	3.50	32.4	19.3	23.0
PVA_{300}-g-PLGA(1)	10.00/6.46/5.20/1.22	50.5	22.2	46.8	2.11	36.0	(2.1)	0.0
PVA-2	5.00/6.46/5.20/0.61	70.3	34.1	67.0	1.97	32.1	(3.2)	0.0
PVA-10	1.50/9.69/7.80/0.34	236.1	116.4	156.2	1.34	23.2	12.9	0.0
PVA-20	0.75/9.69/7.80/0.18	388.1	175.1	239.9	1.37	26.0	21.7	0.0

(a) Feeding (mass/grams): m(modified PVA) / m(lactide) / m(glycolide) / m($SnOct_2$)

(b) Maximal possible M_n calculated from backbone substitution and side chain length calculated through out ^{1}H NMR data

(c) M_n , M_w and polydispersity measured by GPC-MALLS

(d) Glass transition temperature T_g of the second run, method: heating and cooling -10 to 200°C, 10°/min,

(e) average side chain length calculated from ^{1}H NMR data, short SCL: in brackets, because small non clear signals used, if not values proofed by NNE

(f) amine substitution of the backbone

2.3.2 Nomenclature

The synthesized polyesters consist out of four different components. The source-based IUPAC nomenclature for these polymers lead to the designation: Poly(vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(DL-lactide-co-glycolide), Poly(vinyl 3-(dimethylamino) propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(DL-lactide-co-glycolide) and Poly(vinyl 2-(diethylamino)ethylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(DL-lactide-co-glycolide). As abbreviation we propose a system based on proton NMR of the backbone and the side chain length calculated from feeding. For the amine the abbreviations DEAPA (3-diethylaminopropylamine), DEAEA (2-diethylaminoethylamin) and DMAPA (3-dimethylaminopropylamine) are used, followed by the average of amine carrying monomer units of the PVA backbone chain for example: DEAPA(6)-PVA_{300}-g-PLGA(10). One third of the average of amine carrying monomers of the backbone is the same like the degree of amine substitution (DS) of the PVA (figure 1, step A). To further simplify the abbreviation one letter designates the amine substitution, P for DEAPA, M for DMAPA and E for DEAEA substitution, followed by the two numbers in brackets are used, for example P(6)-10, M(7)-2 or E(6)-20. In cases without amine substitution PVA is used instead the letter. If the polyester compositions differs from D,L-lactide and glycolide in 1:1 ratio this will be symbolized by a downshifted letter combination, for example E(12)-10_{LLG}. LLG means L-lactide and glycolide in 1:1 ratio, L means pure D,L-lactide , LL pure L-Lactide and so on. Changes in the ratio could also be added like M(x)-y_{LG75} for a ratio D,L-LA: GA of 75:25. The downshifted number always symbolizes the amount of the first component.

2.3.3 Sample Characterization.

^{1}H and ^{13}C NMR spectroscopic data was collected using a JEOL Eclipse+ 500 and a Joel GX 400 D at a frequency of 500 respective 400 MHz for ^{1}H NMR and 126 respective 101 MHz for ^{13}C NMR at 50 °C in d_6-DMSO (euriso-top, <0.02%

HDO+D2O). 40 to 50 mg sample was used for each measurement. ^{1}H NMR was performed with 64 scans. The ^{13}C NMR was performed with 4096 and 60 000 scans.

The amine substitution (AS) was evaluated by calculating the ratio between the integral of the CH_3-end group of the amine and the integral of the methylene protons of PVA on the basis of the degree of polymerization. $(100/(I_{PVA}/2))*(I_{Amine}/3) = DS$; $DS*3 = AS$

The side chain length (SCL) was calculated using the integrals of the lactide and glycolide end groups (I_{end}) and there central groups (I_{cent}) and adding one for the end group. $[(I_{cent\_LA\_CH} + (I_{cent\_GA}) / 2) / (I_{end\_LA\_CH} + (I_{end\_GA} / 2)] + 1 = SCL$ or $[((I_{cent\_LA\_CH3}/3) + (I_{cent\_GA}) / 2) / ((I_{end\_LA\_CH3}/3)+(Iend\_GA)/2)] + 1 = SCL$

Fourier-Transform- Infrared spectroscopy (FT-IR) was performed on a Nicolet 510P FTIR spectrometer (range between 4000 and 400 cm^{-1}, resolution: 2 cm^{-1}). All powder samples were compressed into KBr pellets.

CHN-analysis was performed with a Hewlett-Packard autoanalyser 185.

Differential scanning calorimetry (DSC) was performed on a Perkin-Elmer DSC7 calibrated against indium and Gallium. Using ca. 5 mg polymer the sample was scanned between -10 and 200 °C at a heating rate of 10 °C/min. Glass transition temperatures were calculated from the second run.

Thermo gravimetric analysis: Ca. 5 mg polymer was used for thermo-gravimetric analysis in a TGA7 (Perkin Elmer) (under nitrogen, heating rate of 20 °C/min, run 30 to 600 °C).

Gelpermeation chromatography in combination with a multi-angle-laser-light scattering detector (GPC-MALLS) was employed for the determination of absolute molecular weights and weight distributions using a Duratec DDG-75 degasser, a Merck-Hitachi L-6000 pump and an AS-2000A autosampler, a Merck T-6300 Column Thermostat, a Wyatt DAWN Eos multiangle-laser-light-scattering detector (normalized with PSS (Polymer Standard Service, Mainz) PMMA 200k standard). The separation was performed using an Optilab DSP together with a PSS SDV linear M (8x300, 5µ) column with a pre-column of the same type (8x50, 5µ). The

measurements were performed at 60 °C column temperature at a flow rate of 0.5 mL/min using dimethylacetamide (DMAc) and 2.5 g lithium bromide /L (LiBr) as eluent. The molecular weights of the samples were determined using the Wyatt software Astra V4.73. To calculate the molecular weights total mass recovery was used (third order fit).

Degradation study. For these investigations a combination of two columns, a Lichrogel PS 40 and a Lichrogel PSMix (each 8x300, 10 µ, Merck Hibar PrePacked Column), and dichloromethane with an addition of 0.3% triethylamine as eluent was used. The other components of the GPC arrangement stayed the same only the Optilab DSP was substituted against a Differential Refractometer Ri-71.

Wide-angle-X-ray diffraction WAXD were performed to determine the crystallinity of 12 samples (PVA, P(6), E(6), M(7), M(32), P(6)-2, P(12)-10, E(70)-10, M(7)-10, M(7)-20, PVA-10, PVA-20). Cu-K_α radiation generated by a Siemens D5000 was used for X-Ray diffraction analysis (WAXD) of powdered polyester samples. The analysis of the X-ray diagram leads to information about crystallinity.

Transmission electron microscopy (TEM). For TEM studies thin films were prepared by spreading a droplet of the polymer solution onto a water surface. Small parts of the films thus formed were transferred to holey carbon coated copper grids and dried in vacuum for three days. Afterwards the dried films were stained within three days using an aqueous solution of 2% osmium tetroxide. A Jeol high-resolution transmission electron microscope JEM 3010 with LaB6- cathode and integrated 2k x 2k CCD-camera was used to study the films at an acceleration voltage of 300kV and low radiation dose.

Degradation studies of polymer film samples. Polymer films were cast from a 5% (w/v) solution in dichloromethane using Teflon™ moulds. After 72 h of drying at a temperature of 4°C the samples were recovered and discs with a diameter of 17 mm were punched from the polymer films in a semi-dry state using a cork bore. Residual

solvents were then removed in vacuo at room temperature until constant weights were obtained.

To determine the in-vitro degradation profiles, weighed film samples (ca. 30 mg, n=3) were placed in 10 ml of phosphate buffered saline (PBS, ph 7.4, 0.15 M) and kept at 37°C in an incubator. Glasses were agitated carefully once a day. After 2, 7, 14 and 21 d, samples were recovered, blotted dry with Kimwipes™ and wet weight was measured gravimetrically. Wet samples were then frozen at -80 °C, freeze-dried in vacuo for 72 hours and dried in vacuo at room temperature until mass constancy. Polymer mass loss was calculated from the following formula: Mass loss (%) = 100 – (mass (dry) x 100/ original mass).

2.4 Results and Discussion

Synthesis of amine modified PVA-g-PLGAs. Recently we reported the synthesis of negatively charged, sulfobutylated poly(vinyl alcohol)-graft- polyester for the special delivery of cationic drugs.[39] In this work we discuss the synthesis of oppositely charged amphiphilic polyesters. The idea was to create a modular system consisting of three different, freely combinable components to create a system that allows the synthesis of tailor-made polyesters. The main target of this study was to establish a relationship between polymer structure and polymer functional properties, such as solubility and polymer degradation rate. To this end a total of 52 different derivatives were synthesized. These biodegradable polyesters should be completely eliminated from the body after a predetermined period of time. Elimination can occur via metabolism or excretion of water-soluble breakdown products with molecular weights < 30 000 g/mol via urinary excretion.[32] Therefore, we selected poly(vinyl alcohol) (PVA) with a molar mass of 14 700 g/mol (M_n) (P=300, degree of hydrolysis 87-89%) as backbone. In a two step process diamines were coupled to this polymer. To avoid cross-linking, we used one-side protected, commercially available diamines N^1,N^1-diethylpropane-1,3-diamine, N^1,N^1-dimethylpropane-1,3-diamine and N^1,N^1-

diethylethane-1,2-diamine activated with carbonyldiimidazole (CDI) (scheme 1).[41] The amines were injected into solution/suspension of CDI in THF to prevent double reaction. The resulting amine-carbonylimidazole (amine-CI) is less reactive than CDI. No further purification of the resulting amine-CI / imidazole mixture was carried out and ^{1}H NMR spectroscopy was used to determine the portion of amine-CI in the mixture. Based on these calculations amine modified PVAs with 2, 4, 11 and 23 % amine substitution were synthesized (table 1). To achieve higher degrees of amine substitution it is necessary to use an excess of amine-CI (table 1). The resulting amine-modified poly(vinyl alcohol)s are water soluble. Three different amines were used to evaluate the influence of the charge - backbone distance and the accessibility of the amine function onto the different polymer properties. The postulated structure of the amine-modified PVAs was in accordance with NMR and FT-IR measurements (figure 2).

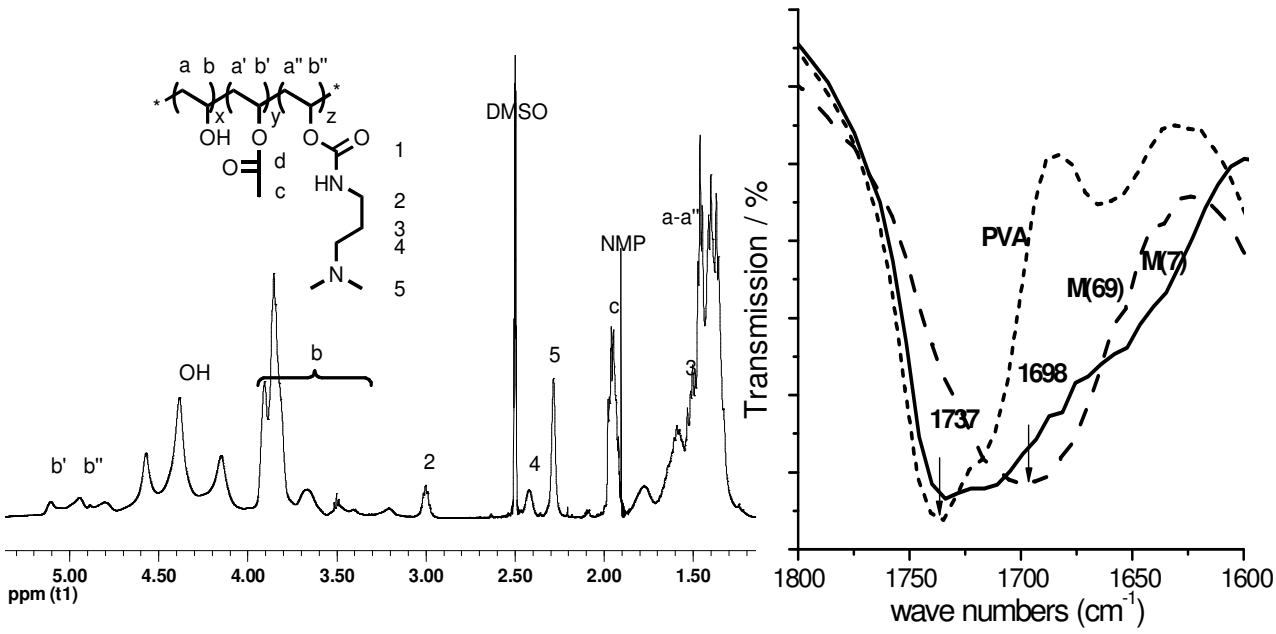


Figure 2. *left: ^{1}H-NMR of the poly(vinyl alcohol) M(7); right: FT-IR spectra: Carbonyl band region of M(7) and M(69) compared to PVA*

Both ^{1}H and ^{13}C NMR spectroscopy show the signals of PVA and amine substitution. The intensity of the amine signals increases with rising degree of amine substitution in the PVA backbone. Due to ultra filtration step this presents clear evidence for the

successful modification of the polyol backbone. Also FT-IR spectroscopy supports this assumption. The strong band of the carbonyl function at wave numbers of 1737 cm^{-1} caused by the ester carbonyl function of acetate slowly disappears with higher amine substitution and is replaced by the band of the urethane carbonyl bond (1698 cm^{-1}) (figure 2). This is in accordance to NMR measurements showing the disappearance of acetate signals with increasing amine substitution. In TGA measurements the different amine substitution of the polyols is clearly illustrated by the corresponding degradation step in the range of 250 and 350 °C. The mass loss in this area is in very good agreement with the degree of amine substitution (table 3).[42]

Table 3: *TGA measurements degradation caused by amine substitution*

Polymer	Amine substitution[a]	Mass loss[b] / %	Calculated mass loss[c] / %
E(6)	2.0	5.5	7.3
E(12)	3.9	10.6	13.6
E(20)	6.7	21.5	22.1
E(33)	10.9	29.9	33.0
E(70)	23.3	54.8	55.1
M(7)	2.3	4.8	7.6
M(13)	4.4	11.8	14.1
M(21)	7.1	20.0	21.9
M(32)	10.8	36.9	31.1
M(69)	23.0	60.7	53.1
P(6)	2.1	9.0	8.2
P(12)	4.0	12.8	14.9
P(18)	5.9	38.2	21.2
P(33)	10.9	40.5	35.5
P(68)	22.7	58.5	57.0

(a) degree of amine substitution / % calculated form ^{1}H NMR measurements

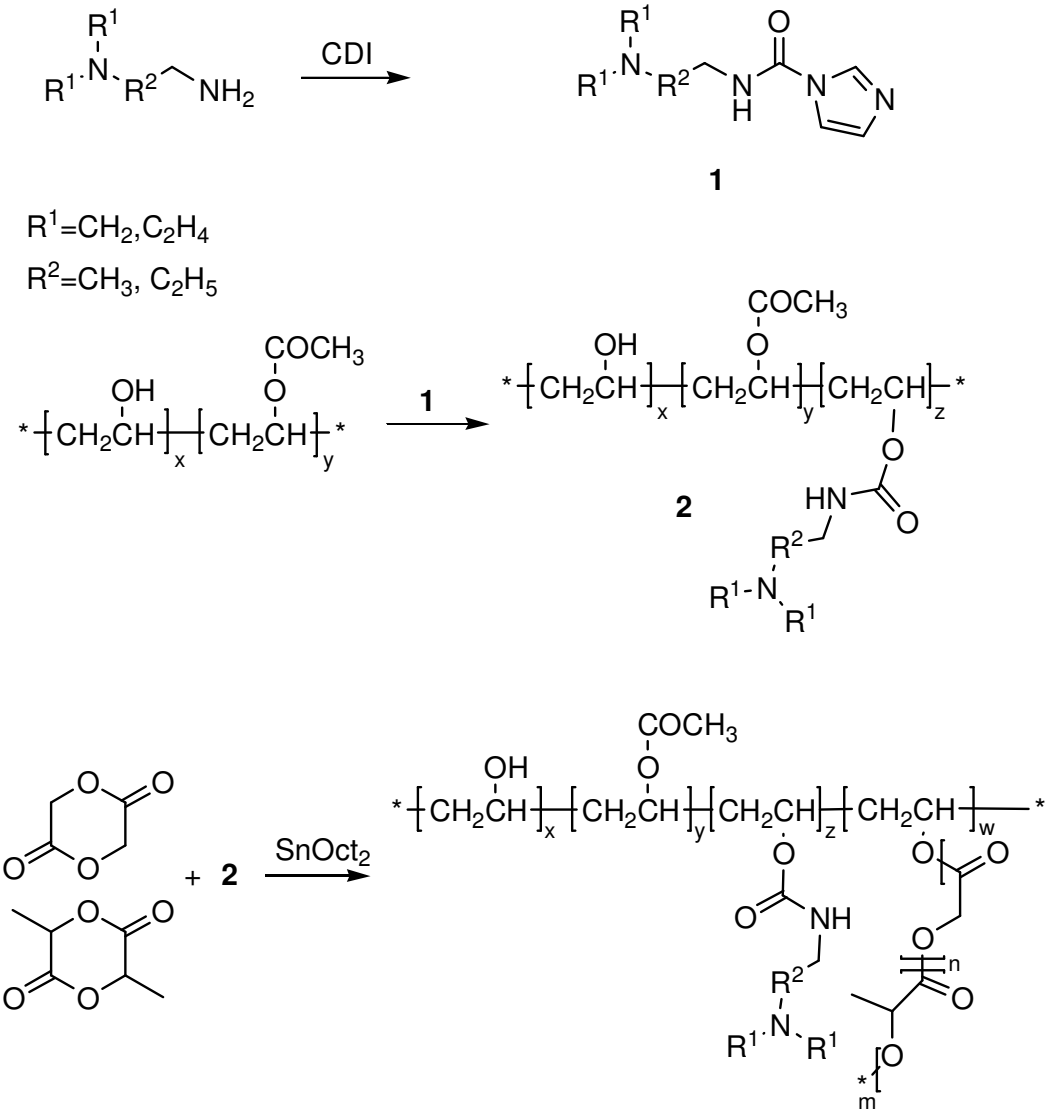
(b) Mass loss during the first degradation step

(c) mass loss calculated from NMR data

Moreover, CHN-analyses fit with theoretical values. These results are in good agreement with NMR and TGA data. GPC measurements demonstrate a monomodal distribution of molecular weights in good correspondence to values calculated from NMR measurements (table 1).

To investigate the solubility properties two different polymer types were created: The more hydrophilic polyesters with short side chains (1:1 and 1:2) (Type I) and the more lipophilic type with longer side chains (1:10 and 1:20) (Type II) (scheme 1, figure 1). These combinations finally result in 52 (48 charged and 4 uncharged) polyesters representing the possible variations of polymer properties shown by this polymer class.

Scheme 1. *Three step process of the polyester synthesis*



Bulk polymerization was used to synthesize the polyesters. In all cases a yellow, amber or dark red colored solidified melt was obtained. All polyesters of type II show solubility in acetone. E(70)-10 and P(69)-10 mainly showed with small amounts of solvent only swelling but could be completely dissolved in DMSO. The polyester solutions partly show turbidity. Apart from P(68)-10 and M(69)-10 they were precipitated in alcohol/water mixtures or in water to remove unreacted PVA. In case of P(68)-10 and M(69)-10 isopropanol was used because water and alcohol/water mixtures did not demonstrate adequate precipitation characteristics. These findings demonstrate that the solubility shifts with increasing amine substitution of the backbone. Compared to the polymers of type II the type I polyesters showed totally different solution behavior. Apart from the two neutral polyesters they were not soluble in acetone but could be solved in methanol, water, acetic acid or in a 1:1 mixture of DMSO and water. Precipitation with isopropanol or more non-polar solvents like acetone, THF or diethyl ether were done. Especially the polyesters with short side chains and high amine substitutions (P(68)-1, P(68)-2, M(32)-1, M(32)-2, M(69)-1, M(69)-2, E(33)-1, E(70)-1 and E(70)-2) showed problematic solution behavior. The solution profiles are summarized in figure 3. The polyesters could be classified into four categories: (I) short side chain length (SCL) and high amine substitution (AS), short SCL and low AS (II), long SCL and high AS (III) and finally long SCL and low AS (IV). Figure 3 demonstrates the solubility within these groups depending on the polarity of the solvent. Polymers of group I are water soluble and do not show solubility in less polar solvents like methanol or acetone. Within group II the greatest change in solubility takes place. It shifts from partly water and methanol soluble polymers to partly methanol and acetone soluble ones. Within group III and IV only acetone solubility is shown.

The PLGA grafted polyesters were studied by NMR spectroscopy. Signals of the backbones and the lactide/glycolide side chains could be identified in ^{1}H and ^{13}C NMR spectra (figure 4).[12,29] Using carbon NMR and CHN analysis it could be demonstrated that the amine is still bound to the PVA backbone after bulk polymerization. In comparison to the corresponding PVA backbone the signals of protons bound to the amine group and especially the signal of the protons in direct

neighborhood to the amino function shift down field suggesting an ionization of the amino group within the polyesters. This ionization could be caused by free lactic and glycolic acid molecules during synthesis or purification. Figure 4a shows the signal of the urethane carbonyl carbon at 155.4 ppm. This signal demonstrates that the link between the PVA backbone and amine still remains intact.

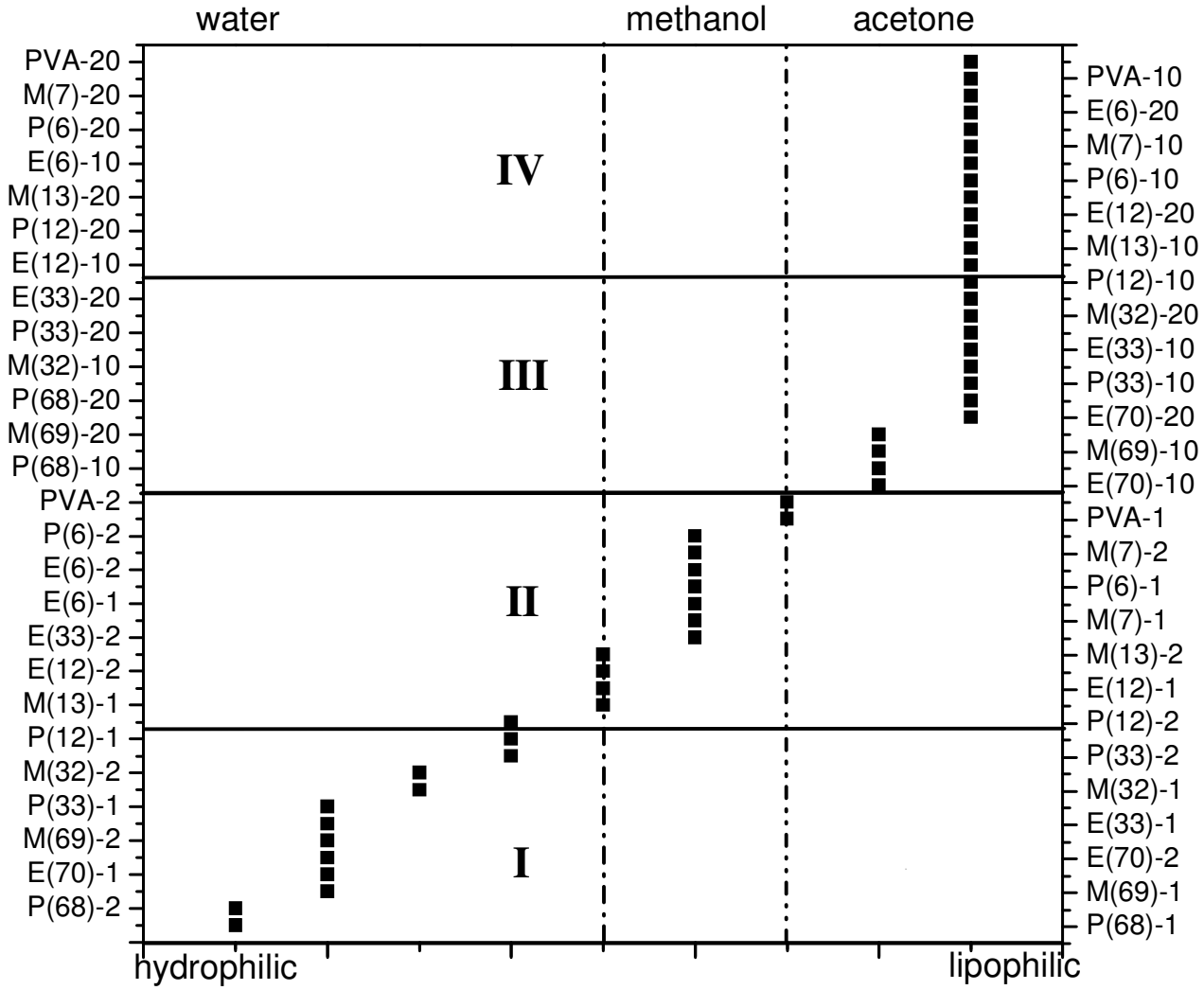


Figure3. *solubility of the polyesters in dependence of side chain length and amine substitution*

Using NMR spectroscopy the change of the polyester side chain could be observed (figure 4b). The signals of the methylene, methine and methyl protons of inner glycolic and lactic acid units increased within polymers of the same backbone. Figure 4b show a type I and II polyester compared to the corresponding backbone and linear polyester. It could be clearly demonstrated that both graft polyesters are showing

signals of backbone (between 5.00 - 3.50 ppm methine protons and OH of PVA), 2.90-2.45 ppm (amine (solvent at 2.50 ppm)), 2.00 – 1.30 ppm (acetate and methylene protons of PVA)) and side chains (between 5.3-5.1 ppm (LA $_{\text{central groups CH}}$), 4.94-4.75 ppm (GA $_{\text{central groups}}$), 4.29-4.18 ppm (LA $_{\text{end group CH}}$), 4.15-4.06 ppm (GA $_{\text{end group}}$), 1.53-1.40 ppm (LA $_{\text{central groups CH3}}$), 1.36-1.28 ppm (LA $_{\text{end group CH3}}$).

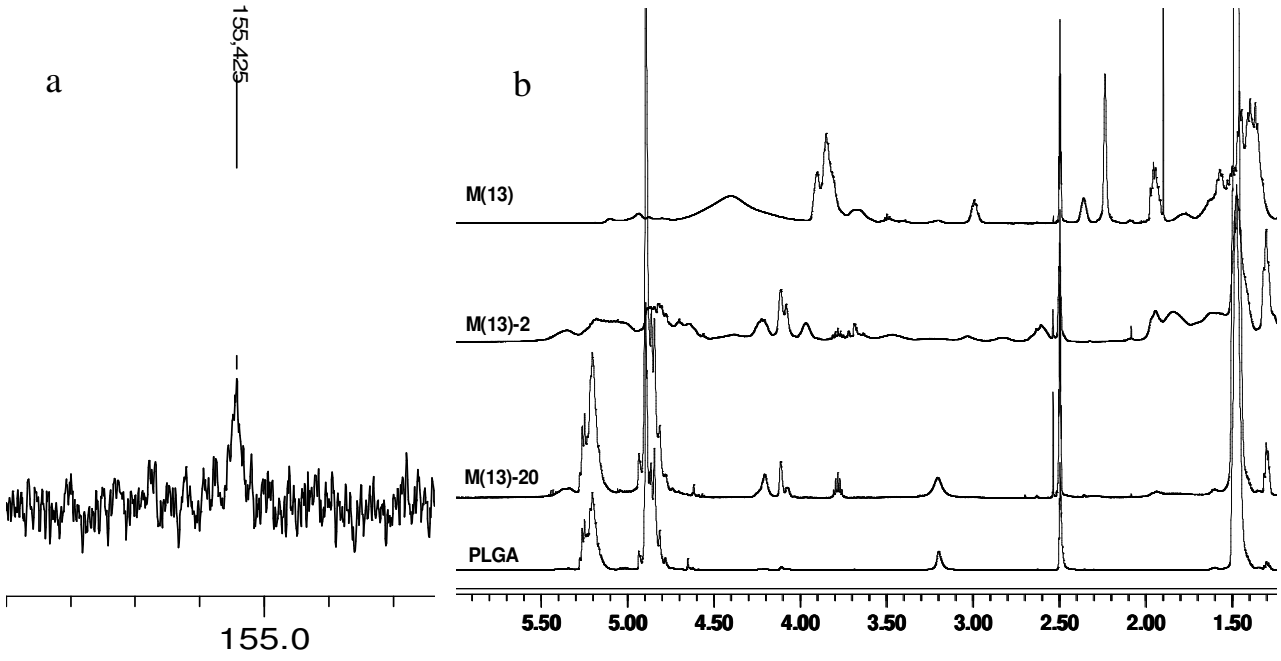


Figure 4. *a. ^{13}C NMR of P(68)-10, urethane carbonyl signal; b. Comparison of ^{1}H NMR spectra of M(13)-2 and M(13).20 to their backbone M(13) and a commercial available linear PLGA.*

The relative short side chains allow the calculation of the average chain length because both the central acid units and also the corresponding end groups are recognizable by ^{1}H NMR (table 2). These calculations show that the NMR measurements yield compositions close to those calculated from the feed ratio (y value used in nomenclature). Especially the type II polyesters demonstrate good correspondence between measured and calculated values. The average of the SCL in 1:1 polymers is ca. 2 and that of 1:2 ca. 3 demonstrating that nearly one respective two cyclic ester rings are opened in the ring opening polymerization (ROMP) and

attached to the backbone. Only the polyesters M(69)-10 and P(68)-10 show chain lengths close to 7. This may be caused by an inhibition effect on the ring opening polymerization or steric effects caused by the amine groups. Also a spacer length of three methylene groups appears to have a higher effect than only two groups in E(70)-10. In case of competition between tertiary amino functions and lactide/glycolide during binding to the catalytic center of tin, the propyl amine groups seem to have a higher affinity.[43] In agreement with van der Velden et al. the signals of the PVA methine protons in direct vicinity to free hydroxyl groups resonate in the area between δ = 4.00 and 3.33 ppm.[44] In this region no other chemical shift of the polymer could be observed. Four different chain lengths are calculated from the feed (Step B in figure 1: lengths of 1, 2, 10 and 20 monomers). Due to these values and based on the degree of polymerization of PVA(P=300) the following composition could be calculated based on ^{1}H- NMR spectroscopy: Chain length one: P[$VACB_{0\text{-}70}$-$VAc_{0\text{-}36}$-$VA_{165\text{-}195}$-$VPLGA_{75\text{-}105}$] , chain length two: P[$VACB_{0\text{-}70}$-$VAc_{0\text{-}36}$-$VA_{120\text{-}150}$-$VPLGA_{105\text{-}135}$], chain length ten: P[$VACB_{0\text{-}70}$-$VAc_{0\text{-}36}$-$VA_{30\text{-}90}$-$VPLGA_{180\text{-}225}$] and chain length twenty: P[$VACB_{0\text{-}70}$-$VAc_{0\text{-}36}$-$VA_{15\text{-}105}$-$VPLGA_{150\text{-}240}$]. In type I polymers 25-35% for 1:1 and 35-45 % for 1:2 of the monomer units within the backbone carry a PLGA side chain. This means that a substantial number of free hydroxyl groups (40-65%) contribute to the more hydrophilic nature of these polymers. In type II polyesters 50 to 80 % of the backbone monomer units are carrying a polyester side chain. Due to this only 5 to 35 % free hydroxyl groups are still remaining in the backbone.

Using these measured chain length and the known structural composition of the backbone a number average of the molar mass could be calculated. Results of GPC-MALLS show rough agreement with these calculations. Also the order of molecular weights corresponds to expectations from feed composition. The polyesters show monomodal distributions, but mainly the type II polyesters contain a low molecular weight part which could be observed using the refractive index (RI) detector but not via light scattering (LS). The concentration of these oligomeric substances increases with higher degrees of amine substitution in the backbone. This finding may be a result of fast degradation caused by amine substitution. In most cases the

polydispersity is smaller than that mentioned in literature for linear PLGAs initiated with tin octoate (Mw/Mn ≈ 2.3 -2.4).[45]

Apart from molecular weight characterization, GPC-MALLS could also be used to obtain information about the molecular conformation of the polymers. By plotting the r.m.s. (root mean square) radii of gyration as a function of molar mass in a double logarithmic way (figure 5) the gross molecular conformation can be investigated according to

$$\log r_i = k + a \log M_i \qquad (1)$$

Where, r_i is a radius of gyration, k the interception on the y axis, a the slope and M_i a molar mass.[46,47] The slope a contains information concerning the molecular conformation of the polyester.

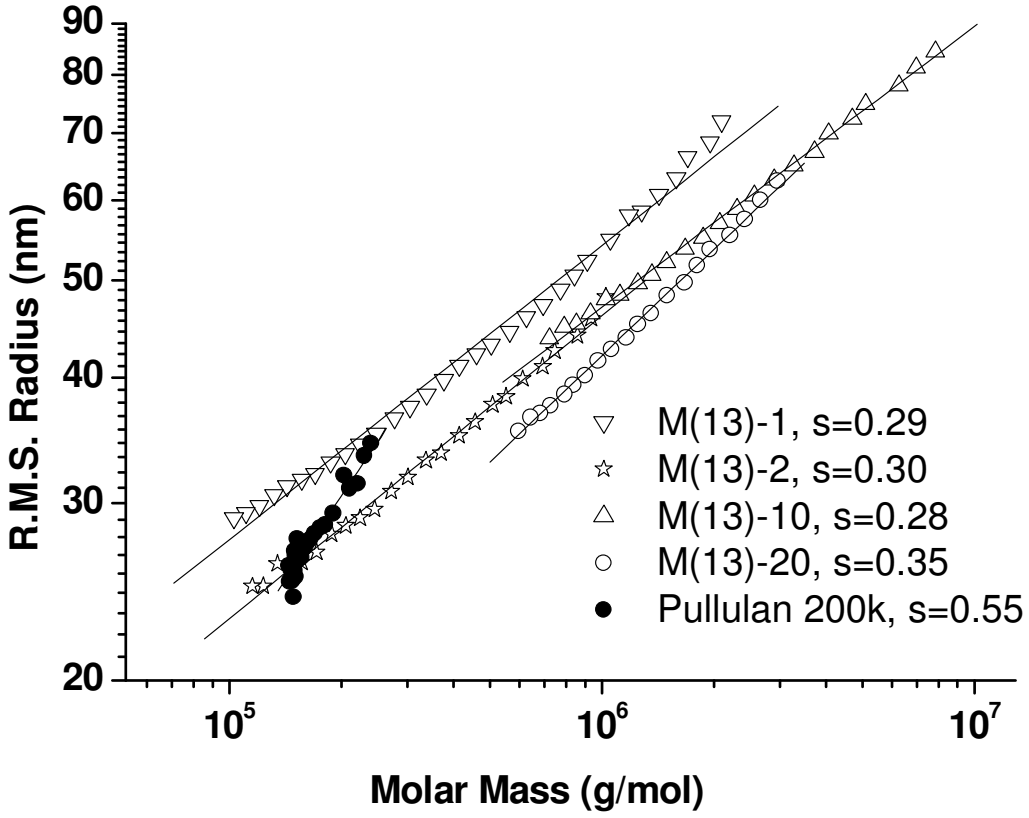


Figure 5. *Double logarithmic plot of R.M.S. radius of gyration against molar mass, the slopes express high branching: M(13)-1, s=0.29; M(13)-2, s=0.30; M(13)-10, s=0.28; M(13)-20, s=0.35; random coil: Pullulan, s=0.55;*

Most real random coils have slopes in the range 0.55 - 0.60.[46,48] Branched polyesters show smaller slopes, suggestive of their more compact structure.[46] In figure 5 the linear, non-branched pullulan is compared to M(13)-y polyesters. Pullulan shows a typical slope value of random coils (0.55). In contrast the slopes of the polyesters are much smaller (0.28-0.35) demonstrating their highly branched character.[46,47]

Beside the molecular structure in solution, the microstructure and thermal behavior of the branched polyesters is important for their performance as drug delivery platform. Differential scanning calorimetry (DSC) of the polyesters shows only one glass transition. In the first run relaxation processes superpose the glass transition (data not shown). The glass temperature (T_g) strongly depends on the side chain length. The non-grafted backbone polymers show the highest T_g. Grafting with lactide and glycolide lead to a decrease with a minimum at a backbone side chain ratio of one to ten. Higher ratios invert this trend and lead to an increase of glass transition (figure 6).

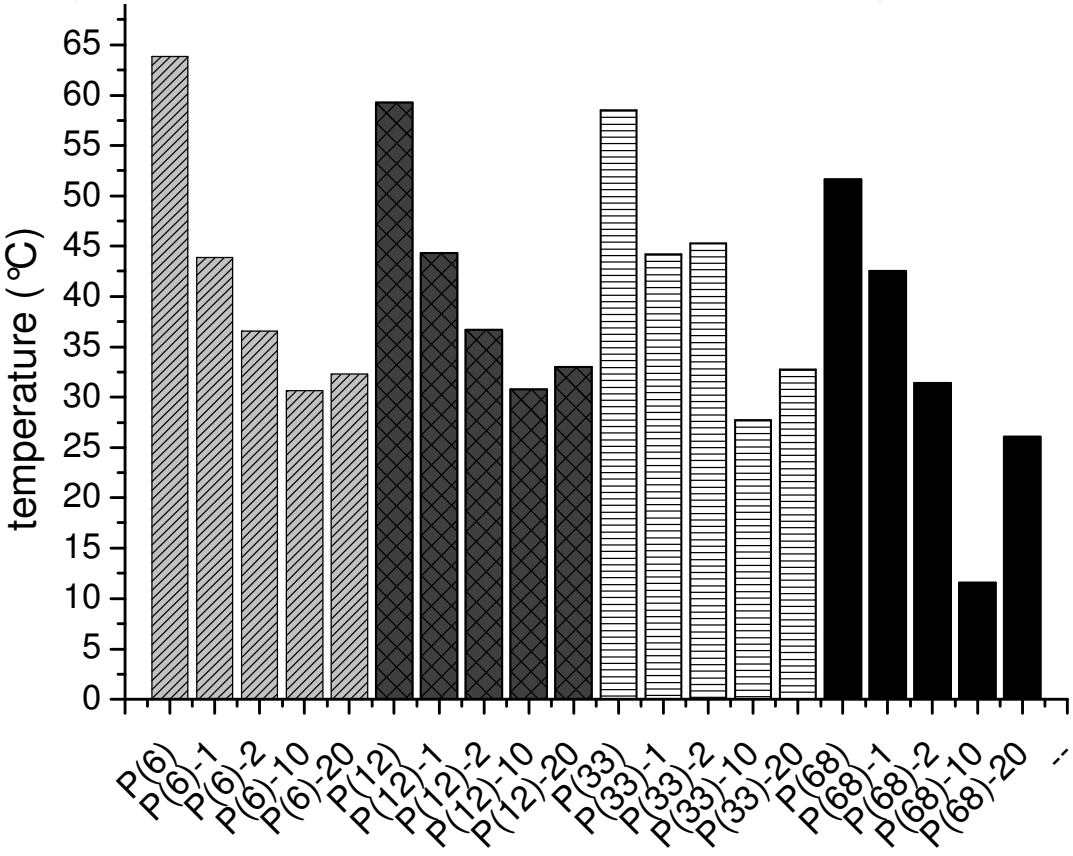


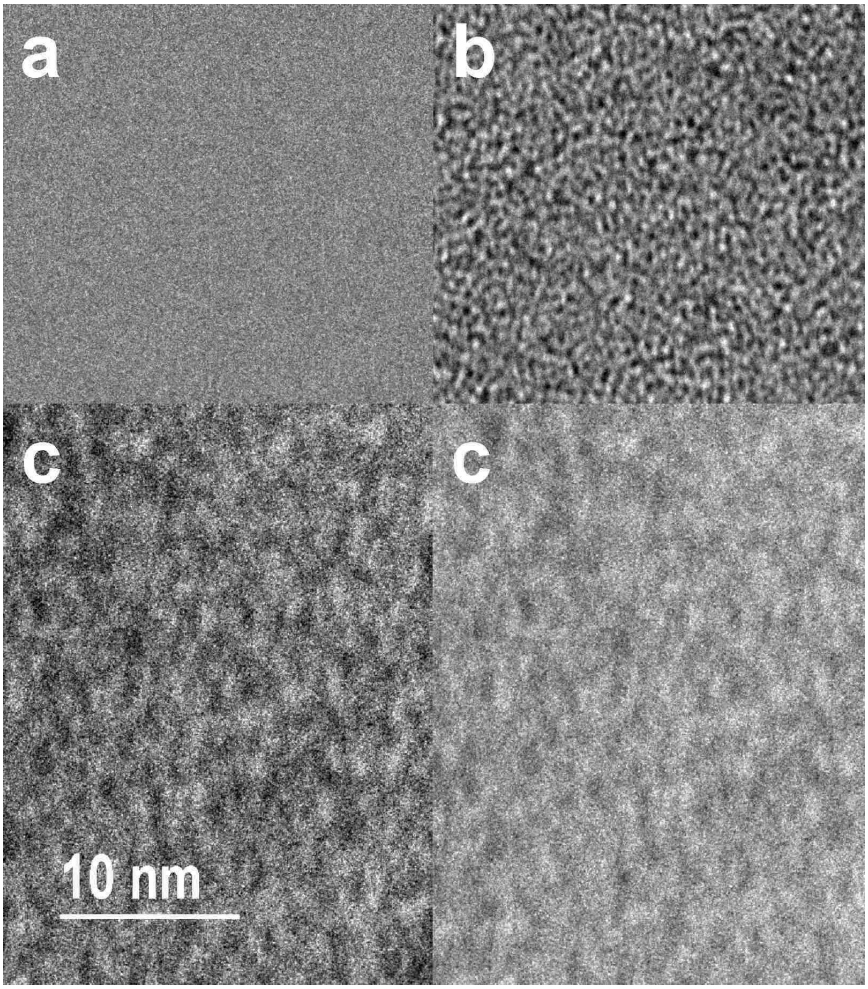
Figure 6. *Glass transition temperatures of the P(x)-y polyesters with 3-(diethylamino)propylamine groups in the backbone, Second run, method: heating and cooling runs: -10 to 200°C, two isothermal steps at -10 °C*

It seems that the side chains have plasticizing effects on the polymer composition.49 This leads to easier chain movement of polyester segments to the point where the side chains are long enough to hinder their own movements. A chain length of around 10 monomers marks this turning point. The amine substitution also decreases T_g within a series of polyesters with increasing degrees of substitution, suggestive of a plasticizer effect. The T_g's measured of type I polyesters correspond to values of linear PLGA copolyesters known from the literature. In contrast the type II polyesters show smaller glass transition temperatures.[45]

Neither DSC nor WAXD (wide angle x-ray diffraction) show crystalline polymer segments. In WAXD only a halo indicative of amorphous materials could be observed (figure 7).

No phase separation was noted in the second DSC run (only one T_g). In contrast to PLLA/PVA blends, full miscibility of backbone and PLGA side chains was observed.[50] TEM (transmission electron microscopy) micrographs, however, seem to show a micro - phase separation within the type II polyesters (figure 7). After staining with osmium tetroxide, dark and bright structural regions with dimensions in the order of approximately 1-2 nm could clearly be distinguished.[51] The stained areas seem to correlate with segments of the polyester containing free hydroxyl groups. These functional groups are found in small amounts within the PVA backbone and at the end of every PLGA side chain. The unstained areas result from hydrophobic segments of the grafted backbone and the central groups of the PLGA chains. Due to this phase separation hydrophilic segments can assumably govern a fast water uptake leading to an accelerated degradation and drug release.

Because of the proposed application as parenteral drug delivery system their in-vitro degradation profile is of critical importance. The degradation behavior of type II polyesters is presented in figure 8. The polymer erosion, characterized by the weight change as a function of degradation time in physiological buffer solution of M(13)-10 is compared with M(7)-10, M(7)-20 and M(13)-20. M(13)-10 demonstrates a degradation half-life of 10 days.



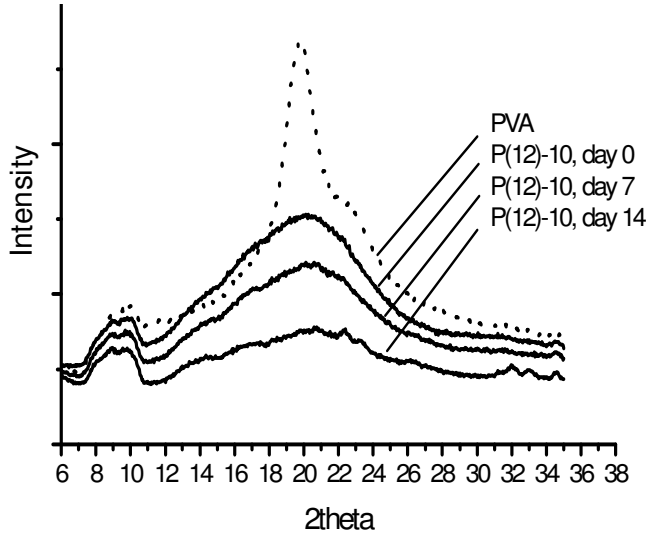


***Figure 7**. left: TEM P(12)-10: (a) carbon grid, (b) free space, (c) P(12)-10 as film after four days of staining with OsO_4 solution(different gamma correction), right: WAXD of the P(12)-10 after 0, 7 and 14 days of degradation showing only the amorphous halo*

In view of the high molecular weight this is an extremely short degradation time as compared to linear PLGAs.[52] During degradation oligomeric substances are formed (figure 8b). The degradation mechanism is currently under investigation, but the data suggest a rapid bulk hydrolysis of the polyester possibly catalyzed by the amino groups . The degradation depends on side chain length and amine amount in the backbone. The degradation rate could be reduced by elongation of the side chain length while increase of the amine substitution leads to an acceleration. As shown in figure 8a an increase of the amine substitution by a factor of 2x leads to a reduction in the half-life from 20 d to 10 d. The study of P(33)-10 even results in the extremely short half-life degradation time of one day. This amazingly short degradation time seems to be the result of the amino functions attached to the PVA backbone, possibly catalyzing PLGA hydrolysis during degradation.

Possibly, this high degradation rate is advantageous for drug delivery applications, since the protein destructing acidic microenvironment known to be formed in slow degrading PLGA systems will most likely be avoided.[7,52]

2.5 Conclusion.

The successful synthesis of amine-modified poly(vinyl alcohol)-graft-poly(lactide-co-glycolide)s could be demonstrated by NMR and GPC-MALLS. Depending on the ratio of the three components (PVA, amine, lactide/glycolide) the solubility of the resulting graft polyester could be modified to large degree ranging from hydrophobic to water-soluble materials. Structure-property relationship were noted for polyesters with short (type I) and long (type II) SCLs. In case of type II polyesters the solubility shifts from more hydrophilic solvents (methanol) to less hydrophilic (acetone). Using GPC-MALLS the molecular weights and the three dimensional structure of the polymers has been investigated. Polyesters with long side chains (type II) are characterized by molecular weights higher > 100 000 g/mol. A degree of branching was demonstrated by multi-angle laser light scattering for type II polyesters. The glass

transition is mainly influenced by side chain length and also in a minor way by amine substitution

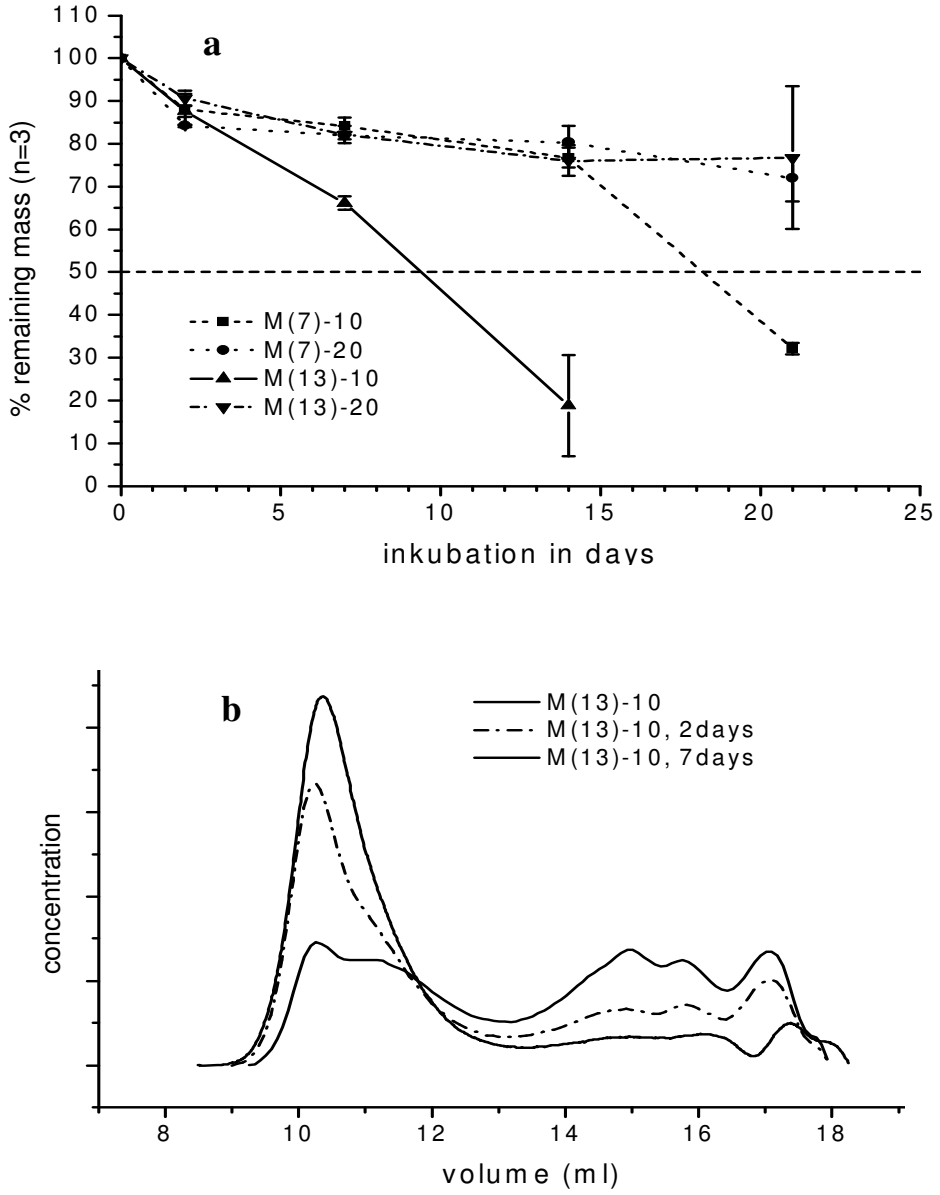


Figure 8. *Degradation a. mass lost of M(7)-10/20 and M(13)-10/20 during incubation time; b. GPC elugrams in of M(13)-10 after 0, 2 and 7 days of incubation.*

Surprisingly these biopolymers show despite their high molar masses compared to linear PLGAs extremely short degradation times. An important role of the amine group for the degradation of this polymer class was demonstrated. The amino function seems to be the main factor for a fast degradation. Elongation of the side chains has the opposite effect. The potential of these biopolymers as platform for biodegradable

delivery systems can be seen in the opportunity to adjust the polymer structure to specific therapeutic applications requiring rapidly degrading devices. Further investigations on drug release and degradation mechanism are currently under way.

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Chapter 3

Chapter 3: A two dimensional NMR study of Poly(vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol)

In preparation for Macromolecules (see Appendix)

3.1 Summary

Binding one-side protected diamines to poly(vinyl alcohol) (PVA) followed by lactide/glycolide grafting to synthesize [Poly[vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide)] is a new synthetic approach for tailor-made polyesters for drug delivery. In order to determine the microstructure of the amine-modified PVA backbones of these polyesters COSY (correlated spectroscopy), ^{1}H-^{13}C HMQC (heteronuclear multiple-quantum correlation) and HMBC (heteronuclear multiple-bond correlation) spectroscopy were used for a complete assignment of ^{1}H and ^{13}C spectra. 54 % VOHVAMVOH, 30 % VAVAMVOH, and 16 % of VAVAMVA were determined as the microstructure the amine substitution of the polymer chain. Average block length of 9 VOH was discovered for the PVA backbone. The methine region in ^{13}C spectra possesses configurational and constitutional data on the microstructure of the polymers. Pentads centered to rr, mr and mm could be assigned to VOHVOHVOH triad and mr, rr to triad of VOHVOHVAM. Therefore, we may propose that mainly isotactic triad react with the activated diamine. Furthermore, the three microstructures of substitution are reflected by the three carbonyl 13C signals of both amine and acetate. Using ^{1}H-^{13}C HMBC the covalent bond between PVA backbone and amine was confirmed by observed cross peaks between carbonyl ^{13}C of the urethane bond and methine of the PVA backbone. Furthermore, the tacticity assignment of Bar et al. (*Polymer Preprints* **2001**, *42*, 43-44) on pure PVA could be approved by the observation of $^{3}J_{CH}$ coupling between non-substituted methine ^{13}C and non-substituted methine ^{1}H in HMBC.

3.2 Introduction.

The development of tailor-made polymers for gene and other drug delivery is one of the major aims in pharmaceutical technology. Due to the conclusion of human genome project more and more gene and protein based drugs will be developed.[1] The bioactivity of these large molecules is based on their intact chain sequence respective in their intact tertiary structure. Both, the tertiary structure of proteins and the integrity of the DNA/RNA chain could be easily damaged by external influences, like enzymes, acids, bases or interacting molecules.[2] To stabilize these molecules carriers are necessary.[3]

Such carriers should have the ability to protect the drug. No loss of bioactivity should appear during manufacturing, application and release of the drug. Furthermore, the loading of the carrier system in sufficient concentration is necessary to ensure an effective delivery for therapeutic application.[4,5]

Recently, we described the synthesis of such a system having amine-modified poly(vinyl alcohol) backbones and poly(lactide-co-glycolide) side chains.[6] In this publication the synthesis and characterization of a great number of different polyesters was described. By physico-chemical characterization and degradation studies structure – property relationships of these polymers were investigated. Furthermore, the abilities of the polyesters as custom tailor-made drug carriers were demonstrated. In this publication the covalent bond between backbone and amine was proved by ^{1}H-NMR and FT-IR spectroscopy. These methods can only verify the presence of amine in the polymer sample. They do not show information about the linkage and the binding site between both components. To ensure a complete characterization of the used amine modified PVA backbones we use two-dimensional (2D) NMR techniques to characterize the constitution and configuration of the poly[vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol].

Many NMR studies about poly(vinyl alcohol) (PVA) and its copolymer with vinyl acetate have been performed.[7-13] Nevertheless, only a few PVA NMR studies use multidimensional techniques.[14,15] And no information about a combined study of ^{1}H-

^{1}H COSY, ^{1}H-^{13}C HMQC (Heteronuclear Multiple-Quantum Correlation), and ^{1}H-^{13}C HMBC (Heteronuclear Multiple-Bond Correlation) can be found in the literature to reveal data on PVA's and especially amine-modified PVA's constitutional and configurational microstructure.

Therefore, in this paper we present results using ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, and HMBC techniques to investigate the microstructure of the amine-modified PVA. A complete assignment of NMR signals is given. Using ^{1}H-^{13}C HMBC we demonstrate the covalent bond between PVA backbone and amine

3.3 Experimental Section

Materials. 2-diethylaminoethylamine (DEAEA) (*purum*, >98%) , 3-diethylaminopropylamine (DEAPA) (*purum*, >98%), 3-dimethylaminopropylamine (DMAPA) (*purum*, >98%), poly(vinyl alcohol) (MW 15000 g mol^{-1}; degree of polymerization 300 (P=300); degree of hydrolysis 86-89%), carbonyl diimidazole (*purum*, ~97%), N-methyl pyrrolidone (NMP) (absolute), Dimethylacetamide (DMAc)(for HPLC, 99.8%) and 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (*puriss.*, absolute, over molecular sieve) were purchased from Fluka GmbH (Germany) and used as received. Tetrahydrofuran (THF) (BASF, Germany) was dried over sodium and distilled under nitrogen before use. All other chemicals including lithium bromide (extra pure) (Merck) were used as received without further purification.

Synthesis. The synthesis of Poly[vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide) is discussed else where. 6 In brief, using carbonyl diimidazole chemistry the diamines were activated in THF solution. After distilling the THF off the calculated amount of the reaction mixture was injected into a solution of PVA in NMP at 80 °C and stirred for 4.5 days at the

same temperature. The resulting polymers were purified by ultrafiltration (YM1 membrane, cut off 1000 g mol-1, Millipore) and dried by lyophilisation (Edward Freeze Dryer Modulyo, standard conditions).

Nomenclature. As abbreviation for the synthesized PVAs A(x) is used. (A indicates the type of amine substitution (P=DEAPA, M=DMAPA, E=DEAEA), x is the number of monomers in the backbone carrying amine substitution.)

NMR Experiments. 50 to 100 mg polymer were solved in 0.75 ml d_6-DMSO (euriso-top, <0.02% HDO+D_2O). ^1H (400.13 MHz), ^{13}C (100.21 MHz), and ^1H-^{13}C correlation spectra were recorded on a Bruker DRX-400 spectrometer. COSY experiment was performed on a Bruker DRX-500 spectrometer. ^1H and ^{13}C were referenced to the d_6-DMSO solvent signal. A 5 mm multinuclear gradient probe and gs-HMQC[16] and gs-HMBC[17] pulse sequences were used for the ^1H-^{13}C correlation experiments. While the HMQC experiment was optimized for C-H coupling of 140 Hz, with decoupling applied during acquisition, the HMBC experiment was optimized for coupling of 8 Hz, without decoupling during acquisition. HMBC and HMQC data were acquired with 512 points in *F2*, the number of increments for *F1* was 256, 64 scans were used for HMQC and HMBC experiments, respectively, and four dummy scans were used for both experiments. COSY spectra were recorded with 1024 points in *F2* and 256 increments for *F1*. A relaxation delay of 1 s was used for all 1D experiments and 2 s for all 2D experiments. The typical experiment time was about 12 h for HMQC and HMBC, respectively. All the measurements were performed at 340 K.

Gelpermeation Chromatography was carried out using a Merck-Hitachi system coupled to a Wyatt DawnEOS Light Scattering detector in DMAc + 2.5 g L^{-1} LiBr (flow rate = 0.5 mL/min) at 60 °C. [6]

3.4 Results and Discussion

Recently we reported the synthesis and characterization of a new polyester system developed for the tailor-made production of drug delivery systems for hydrophilic substances and fast degradation.[6] Characteristic data of these polymers are listed in Table 1.

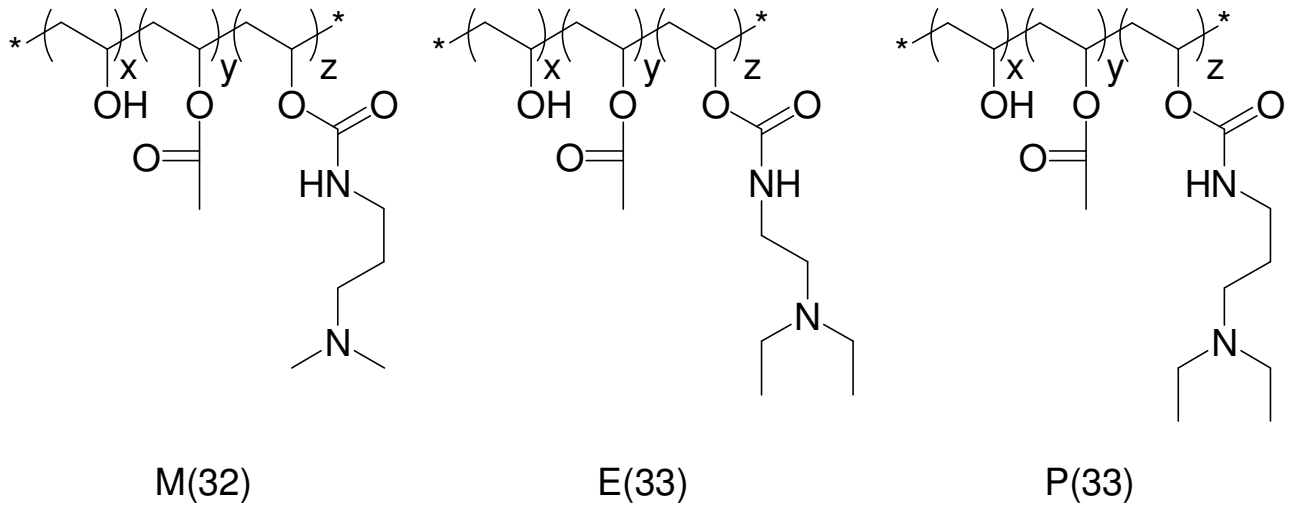
Table 1. *Poly(vinyl dialkylaminoalkylcarbamate-co- vinyl acetate-co-vinyl alcohol)s: Molecular weights and degree of amine substitution*

Polymer	M_w (g mol^{-1})	M_w/M_n	DS: amine (%)[a]	DS: acetate (%)[a]
M(32)	14400	1.2	10.8	4.1
E(33)	17200	1.2	10.9	4.1
P(33)	19800	1.2	10.9	0.8
PVA	14000	1.2	0.0	11.8

[a] degree of substitution (DS) calculated from ^{1}H-NMR

Nevertheless, a detailed structure characterization of these polymers has not yet been published. In this paper we choose two polymers M(32), E(33) and P(33) (Scheme 1) as model systems and show a complete structure analysis by using 1D and 2D NMR techniques. Both polymers were synthesized out of the same commercial available PVA (table 1). The degree of polymerization of this PVA was 300. They have 10.8 respectively 10.9 % amine and 4.1 % acetate substitution. The number 32 respectively 33 directly relates to the amine substitution and the degree of polymerization.

Scheme 1: *Structure of M(32), E(33)and P(33)*



M(32) E(33) P(33)

^{1}H and COSY studies. The ^{1}H spectrum of M(32) and its assignment is shown in Figure 1. The peaks for DMAPA are: NH, 6.78, 6.70, 6.63 ppm; CH_2(4), 2.99 ppm; CH_2(2), 2.22 ppm (t, 7.10 Hz); CH_3(1), 2.12 ppm; and CH_2(3), 1.53 ppm (quintet, 7.30 Hz). The CH_3 group of acetate shows a peak band of 1.99 – 1.94 ppm. As shown by the scheme attached to Figure 1, the methylene and methine of the PVA backbone are denoted as a and b, respectively. The acetate-substituted ones are denoted as a' and b', while the amine-substituted ones denoted as a'' and b'', respectively. The non-substituted methine b is further divided into two components b_1 and b_2, where b_2 stands for the methine position directly next to substitutions. The peaks for the PVA backbone appear as follows: CH(b'), 5.10 ppm; CH(b''), 4.92, 4.87, 4.82 ppm; OH, 4.40 ppm; CH(b_1), 3.91 – 3.86 ppm; CH(b_2), 3.72 – 3.68 ppm; CH_2(a'), 1.79 ppm ; CH_2(a''), 1.64 ppm; and CH_2(a), 1.47 – 1.39 ppm. The correctness of the assignment for DMAPA is confirmed by 2D experiments COSY (Figure 2) and HMBC (Figure 6).

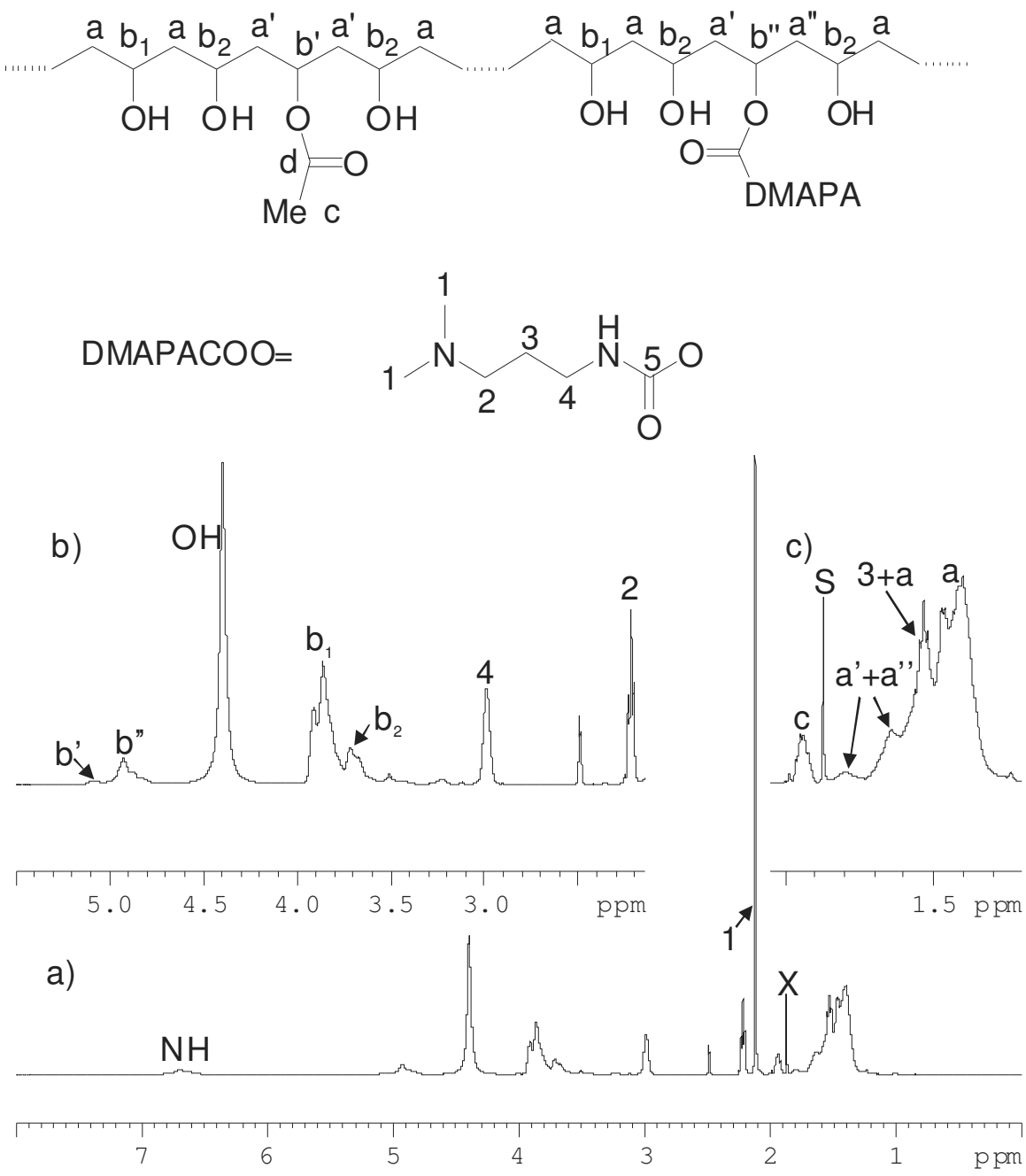


Figure 1. *(a) [1]H NMR spectrum of the amine modified PVA copolymer M(32). (b) and (c) are extended inserts. The labels S stands for the solvent DMSO and X for impurity for all the figures.*

The COSY cross-peaks observed for DMAPA show connections between NH and 4, 3 and 4, and 3 and 2. The assignment of 1 is further confirmed by the observation of HMBC cross peaks between 1 and 2 (2.12 ppm – 56.4 ppm and 2.22 ppm – 44.7 ppm,

respectively). The assignment of the PVA backbone is referenced to van der Velden et al. and Budhlall et al.[13,18] and further confirmed by the COSY experiment. In contrast to our work both authors investigated systems with only two different structural components. Here the amine modification leads to a three-component system.

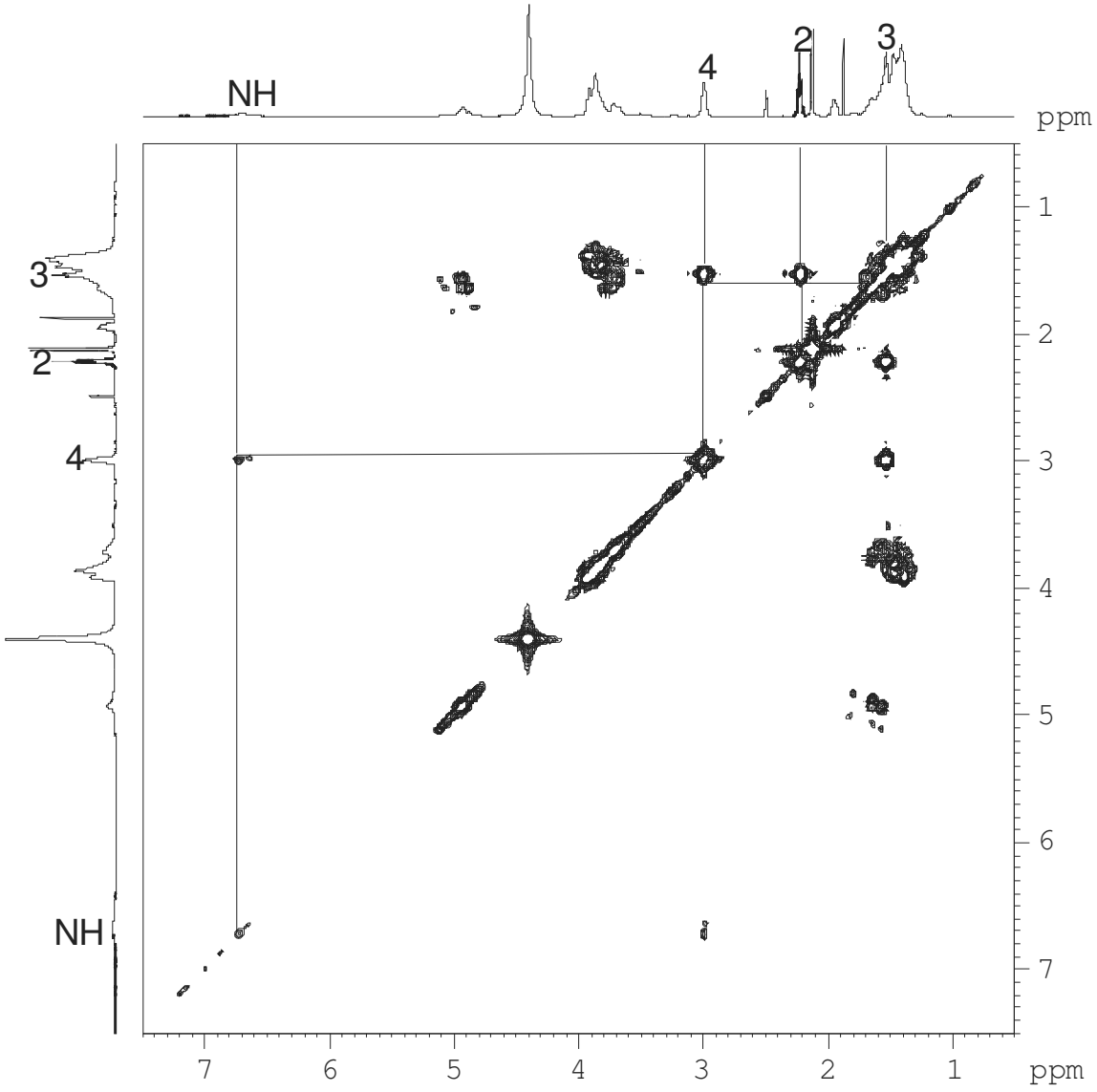
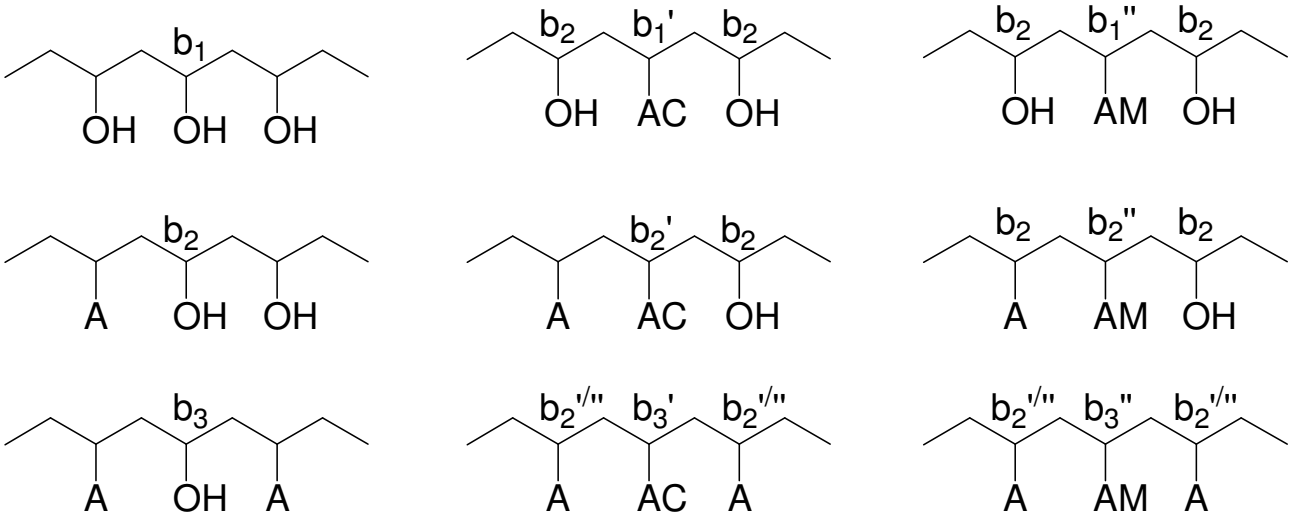


Figure 2. *COSY spectrum of M(32).*

The assignment of the different substitution (a' vs. a'' and b' vs. b'', see scheme 2) is according to the different percentages: 10.8 % of amine and 4.1 % of acetate.[6] The ^{1}H signals of a, b_1, b_2, and b'' show resolved multi-components. These multi-components are due to either compositional or configurational microstructure of the copolymer. By

using ^{1}H and ^{13}C spectra van der Velden presented a compositional analysis of the microstructure of poly(vinyl acetate-co-vinyl alcohol).[13] Here we present a detailed microstructure analysis of M(32) by using COSY technique.

Scheme 2. *Different triads of the amine-modified PVA*



A = acetate or amine substituent
AC = acetate substituent
AM = amine substituent

As van der Velden mentioned three different triads exist for the substituted methine. The possible triads for the non-substituted, acetate- and amine-substituted methine of M(32) are shown in Scheme 2. For the non-substituted methine only two components b_1 and b_2 are observed. Due to the block property of the comonomers [13] triad b_3 has very little contribution and is not detectable. Since M(32) has only 4.1 % of acetate substitution, the triads b_1', b_2', and b_3' are weak and overlap into a broad peak b'. The amine-substituted methine shows three components: b_1'', b_2'', and b_3'', where b_1'' is assigned to the signal at the lowest field position and b_3'' to the one at highest field. [13]. A deconvolution of the ^{1}H spectrum shows 54, 30 and 16 % for b_1'', b_2'', and b_3'', respectively. This means the amines exist in the copolymer in 54 % as separated substitution (triad VOHVAMVOH), in 30 % as VAVAMVOH, and only in 16 % as pure vinyl alkylcarbamate or mixed vinyl acetate vinyl alkylcarbamate triads

VAVAMVA. This assignment is confirmed by analyzing the ^{1}H signal integrals of the different methines. Thus the integrals obtained are 16.2 % for (b'+b''), 65.0 % for b_1, and 18.8 for b_2, with the sum normalized to be 100 %. As shown in Scheme 2, for triads VOHVAMVOH, VAVAMVOH, and VAVAMVA, the ratio b''/b_2 are 0.5, 1.0, and 1.5, respectively. The same ratio exists for the acetate triads. Thus, by using the corresponding percentage for the different triads deduced from a deconvolution of b'', the total ratio of (b'+b'')/b_2 (further denoted as block ratio of substitution) is calculated to be 0.81. This calculated result agrees well with an experimental value of 0.86. A block ratio of 0.5 means only separated substitution, and a ratio higher than 0.5 means a block structure of the substitution. By analog, the ratio of b_1/b_2 can be defined as a block ratio of PVA polymer. An experimental value of 3.5 is obtained and thus implies an average length of 9 for the PVA block.

^{13}C and HMQC studies. Up to now we discussed the microstructure of M(32) by using ^{1}H and COSY spectra. By taking advantage of the rather well resolved methine spectra, compositional microstructure up to triads has been verified. Since ^{13}C spectra have much larger chemical shift range than ^{1}H spectra, they are expected to show more resolved signals and to provide information of configurational microstructure. The ^{13}C spectrum of M(32) is shown in Figure 3. Using ^{1}H-^{13}C HMQC techniques (Figure 4) and the proton assignment (Figure 1), the aliphatic ^{13}C signals of the side chains can be completely assigned. The acetate methyl group appears at 20.7 ppm; the signals at 27.1 and 38.6 ppm are assigned to the amine methylene groups at positions 3 and 4, respectively; the methyl groups (1) of amine appears at 44.7 ppm; and the signal at 56.4 ppm is assigned to the amine methylene 2. Carbon NMR spectra of the methylene and methine groups of the non-substituted PVA backbone show resolved signals due to configuration of the substructures (multiples of a and b), while weak broad peaks are observed mainly for the amine-substituted CH_2 and CH. The weak signal bands at 43.7 – 41.4 ppm and 69.1 – 68.7 ppm are thus assigned to a'' and b'', respectively.

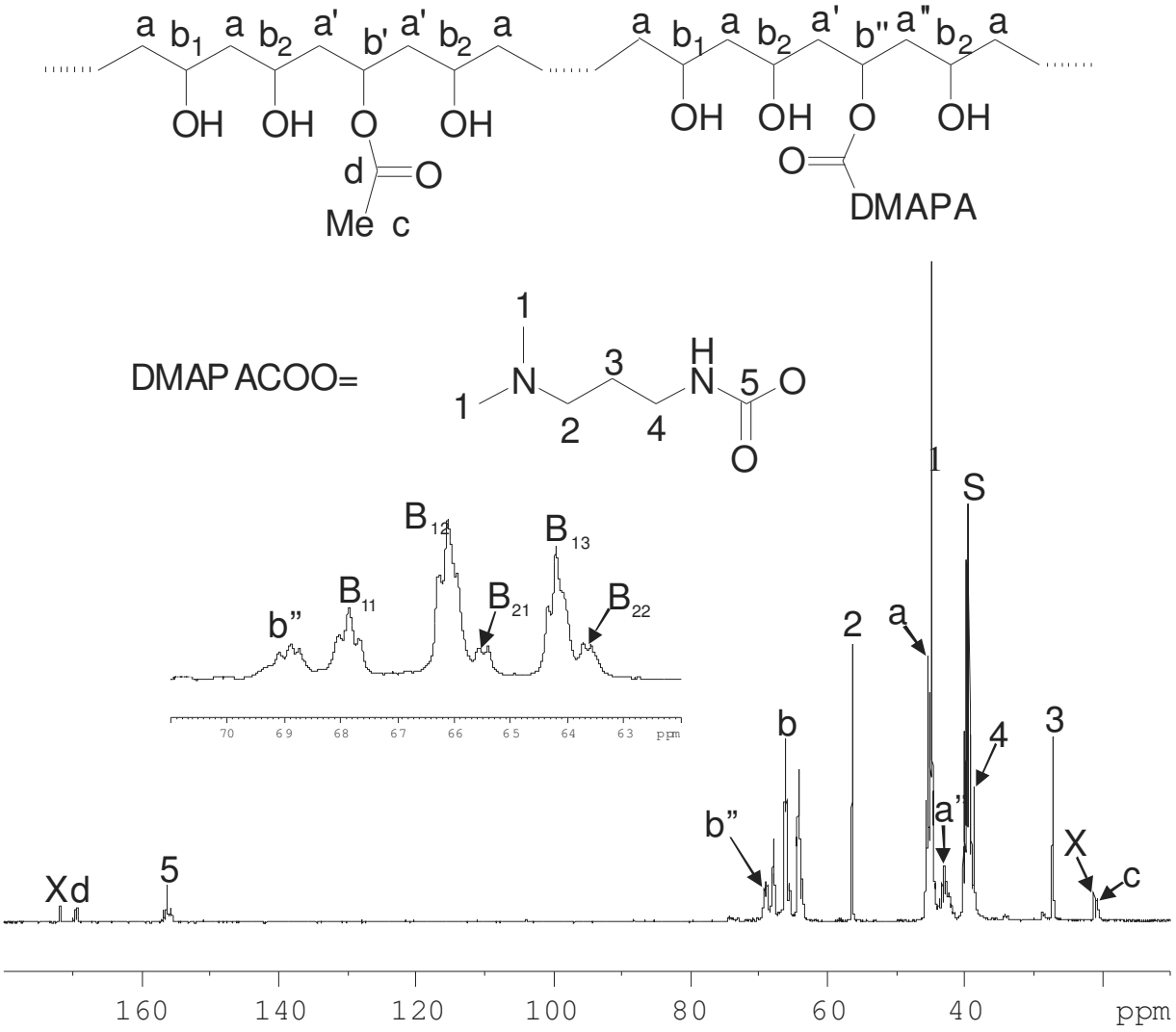


Figure 3. *^{13}C spectrum of M(32).*

Recently, Brar et al. reported a complete tacticity assignment to the methylene and methine ^{13}C signals of unmodified pure vinyl alcohol by applying the 2D spectroscopy HSQC-TOCSY. [14] The four peaks for the methylene are thus assigned to the following tacticities: rrr at 45.6 ppm, (rmr + mrr) at 45.3 ppm, (mmr + mrm) at 44.9 ppm, and mmm at 44.5 ppm. The spectrum of the M(32) methine shows additional peaks (B_{21} and B_{22}, see Insert to Figure 3) as compared with the corresponding signals of the pure PVA. The integral of these two peaks has to be taken into consideration (as a component to the non-substituted methine), in order to have the ratio between the substituted and the total methine signals correct for the real degree of substitution. This means that peaks B_{21} and B_{22} are due to certain sort of non-substituted methine.

Further, a ^{13}C measurement on a commercial PVA (Fluka, 12 % acetate) detected no peaks of B_{21} and B_{22}. Therefore, peaks B_{21} and B_{22} are due to non-substituted methine related to the amine modification. In order to have a clear assignment of these peaks a special HMQC spectrum with 1024 increments in F_1 dimension is recorded. With high resolution in F_1, this HMQC spectrum provides with clearly resolved methine cross peaks (Figure 5).

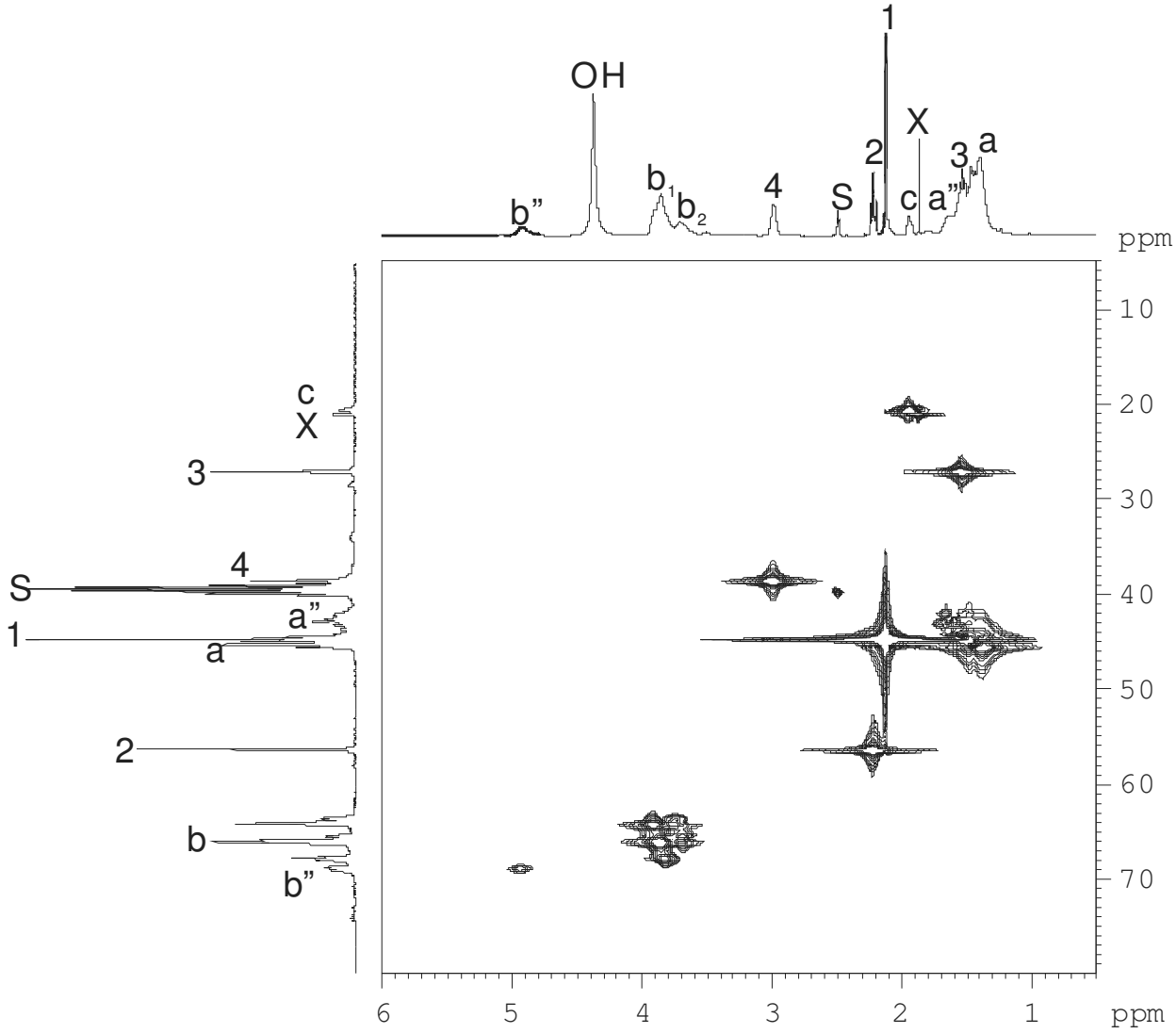


Figure 4. *^{1}H-^{13}C HMQC spectrum of M(32).*

The major peaks show cross peaks with b_1 (the unmodified triad VOHVOHVOH) and the weak peaks B_{21} and B_{22} are correlated to b_2 (triad next to modification VOHVOHVA). Therefore, signals due to compositional and configurational

microstructures can now be assigned separately. The triads VOHVOHVOH overlap with VOHVOHVAC and have the following signals: rmmr, mmmr, and mmmm appear at 68.0, 67.9, and 67.7 ppm; rmrr, (mmrr + rmrm), and mmrm have signals at 66.3, 66.1, and 65.9 ppm; and rrrr, mrrr, and mrrm signals appear at 64.3, 64.2, and 64.1 ppm. The weaker signals due to the hetero triads VOHVOHVAM are assigned as mr at 65.5 and 65.4 ppm, and rr at 63.7 and 63.6 ppm. No mm tacticity is observed for triad VOHVOHVAM. The cross peaks related to the ^{1}H signal of methine b_2 for both triads VOHVOHVAC and VOHVOHVAM are resolved into four peaks H, I, J, and K (Figure 5).

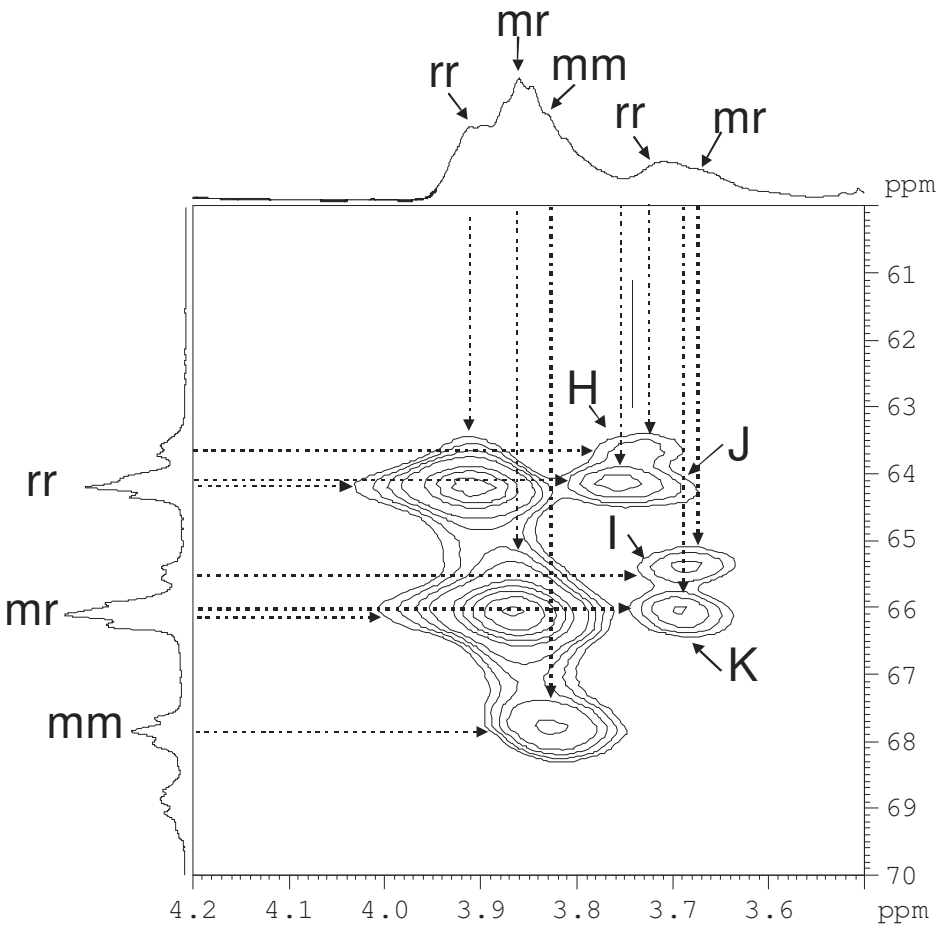


Figure 5. *^{1}H-^{13}C HMQC spectrum of M(32) in the regions of (F_2) 4.2 – 3.5 ppm and (F_1) 70 – 60 ppm.*

The cross peaks H and I are due the triad VOHVOHVAM, while cross peaks J and K are due to the triad VOHVOHVAC. The assignment of the triad VOHVOHVAC is

not straightforward, because its ^{1}H signals overlap with that of the triad VOHVOHVAM and ^{13}C signal overlap with that of the triad VOHVOHVOH. The ^{1}H spectrum of b_1 and b_2 shows signal bands for different tacticities up to triads. By using HMQC cross peaks and the ^{13}C assignment, the tacticity of b_1 and b_2 are assigned and labeled on Figure 5. It is noteworthy to mention that there is no cross peak of hetero triads with tacticity of mm. We propose that mostly isotactic triads react with the activated diamine. Due to a lower sterical hindrance in comparison to the other configuration in mm configuration the hydroxyl groups can easily form hydrogen bonds with themselves, with the catalyzing DMPU and the amine-CI and there is the lowest sterical hindrance. We hypothesize that this promotes the formation of the urethane bond between PVA and amine. The observation of only one cross peak due to b'' confirms these considerations.

HMBC studies. In ^{1}H-^{13}C HMBC long-range couplings of the protons with carbons in beta, gamma or delta positions are detected. This technique enables the recognition of couplings across hetero atoms like nitrogen and oxygen. The quaternary ^{13}C signals can thus be assigned, and of even greater importance the covalent bonds between PVA backbone and diamine can be proved.

Figure 6 shows the HMBC spectrum of M(32). Cross peak L shows a $^{3}J_{C-H}$ coupling between methylene protons 4 and the carbonyl carbon 5 of amine. The ^{13}C signals at 156.8, 156.3, and 155.8 ppm are thus assigned to the carbonyl carbon 5. Cross peak M shows a $^{3}J_{C-H}$ coupling between methyl proton c and the carbonyl carbon d of acetate. The ^{13}C signals at 170.0, 169.6, and 169.3 ppm are thus assigned to carbonyl carbon d. In case of a covalent urethane bond between PVA backbone and diamine cross peak between methine proton b'' and carbonyl carbon 5 shall be detectable in ^{1}H-^{13}C HMBC measurements. This is confirmed by the observation of cross peak N in Figure 6.

Both of the carbonyl carbons 5 and d have three signals. These multiple components of the ^{13}C signals reflect the compositional microstructures of the amine-modified copolymer PVA. This is confirmed by the fine structure of the HMBC cross peak

between the amine-substituted methine proton b'' and the carbonyl carbon 5 shown in Figure 7a. From ^{1}H and COSY spectra we have assigned b_1'' to VOHVAMVOH, b_2'' to VAVAMVOH; and b_3'' to VAVAMVA. The strongest peak at 156.3 ppm shows a HMBC cross peak with the largest component b_1'' of the methine proton (figure 7a). Because of the low acetate concentration, a HMBC cross peak between acetate carbonyl d and the corresponding acetate-substituted methine b' is too weak to be detected. Nevertheless, three ^{13}C signals of the carbonyl d are observed and are thus assumed to be due to the three different compositional microstructures.

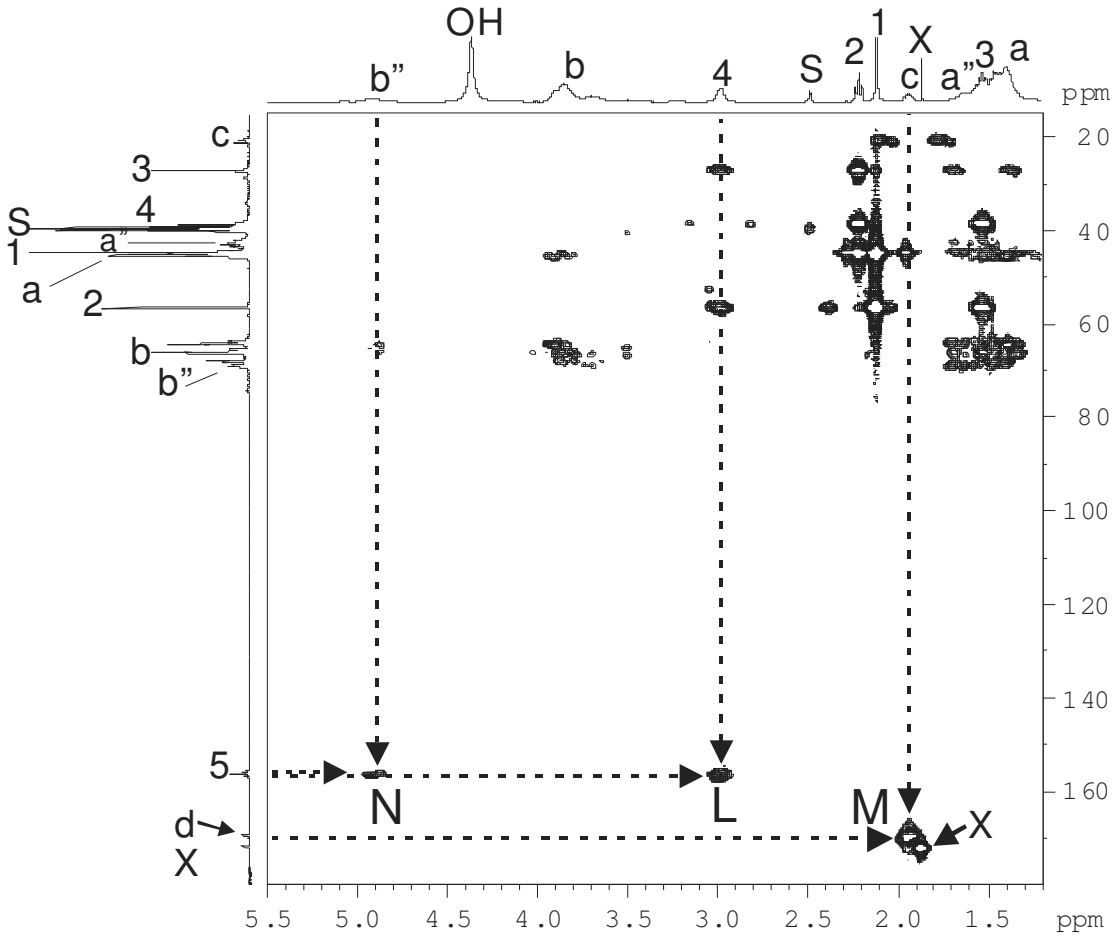


Figure 6. *^{1}H-^{13}C HMBC spectrum of M(32).*

Brar et al. used ^{13}C edited ^{1}H-^{1}H total correlation (HSQC-TOCSY) to determine for the first time the tacticity of an unmodified pure PVA completely. [14] In the course of our study on the modified PVA, we noticed another possibility of doing this, that is, by observing the $^{3}J_{CH}$ of the HMBC correlation. This is shown in Figure 7b. The

carbon of a pentad methine can have two $^{3}J_{CH}$ couplings with the methine protons of its first neighboring comonomers. Thus the ^{13}C of the rr centered pentads mrrm, mrrr, and rrrr show $^{3}J_{CH}$ cross peaks with protons of triads rr and mr; the mr centered ones mmrm, (mmrr + rmrm), and rmrr arise correlation with all the three triads mm, mr, and rr; and the mm centered pentads mmmm, mmmr, and rmmr give cross peaks with triads mm and mr. These HMBC cross peaks confirm the tacticity assignment.

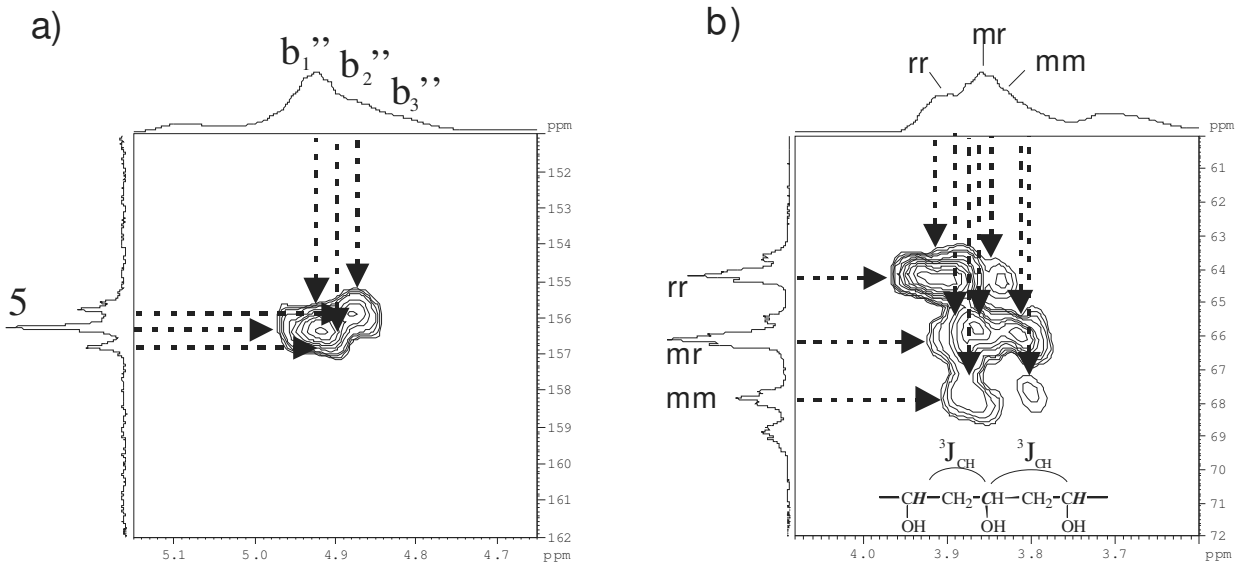


Figure 7. *^{1}H-^{13}C HMBC spectrum of M(32) in the regions of (F_2) 5.15 – 4.65 ppm and (F_1) 162 – 151 ppm (a), and in the regions of (F_2) 4.1 – 3.6 ppm and (F_1) 72 – 60 ppm (b).*

The spectra of DEAEA-modified PVA E(33) shows that the microstructure of both polymers are very similar. Same tacticity and triad formation could be observed. There are also cross peaks of covalent urethane bonds between PVA and amine (Figure 8, lower right corner). Only the cross peaks of the amine group are different. These peaks appear as follows: 5 (156.9-154.9 ppm), 4 (3.04 ppm-38.5 ppm), 2 (2.50 ppm-46.5 ppm), 3 (2.47 ppm-51.8 ppm) and 1 (0.98 ppm-11.6 ppm) (figure 8, attached scheme). In the spectrum of the polymer the proton signals of 2 and 3 overlap in ^{1}H-NMR and cannot be separated by this method. Using ^{1}H-^{13}C HMBC it is easy to

decide which proton is better shielded in NMR. In Figure 8 (upper left) 3 and 2 show only strong cross peaks. This indicates that mainly $^{2}J_{CH}$ coupling with the direct neighborhood appears. Due to this observation cross peaks between 2 (^{1}H NMR) and 1 (^{13}C NMR) respective between 3 (^{1}H NMR) and 4 (^{13}C NMR) can be identified. It results that the signal at 2.50 ppm is caused by 2 and the signal at 2.47 ppm by 3. 3 is slightly better shielded than 2. A block length of 9.8 VOH could be observed for this polymer.

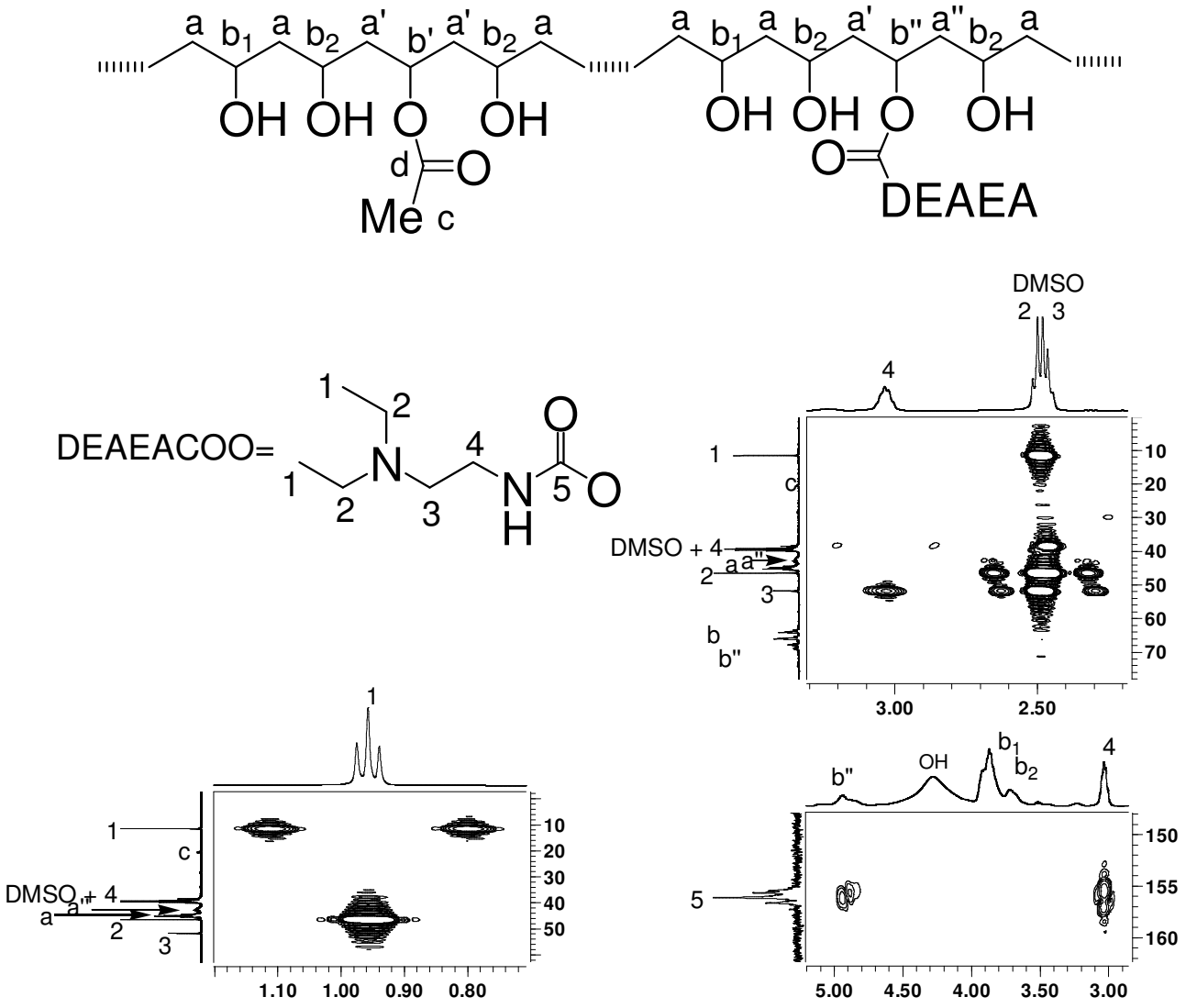


Figure 8. *^{1}H-^{13}C- HMBC crosspeaks of the amine substitution of E(33)*

3.5 Conclusion.

Using COSY and the ^{1}H-^{13}C HMQC and HMBC spectroscopy a complete assignment of the ^{1}H and ^{13}C spectra of the amine-modified PVA has been done. For M(32) the methine ^{1}H spectrum revealed 54% of VOHVAMVOH, 30 % of VAVAMVOH, and 16 % of VAVAMVA to be the microstructures of the amine modification. On the other hand, an average block length of 9 VOH units was estimated for the PVA backbone (M(32)). The ^{13}C spectra of methine contain components due to both compositional and configurational microstructures of the modified copolymer. Microstructures up to pentads centered on rr, mr, and mm are thus assigned to the compositional triad VOHVOHVOH; whereas only triads rr and mr are observed for triad VOHVOHVAM. We may thus deduce that mainly isotactic triads react with activated diamines. The carbonyl ^{13}C of both diamine and acetate show three signals, which reflect the three microstructures of substitution. The observation of HMBC cross peaks between amine carbonyl ^{13}C and the attached methine proton of the PVA backbone provide experimental evidence for the covalent bond between PVA backbone and diamine, and simultaneously confirms the assignment of the carbonyl ^{13}C signals. In the HMBC experiment, the observation of $^{3}J_{CH}$ coupling between the non-substituted methine ^{13}C (up to pentads) to its neighboring non-substituted methine ^{1}H (of up to triads) confirms the tacticity assignment carried out by Brar et al. on pure PVA.

3.6 References

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Chapter 4

Chapter 4: Design of Amine-Modified Graft Polyesters for the Effective Gene Delivery Using DNA loaded Nanoparticles

Pharmaceutical Research, accepted (see appendix)

4.1 Summary

Purpose: The design of a polymeric platform for effective gene delivery using DNA loaded nanoparticles.

Methods: The polymers were synthesized by carbonyldiimidazole (CDI) mediated coupling of diamines 3-diethylaminopropylamine (DEAPA), 3-dimethlyaminopropylamine (DMAPA) or 2-diethylaminoethylamine (DEAEA) to poly(vinyl alcohol) (PVA) with subsequent grafting of D,L-lactide and glycolide (1:1) in the stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups / monomer units). The polymers were characterized by ^{1}H-NMR, GPC-MALLS (gel permeation chromatography - multiple-angle-laser-light-scattering), and DSC (differential scanning calorimetry). DNA loaded nanoparticles prepared by a modified solvent displacement method were characterized with regard to their zeta (ζ) -potential and size. The transfection efficiency was assessed with the plasmid DNA pCMV-luc in L929 mouse fibroblasts.

Results: The polymers were composed of highly branched, biodegradable cationic polyesters exhibiting amphiphilic properties. The amine modification enhanced the rapid polymer degradation and resulted in the interaction with DNA during particle preparation. The nanoparticles exhibited positive ζ-potentials up to + 42 mV and high transfection efficiencies, comparable to polyethlyenimine (PEI) 25kDa/DNA complexes at a nitrogen to phosphate ratio of 5.

Conclusion: The polymers combined amine-functions and short PLGA chains resulting in water insoluble polymers, capable of forming biodegradable DNA nanoparticles through coulombic interactions and polyester precipitation in aqueous medium. The high transfection efficiency was based on fast polymer degradation and the conservation of DNA bioactivity.

4.2 Introduction

DNA vaccines have been subject to intensive research efforts recently and it has become increasingly clear that adjuvants are necessary to reduce the DNA doses used while reaching protective immune responses.[1] Adjuvants, such as micro– and nanoparticles have been studied intensively as DNA delivery systems providing i) a sustained and predictable DNA release; ii) targeting antigen presenting cells using particles < 10µm and iii) stabilization of DNA in physiological environment.[2] Several encapsulation techniques, mainly using biodegradable PLGA, have been reported, such as spray-drying[3] and modified double emulsion methods,[4] all of which utilizing high-speed homogenization or sonication. These shear forces were found to compromise plasmid integrity and bioactivity.[5,6] Additionally, DNA was damaged in the acidic environment created by PLGA degradation products.[3]

Here we describe a gentle solvent displacement method for the encapsulation of DNA relying on a new class of biodegradable polymers with rapid degradation properties.[7] This method allows the encapsulation of DNA without high speed / shear homogenization using amine-modified branched polyesters. These polymers interact with DNA by electrostatic interactions and facilitate nanoparticle formation due to their amphiphilic character. We systematically investigated these polymers to characterize the influence of polymer structure on functional properties such as nanoparticle size and charge and the protection of plasmid DNA by the transfection efficiency (Figure 1).

4.3 Experimental Section

Polymer Synthesis and Characterization. Biodegradable comb-branched polymers consisting of amine-modified poly(vinyl alcohol) (PVA) backbone

grafted with PLGA side chains in ratio [n(OH)/n(monomer)] of 1:10 and 1:20 were synthesized and characterized as previously described.[7] The amine modifications consisted either of 3-diethylamino-1-propylamine (DEAPA = P), 2-diethylamino-1-ethylamine (DEAEA = E) or 3-dimethylamino-1-propylamine (DMAPA = M). Briefly, after activation of the diamine component using carbonyl diimidazole (CDI) in tetrahydrofuran (Figure 2) the activated components were added to poly(vinyl alcohol) (PVA) (Fluka, degree of polymerization: P=300) in N-methylpyrrolidone and reacted for 4 d at 80°C. Then Lactide and glycolide (1:1) were grafted in stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups / monomer units) by bulk polymerization onto the amine-modified PVA-backbones at 150°C using tin(II) 2-ethylhexanoate as catalyst.

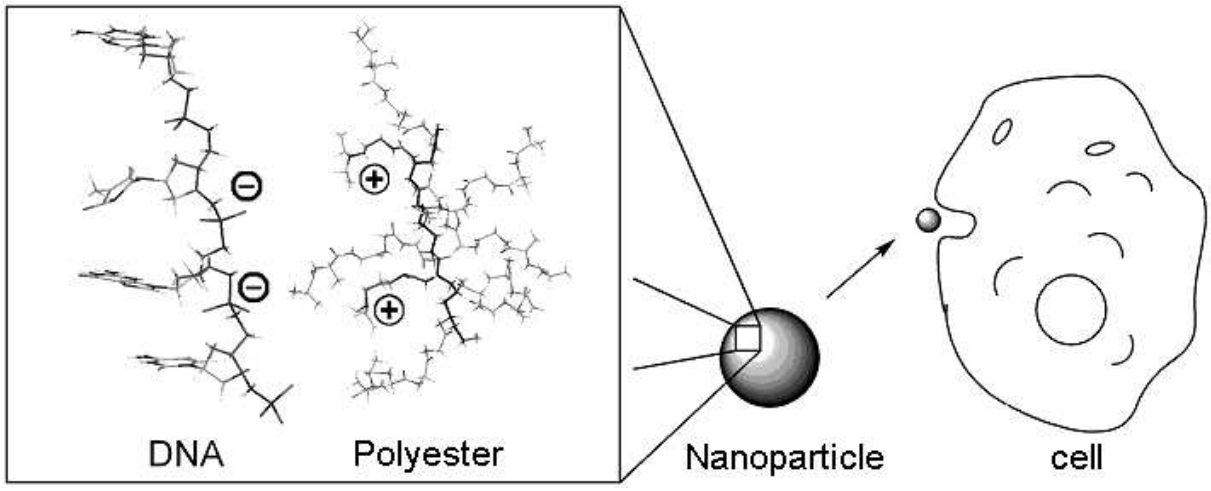

Figure 1. *Schematically depiction of formation, design and cell uptake of polyester/DNA nanoparticles*

The source-based IUPAC nomenclature for e.g. DEAPA modified polymers is the following: Poly(vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(DL-lactide-co-glycolide). As abbreviation we use

A(x)-y. (A indicates the type of amine substitution (P=DEAPA, M=DMAPA, E=DEAEA), x is the number of monomers in the backbone carrying amine substitutions, y is the PLGA side chain length calculated from feed). Resomer®502H (RG 502H) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

^{1}H-NMR spectra were generated in d_6-DMSO with a Jeol Eclipse+500 NMR Spectrometer (JEOL, USA) at 50°C using 64 scans (500 MHz). GPC-MALLS was carried out with a combination of DAWN EOS, Optilab DSP (Wyatt Europe GmbH, Germany) and PSS SDV linear M column (PSS, Mainz, Germany)(flow rate 0.5 mL/min, solvent: dimethylacetamide +2,5 g/L LiBr at 60°C). DSC measurements were conducted with a Perkin-Elmer DSC 7 (USA). Polymer degradation was measured gravimetrically after incubation of polymer films in PBS-buffer at pH 7.4 (37°C) over 21days according to citation 8[8].

DNA Nanoparticle Preparation and Characterization. Nanoparticles were prepared by a modified solvent displacement method.[9] Briefly, 500 µl of an aqueous solution containing 0.5 µg/µl plasmid DNA were added to 2.5 ml of an acetone solution containing 50 mg of the water insoluble polymer. The product was injected into 10 ml stirred 0.1% Pluronic™ F68 (BASF, Germany) in distilled water. The resulting nanoparticle suspension was stirred 3 hours under constant laminar air flow to remove residual acetone. Particle size and ζ-potential measurements were carried out in a Malvern Zetasizer 4 (Malvern, Germany), according to citation 9[9] after calibration with a Malvern –50 mV transfer standard. Scanning electron microscopy (SEM) was performed with a CamScan 4 (Cambridge, UK) after gold sputter coating using an AUTO 306 (Edwards, UK). High resolution transmission electron microscopy imaging (TEM) was performed after cryo-sectioning of the nanoparticles with a JEM 3010 (Jeol, Japan) on a collodium grid.

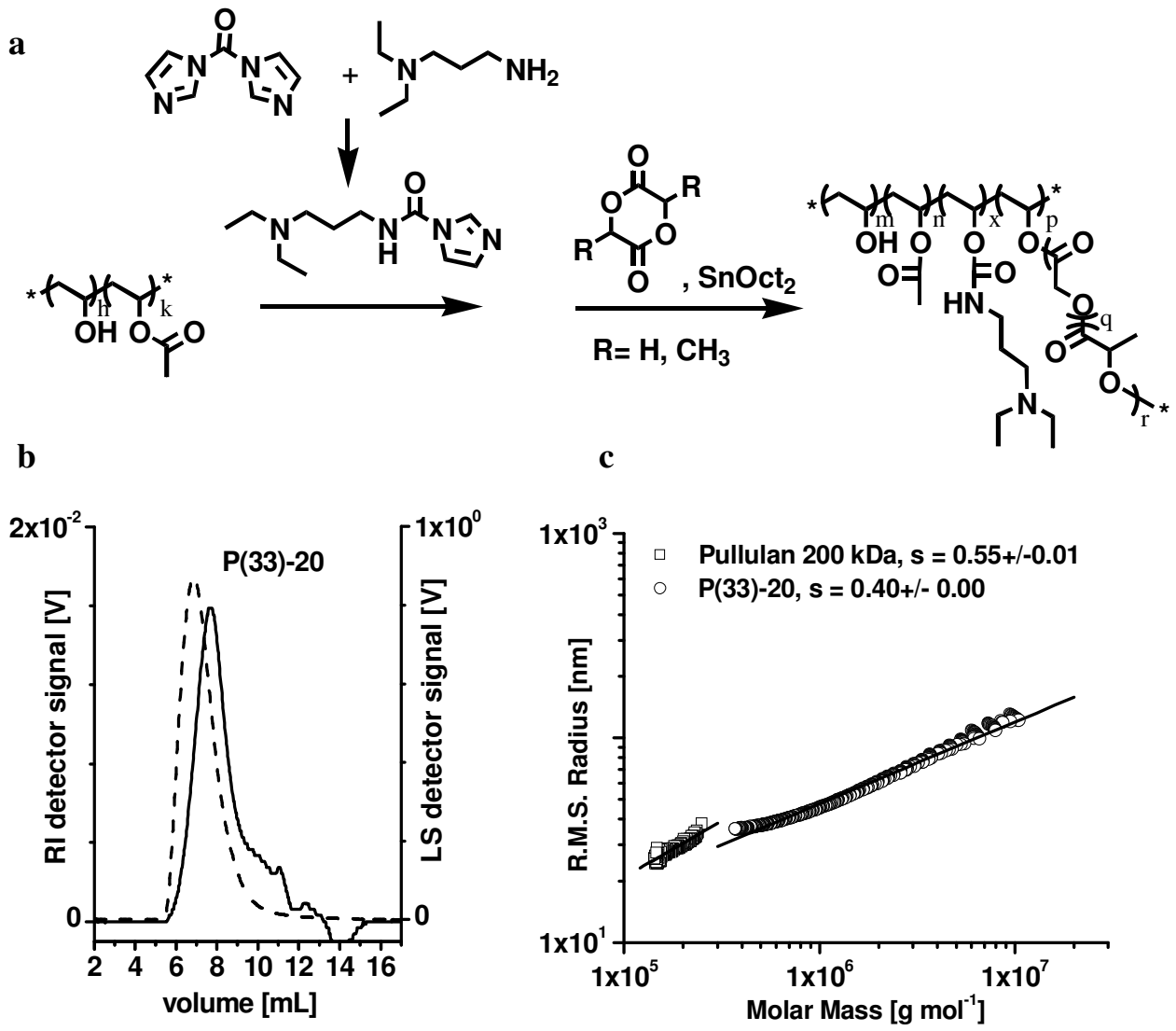

Figure 2. *The synthesis of Poly(vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide) using a three step process with PVA, Amine and CDI as precursors b) GPC elution profile of P(33)-20 (signal of the refractive index detector: straight line, light scattering signal: dashed line) indicating monomodal distribution of molar mass of the polyesters, but also a small, low molecular weight part c) plot of the radius of gyration against MW. The reduced slope of the amine-modified polyester (P33)-20) demonstrated the highly branched structure in comparison to random coiled pullulan.*

***In Vitro* Transfection Efficiency.** L929 mouse fibroblast (DSMZ, Germany) cells were plated at a cell density of 50 000 cells/2 ml in 12 well dishes 24 h prior to transfection. Aliquots of the particle suspension containing 4 µg pCMV-luc theoretical load were added to 0.5 ml glucose 5 % medium pH 7.4. The cells were pre-incubated with the nanoparticle suspension for 5 min, after which 1.5 ml cell culture medium containing 10% fetal calf serum (FCS) was added. The nanoparticle suspension was dispersed in the glucose medium before the addition of the medium, since instabilities of nanoparticle suspension were observed in the medium. The nanoparticle suspension was removed after 4 hours of incubation and replaced with fresh medium containing 10% FCS. Cells were harvested after 48 h and luciferase transfection efficiency was assessed according to citation 10[10]. Results were presented as luciferase / protein ratio [ng/mg].

4.4 Results and Discussion

In this study we present a new class of water-insoluble, amphiphilic polyesters, developed specifically for the use of DNA encapsulation. We hypothesized that the loading efficiency of DNA nanoparticles could be greatly increased by three characteristics of the comb-branched polymers. Firstly, electrostatic interactions are thought to stabilize and protect DNA during the encapsulation process. Secondly, fast polymer degradation rate should allow the release of bioactive DNA and thirdly, tertiary amino functions should facilitate gene delivery. We therefore developed polymers containing an amine-modified backbone for ionic interactions and possible degradation of endsomal/lysosomal vessels and relatively short but multiple, biodegradable PLGA side chains for fast polymer degradation. The unique properties of these polymers were confirmed in the nanoparticle formation process. DNA was solubilized by the polymer in the

acetone solution due to the amphiphilic characteristics in the acetone/water mixtures used for the solvent displacement method, suggesting strong DNA/polymer interaction. The subsequent injection in aqueous medium resulted in nanoparticle formation. The biodegradable DNA nanoparticles exhibited effective gene delivery, demonstrated by high transfection efficiencies *in vitro*.

We synthesized 24 cationic, as well as two neutral derivatives of amine-modified comb-branched polyesters, and characterized their functional properties in relationship to their structure (Figure 2). We grafted relatively short PLGA side chains consisting of approximately 10 or 20 repeating units on the amine-modified PVA-backbone. Consequently, already a small number of hydrolytic cleavage events would result in water soluble polymer fragments, thereby releasing the encapsulated DNA.

The total number of biodegradable PLGA side chains grafted on an amine-modified PVA backbone ranged from 150 - 240, resulting in a cationic and water insoluble polyester. The general characteristics of the polymers properties with different amine substitutions (DEAPA / DMAPA / DEAEA) were similar. The DEAPA substituted polyesters were all soluble in acetone and thus, suitable for the nanoparticles preparation process. Therefore, we selected this type of polymers for further study.

The brush-like structure of the graft-polymers was verified using ^{1}H-NMR spectroscopy, as well as GPC-MALLS depicted in (Figure 2b/2c) (P(33)-20). The degree of PLGA side chain substitution was calculated from the ^{1}H-NMR spectrum showing that only 5 to 35 % hydroxyl groups of the PVA still remained free after reaction. The PLGA side chain lengths (SCL) were calculated from these data, demonstrating good correspondence with the theoretical values (Table 1). However, increasing amine substitution led to a decrease of SCL. A possible explanation could be an inhibitory effect of the amino-function on the tin catalyst which competed with lactide/glycolide

monomers. The molecular weights of the polymers were calculated from a combination of this data, based on the known amine substitution of the PVA backbones. The values for molecular weight (MW) were confirmed by GPC-MALLS (Figure 2b). GPC measurements demonstrated the monomodal MW distribution of the polyesters. The molecular weights did not show an expected trend towards lower MW with increasing amine-substitution because of i) the fast degradation of the polyesters, ii) the resolution of GPC and iii) decreasing acetate content with increasing amine substitution. The nanostructure of the polymers in solution was characterized by the evaluation of the radius of gyration in a double logarithmic scale plotted against the molar mass of the polyesters (Figure 2b). The resulting slope of the linear fit was compared to the slope of random coiled pullulan (0.55). The flatter slope exhibited by the amine-modified polyesters (P(33)-20: 0.40) indicated a compact, highly branched nanostructure of these polymers.

Polymer degradation at 37°C in PBS buffer at pH 7.4 was greatly accelerated as compared to common linear PLGA. NMR studies demonstrated the reduction of the SCL of P(12)-10 from originally 10.8 units to 8.6 units in seven days and to 5.4 units after another week (Figure 3). These measurements cannot be exclusively explained by physical erosion. As such an erosion would either not show deceased SCL or only a small SCL reduction. This behavior may substantially reduce the exposure time of an encapsulated substance to the detrimental effects of acidic degradation products generated by PLGA bulk erosion. This behavior is remarkable, since the molecular weights of the graft-polyesters are approximately ten-fold higher than the linear PLGA (RG 502H) (Table 1). This property corresponded to our hypothesis of a substantial reduction in time for the drug release.

An increase in the PLGA side chain length from approximately 10 to 20 repeating units increased the degradation time as expected. P(33)-20, for

example, showed a degradation half-life of 13 days, compared to one day for the P(33)-10 analogue (Table 1).

Table 1. *Characterization of the amine-modified polyesters, demonstrating the low glass transition temperatures, extremely high molecular weights (MW) combined with fast polymer degradation at 37 °C in PBS buffer. DNA Nanoparticles were exhibited smaller sizes and high ζ (zeta)-potentials, compared to PLGA nanoparticles.*

Polyester	T_g [°C][a]	MW [kg mol^{-1}]		SCL[d]	Degradation half-life[e,f] [d]	Nanoparticle size[f] [nm]	Zeta Potential[f] [mV]
		M_n[b]	M_n[c] M_w[c]				
P(6)-10	30.6	(107)	211 281	11.2	>21	n.d.	n.d.
P(12)-10	30.8	179	196 263	10.8	9	163 ± 1	22 ± 1
P(33)-10	27.7	179	195 367	9.4	1	152 ± 3	35± 3
P(68)-10	11.5	172	282 799	7.4	n.d.	309 ± 16	42 ± 2
P(12)-20	33.0	422	227 304	19.3	>21	n.d.	n.d.
P(33)-20	32.8	385	375 712	17.2	13	351 ± 7	31± 5
RG 502H[g]	36.5	6.1	6.6[g] 15[g]	84.6	16	602 ± 3	-55 ± 3

(a) Glass transition temperature (heat rate: 10 °C/min, -10 to 200 °C, second run)

(b) MW calculated from the ^{1}H-NMR data

(c) MW from GPC-MALLS (DAWN EOS, Optilab DSP, column PSS SDV linearM, solvent DMAc+2.5 LiBr g/L, 60°C, 0.5 mL/min)

(d) PLGA side chain length calculated from ^{1}H NMR

(e) Time at which 50% mass loss of a polymer film occurs (degradation half-life)

(f) Mean of three independent measurements ± standard deviation

(g) Commercial PLGA (1:1) lactic acid : glycolic acid subunits. MW: specifications supplied by the manufacturer (Boehringer Ingelheim)

The degradation rates increased more than proportionally with increasing amine substitutions of the polymer. For example, the degree of amine substitution in P(33)-10 was three times greater than in P(12)-10, however, P(33)-10 exhibited a nine-fold increase in the rate of degradation. This effect can be explained by the rapid, initial PLGA mass loss of the P(x)-10 polymer in comparison to the slower mass loss of P(x)-20 polymers, attributed to a catalytic effect of the amino-functions, promoting the acidic ester degradation, caused by their protonation. This would lead to new carboxyl-functions restarting the catalytic cycle. Further, the protonated amino-functions will promote water uptake into the polymer affecting an increased rate of hydrolysis.

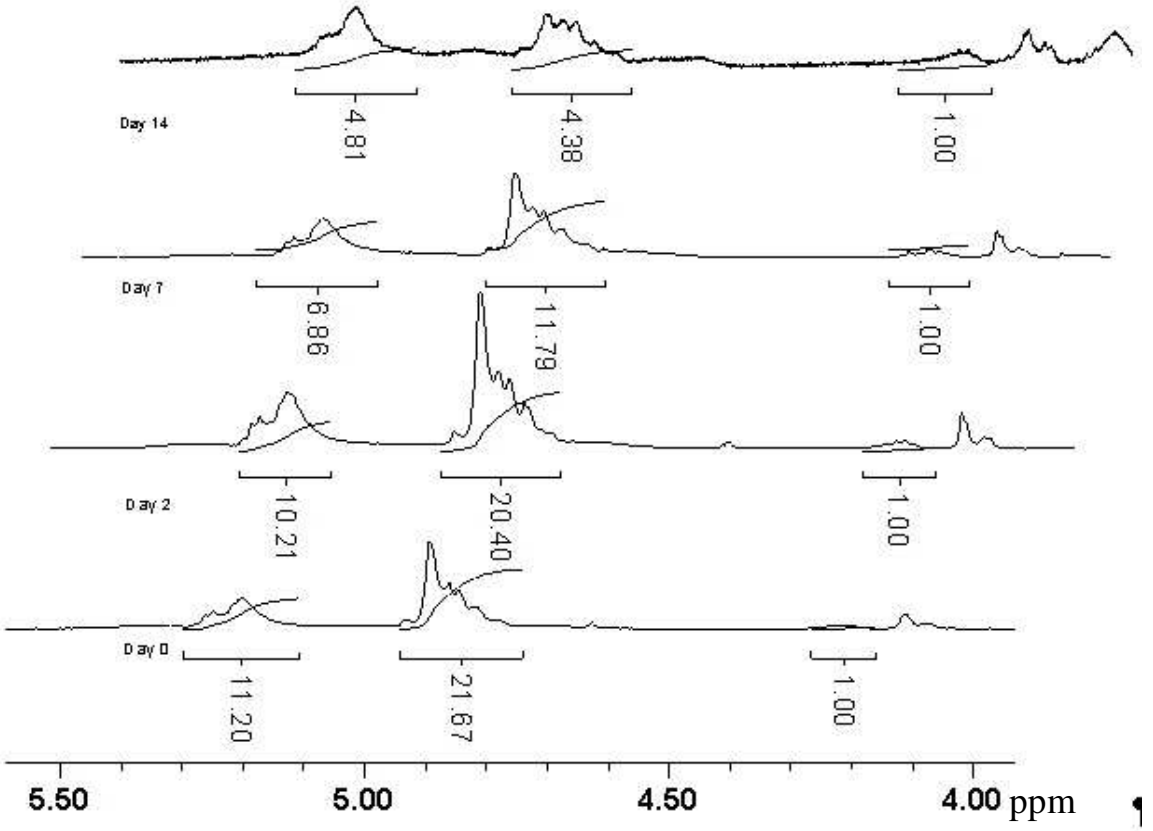

Figure 3. *^{1}H NMR spectra of P(12)-10: degradation after two, 7 and 14 days, Decrease of central methylene group signals in compare to the end group signals of the polyester side chains, A proof for the reduction of side chain length caused by degradation.*

All polymers displayed glass transition temperatures near 30 °C, implying they exist in the glassy state in physiological environment (Table 1). In general, polymers with longer PLGA side chains and reduced amine substituents had higher transition temperatures. Thus, the amine-groups were thought to have acted as a plasticizer in the polymer. The influence of the polymer chain motility has to be further investigated for possible interactions with cellular membranes and the influence on the gene delivery process.

An important feature of the polymer characteristics were the tertiary amine-modifications of the polymers, hypothesized to stabilize DNA within the polymer matrix and to facilitate the gene transfer. Ionic interactions with the polymer were presumably the reason for the solubilization of DNA in the acetone/water mixture. For example, DNA could be completely dissolved in an acetone/water 5:1 [v/v] solution of the polymer, whereas DNA alone precipitated. Therefore, no further homogenization process was necessary to disperse DNA before the subsequent coacervation of the water insoluble polymer in the 0.1% poloxamer solution. Nanoparticles were only obtained with polymers exhibiting amine substitutions of 4% (P(12)-10) and higher, underlining the importance of the amphiphilicity induced by the amine substituents. The structure of the polymers was described to be brush-like, due to the short and numerous PLGA chains. Therefore we did not expect a micellar assembly of the polymers neither in acetone, nor in the non-solvent water. In contrast, water soluble, poly(l-lysine)-g-PLGA polymers had a more distinct amphiphilic structure, containing a shorter hydrophilic backbone with few and long PLGA chains of approximately 210 monomers.[11]

The nanoparticles exhibited hydrodynamic diameters ranging from 152.4 nm (P(12)-10) to 351.3 nm (P(33)-20), whereas PLGA (RG 502H) nanoparticles prepared by the same procedure were approximately 200 nm larger (Table 1).

Hence, despite a 33-fold higher molecular weight, the amphiphilic qualities of the polymers, influencing the viscosity, resulted in nanoparticles of reduced size. Particle sizes measured by photon correlation spectroscopy were confirmed by scanning electron microscopy (SEM) and transmission electron microscopy of nanoparticle cryo-sections (TEM). The particle morphology was examined by these methods as well (P(12)-10, Figure 4). Particles were uniform in size and had smooth surfaces.

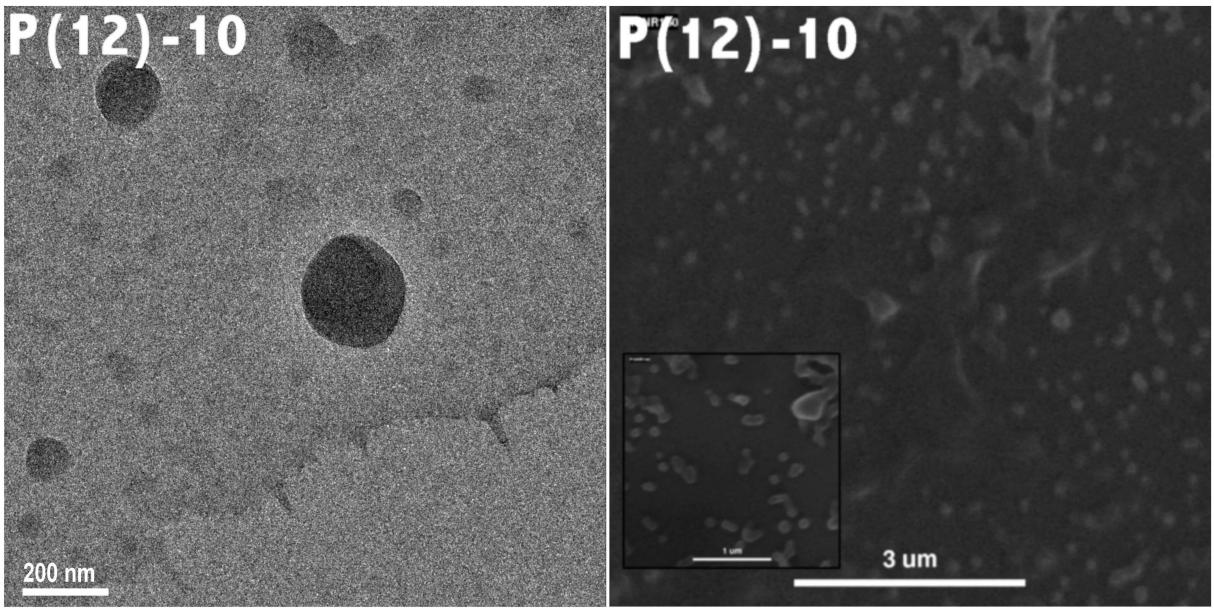

Figure 4. *TEM (left) and SEM (right, 1µm scale of the inlay) micrographs of DNA P(12)-10 nanoparticles confirm the particulate structure and the size measured by photon correlation spectroscopy.*

ζ-potentials of all preparations were clearly positive, with the exception of the linear PLGA, arising from the DNA phosphate groups, which were inverted by the cationic polymers.

All DNA nanocarriers were used *in vitro* for transfection assays, as efficient gene delivery remains a prerequisite for subsequent *in vivo* immunization. By directly using the nanoparticles *in vitro*, we could detect the gene transfer properties of the amine-modified polymers, as well as the DNA bioactivity after

nanoparticle preparation. Free plasmid and DNA complexes with PEI 25kDa, a potent polymeric transfection agent, were used as references to compare the luciferase expression levels with other polymer types.[12] On account of this, we could consider the nanoparticles as a potent transfection agent. All DNA nanocarrier formulations resulted in increased transfection efficiencies compared to free DNA (Figure 5). The efficiency increased exponentially with the amount of amine substitution of the polymer. The 500,000-fold increase in transfection efficiency of the P(68)-10 plasmid nanoparticles, compared to free DNA, was remarkable, especially when considering the fact that the amount of polymer in relation to DNA was reduced by the factor 0.4 to avoid nocuous effects of an excess of cationic charges. Nanoparticles of P(x)-10 polymers clearly displayed higher efficiencies than their P(x)-20 analogues.

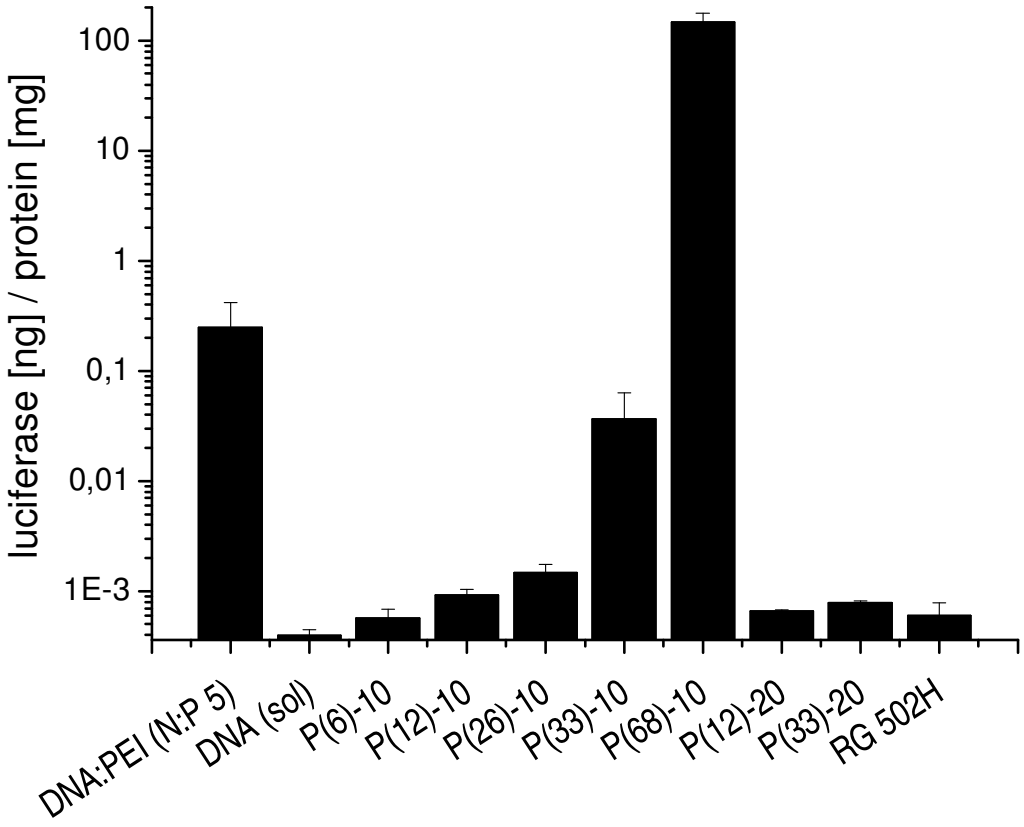

Figure 5. *Transfection efficiency of pCMV-luc DNA, encapsulated in amine-modified nanoparticles was greatly enhanced compared to free DNA, DNA: PEI 25kDa complexes (N/P 5) and a DNA RG 502H particle preparation.*

The careful elucidation of the transfection mechanism of the polymers is yet to be investigated, however, we assume that these findings do not depend on increased ζ-potentials or particle size effects, but must be dependent on the particle structure and DNA polymer interactions as well. The polymers consisted of 3-dimethlyaminopropylamine substituents, representing tertiary amines which may be essential for the cell uptake and endosomal escape of polyplexes by the `proton sponge´ effect.[13]. This effect could be intensified by the fast polymer degradation resulting in an increase of the osmotic pressure in the endosome, as proposed by Koping-Hoggard.[14] However, other mechanisms of endosomal release have eventually to be considered, for example, fusogenic activities, taking into account the low glass transition temperatures and hydrophobic moieties of the polymer,[15] or the `hydrogel effect´ of swelling polymer in the endosome.[16] Therefore, we concluded that the combination of different properties within one biodegradable polymer, resulting in a fast degradation, ionic interactions with DNA and the formation of water insoluble nanoparticles, provided considerable advantages with regard to the transfection efficiency *in vitro*. Further experiments investigating the transfection efficiency under *in vivo* conditions and the encapsulation with other compounds, such as peptides, susceptible to acid degradation are in progress.

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Chapter 5

Chapter 5: Summary and Outlook

5.1 Summary

In the present thesis a novel tailor-made polyester class based on amine-modified backbones and PLGA side chains was synthesized after designing its chemical structure under consideration of all necessary assumptions for a drug delivery system.

Chapter 2: 55 different well defined poly(vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide)s were synthesized to investigate the relationship between structure and properties. A three step process was used to synthesize the module-like structure of the polymers. In step one, one-side protected diamines were activated using carbonyl diimidazole chemistry. The activated diamines were coupled to commercially available poly(vinyl alcohol) (saponification of 88 % , molecular weight 15000) via urethane bonds (figure 1). Changing the ratios of the two components amine substitutions between 0 and >25 % are possible. Due to this synthetic step amino functions respective charges were inserted into the polymer. Using NMR and FT-IR measurements and CHN analysis the successful amine substitution could be proved and the amine content in the backbone was determined. In the third and last step D,L-lactide and glycolide were grafted onto the PVA backbone in a 1:1 ratio. Tin(II) 2-ethylhexanoate was used as catalyst and four different polyester side chain lengths were realized using bulk polymerization process. Using the stoichiometric ratio free hydroxyl groups to monomer units (lactic/glycolic acid) two major polymer types were synthesized to investigate the properties of the graft polyesters. Polyesters with relative short side chain lengths of one and two units (type I) and such with side chain lengths of 10 and 20 units (type II) were synthesized.

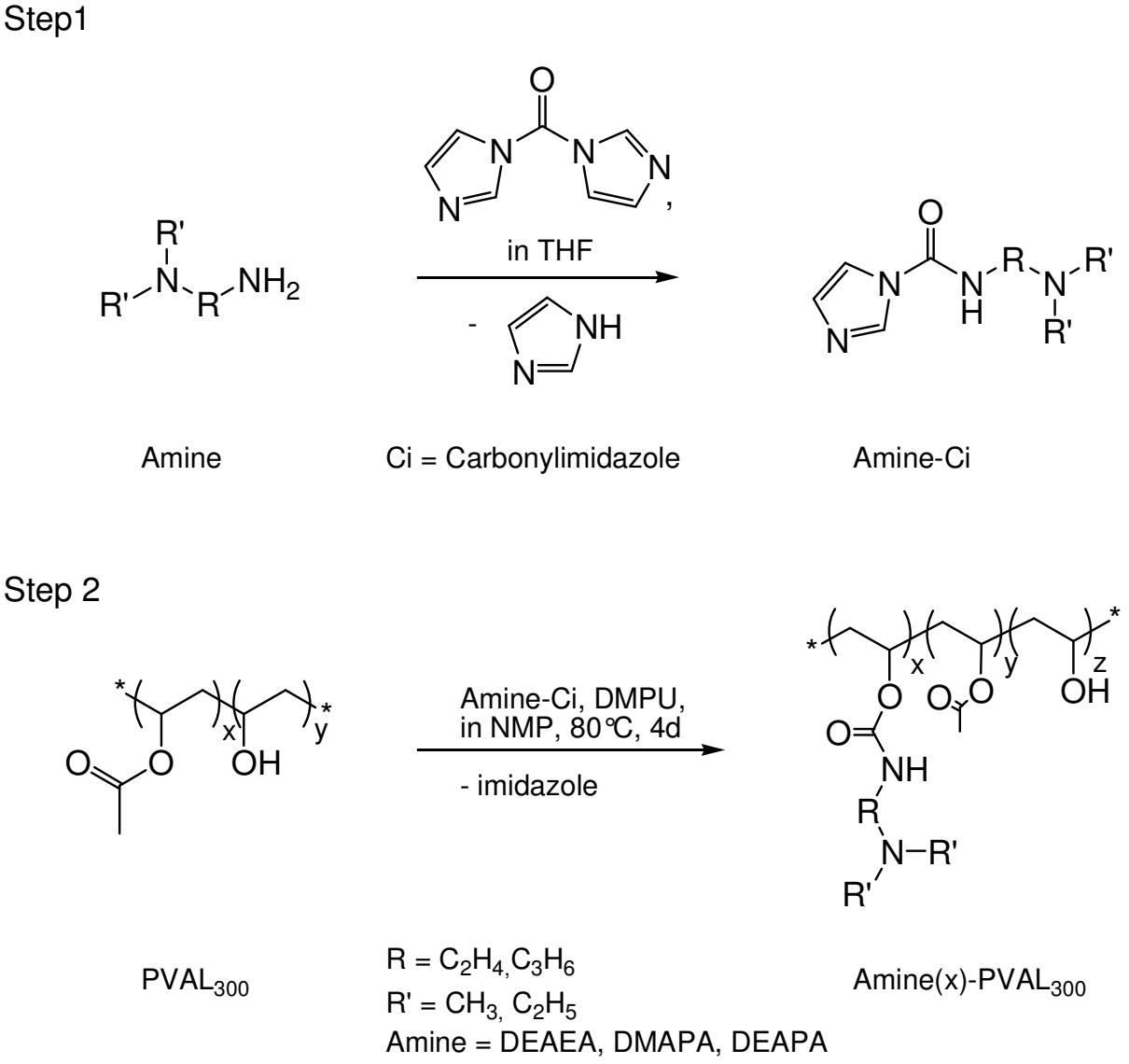

Figure 1. *Step 1: Activation of the amine using carbonyl diimidazole in water free tetrahydrofuran (THF); Step 2: Coupling of amine and poly(vinyl alcohol): Synthesis of the backbone of the new developed carrier system*

To simplify nomenclature the abbreviation A(x)-y is suggested (A indicates the type of amine substitution [P= DEAPA= 3-diethylaminopropylamine, M= DMAPA= 3-dimethylaminopropylamine, E= DEAEA= 2-diethylaminoethylamine], x is the number of monomers in the backbone

carrying amine substitutions, y is the PLGA side chain length calculated from feed)(figure 2)

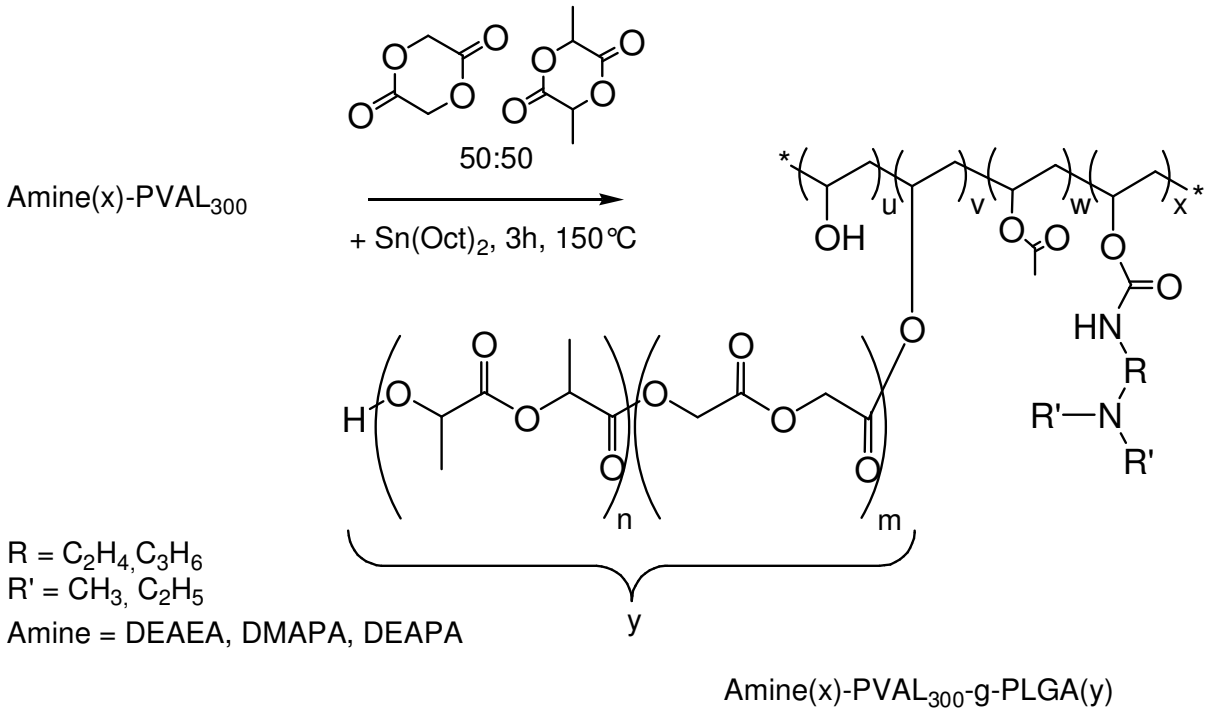

Figure 2. *Structure and synthesis of the graft polyester with hints about the further used nomenclature*

The polyesters show strong structure-depending solution behavior. This is influenced by side chain length and degree of amine substitution. While high amine substitution and small side chains lead to water soluble polyesters small amine substitutions and long side chains result in organic soluble polyesters. In general type I polyesters are more hydrophilic than type II ones.

Using NMR and GPC-MALLS the graft structure of the polyesters were proven. The molecular weights were determined by static light scattering. A double logarithmic plot of r.m.s. radii of gyration against molar masses confirmed the highly branched and compact structure of the synthesized polyesters.

Beside solution behavior the glass transition temperatures (T_g) show also a strong dependency on PLGA side chain length and a weak one on amine substitution. PVAs without PLGA grafting have the highest T_g. All other polymers using the same backbone show smaller T_g's which could be arrange on a parable-like curve. The T_g of all 1:10 polyesters is always the minimum on this parable. Increasing degrees of amine substitution also result in a decrease of T_g of polymers which having the same side chain length. Both observations are a result of disruption of microstructure within the polymer. In PVA this structure is mainly formed by hydrogen bonding. A decrease of T_g means a better segment flexibility within the polymer chain. Having a chain length around ten this flexibility seems to be at the maximum. At this point hydrogen bonding is very low and the side chains are too small to disturb their own movements. Increasing degrees of amine substitution also results in a loss of hydrogen bonding caused by the smaller ability of tertiary amino functions to form hydrogen bonds. The T_g of all type II polyesters are below blood heat which is helpful for their use as drug delivery systems in the shape of microparticles or implants.

The nanostructure of the polyester could be clarified by TEM analysis. Using osmium tetroxide the hydrophilic parts of the polymer were stained and a separation of hydrophilic and lipophilic polymer parts in the range between 1-2 nm was proved.

The strong influence of the amine substitution on the degradation of the polyesters was researched. Modifying one eighth of the free hydroxyl groups of the PVA backbone an extremely short degradation time smaller two days could be reached. The degradation mechanism seems to be a mixture of bulk and surface erosion. The very fast degradation seem to be mainly caused by amine substitution. Because of its hydrophilic and basic nature it promotes the breakdown of the ester bonds.

In conclusion, a new polyester class was developed. Varying side chain length and amine substitution these polyesters can be especially designed for drug delivery problems. Their structure was clarified by NMR-spectroscopy and GPC-MALLS (multi-angle-laser-light-scattering). By variation of side chain length and /or amine substitution the solution and thermal behavior could be adjusted. Aimed amine substitution could be used to control degradation speed of the polyesters. Grafting of short side chain onto amine modified PVA converted a pure bulk erosion into a mixed surface and bulk erosion. To our knowledge this is the first time a degradation time of PLGA polyesters smaller than 15 days could be reached and could be freely modified by changing side chain length and amine substitution. Due to their solution and thermal behavior this polymer class is suitable for manufacturing of microparticles and implants.

Chapter 3: Using two dimensional NMR techniques like ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HMQC a clear assignment of ^{1}H and ^{13}C NMR signals of the amine modified PVAs was established. Microstructures up to pentads centered on rr, mr, and mm were assigned for triad VOHVOHVOH whereas only rr and mr was observed for triad VOHVOHVA. Using ^{1}H-^{13}C HMQC the main location where the reaction between activated diamine and PVA backbone take place was identified as the isotactic triads. The covalent bond between amine and PVA backbone was proven by ^{1}H-^{13}C HMBC using long range coupling of methylene groups of backbone and amine. The microstructure of the amine modification was revealed as 54% VOHVAMVOH, 30% VAVAMVOH, and 16 % of VAVAMVA. A average block length of 9 VOH units was estimated for the PVA chain.

Chapter 4: Because of short side chain lengths very short degradation times could be reached. Especially, P(33)-10 has extremely short degradation times of

less than two days. Using proton NMR a shortening of the PLGA side chains could be measured. This approves degradation of the polyesters. Solvent displacement techniques could be used to get DNA carrying nanoparticles having sizes around 150 nm. TEM and SEM images show oval to spherical shaped particles. It was demonstrated that some of the synthesized Polyesters have interesting transfection abilities in *in-vitro* experiments on L929 mouse fibroblasts. Especially, the particles formed by P(68)-10 feature an extraordinary high transfection even higher than that of PEI/DNA complexes. Holding the side chain length constant it was possible to increase transfection by increasing the amine substitution of the backbone. An opposite effect results at constant amine substitution and elongation of the PLGA chains.
The development of a multi functional DNA-delivery system shall be deemed to be successful.

Conclusion: Due to all measurements and observation the usefulness of the developed polymer class as tailor-made drug delivery system was proven. It possesses interesting degradation and transfection capabilities. These features give the polymer class high potential as carrier system for DNA vaccination and for the delivery of other hydrophilic drugs.

5.2 Outlook

Further studies concerning toxicology and mechanism of degradation are necessary. At this point the synthesized polyesters seem to be less harmful than common transfection argents like PEI or viruses. Precise studies using MTT-test and other tests for toxicology shall be used to confirm this.

The degradation of the polyesters seems to have characteristics of both surface and bulk erosion. Further precise degradation studies using only a small number of polymers are necessary to investigate the degradation mechanism in detail.
It could be assumed that amine-modified PVA-polyesters show a higher drug loading than linear PLGAs. The release characteristics should also be different. Both, loading and release characteristics of the polyesters could not be investigated in detail. Further studies are necessary.
Beside side chain length, type of amine substitution and degree of amine substitution further parameters of the synthesized polymer class can be varied. It is also possible to modify the lactide/glycolide ratio in the side chains or to use ε-caprolactone as third monomer in the grafting process. Further poly(vinyl alcohol)s having different molar masses and degrees of saponification can be used as backbones.
The versatility of the polymer system makes it suitable for a wide range of drug delivery problems. There is no limit to custom tailor–made adjustment of these polymers.

5.3 Zusammenfassung

In der vorliegenden Arbeit ist es gelungen ein modular aufgebautes Polymersystem basierend auf aminmodifizierten PVA und PLGA Seitenketten zu entwickeln, das sich maßgeschneidert an unterschiedlichste Aufgaben des Arzneistofftransports (drug delivery) anpassen lässt.

Kapitel 2:. 55 unterschiedliche, genau definierte Polymere wurden zur genauen Untersuchung der Struktur-Eigenschaftsbeziehungen der Poly(vinyldialkylaminoalkylcarbamat-co-vinylacetat-co-vinylalkohol)-graft-Poly(D,L-lactid-co-glycolid)e synthetisiert. Die Synthese dieser Polymere

erfolgte über einen dreistufigen Prozess. In Stufe eins wurden einseitig geschützte Diamine mittels Carbonyldiimidazol (CDI) aktiviert und in einem zweiten Schritt über eine Urethanbindung an kommerziell erhältlichen, zu 88% verseiften Polyvinylalkohol gekoppelt (Abbildung 1).

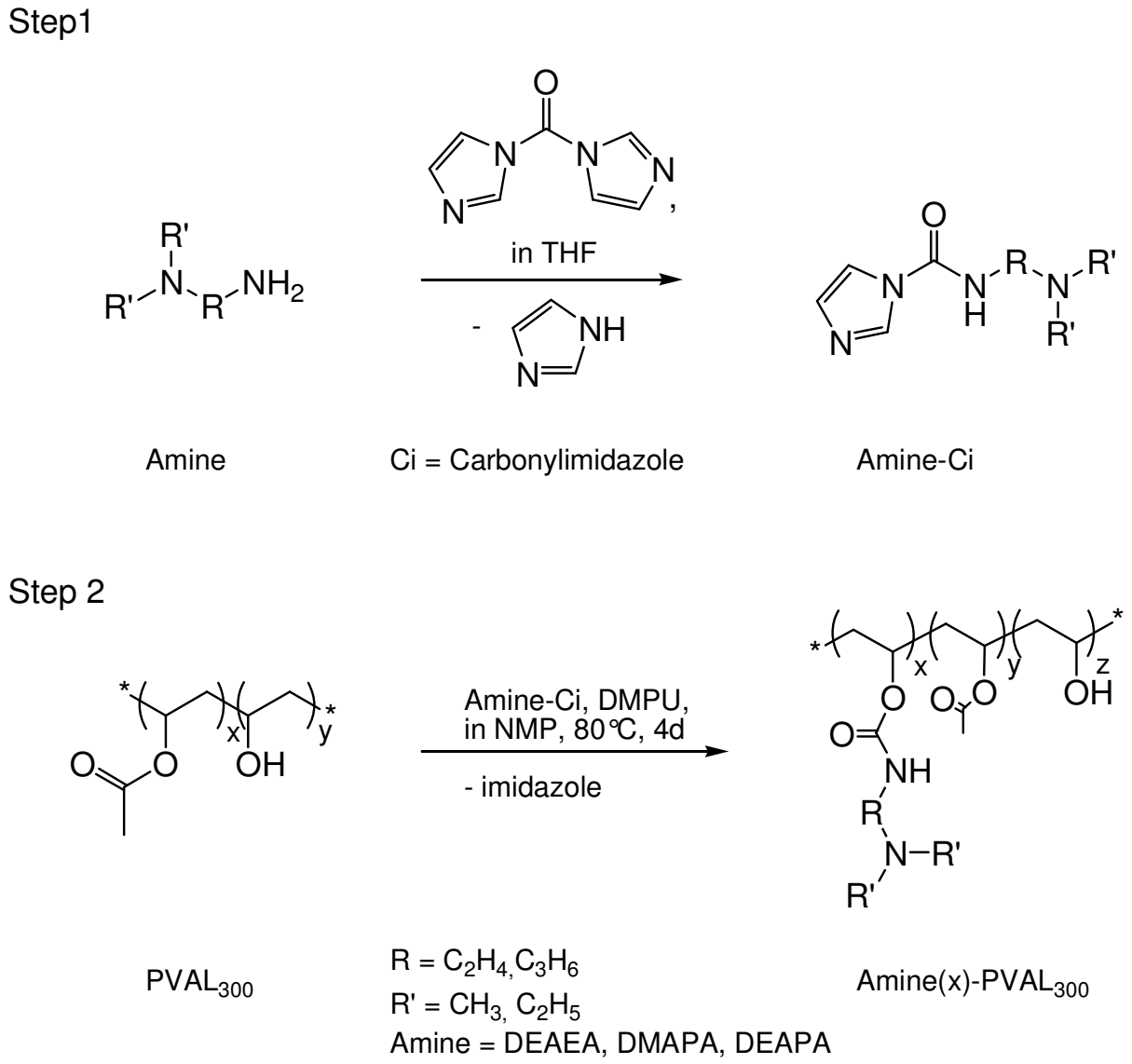

Abbildung 1. *Stufe 1: Aktivierung des Amins durch Carbonyldiimidazol (CDI) in wasserfreiem THF; Stufe 2: Kopplung von Aminen an Polyvinylalkohol: Synthese des Rückgrats des neu entwickelten bioabbaubaren Pfropfcopolyestersystems*

Mittels Änderung des stöchiometrischen Verhältnisses beider Komponenten ist eine Variation der Aminsubstitution innerhalb einer großen Bandbreite von 0 bis >25 % möglich. Durch NMR und FT-IR Messungen sowie CHN-Analytik konnte die Aminsubstitution nachgewiesen und der genaue Substitutionsgrad des jeweiligen PVAs bestimmt werden.

In der letzten Stufe wurden dann D,L-Lactid und Glycolid in einer durch Zinn(II)-2-ethylhexanoat katalysierten Substanzpolymerisation im Verhältnis 1:1 in unterschiedlichen stöchiometrischen Verhältnissen (freie OH-Gruppen : Milch- und Glykolsäure–Monomeren) auf das Rückgrat gepfropft (Abbildung 2).

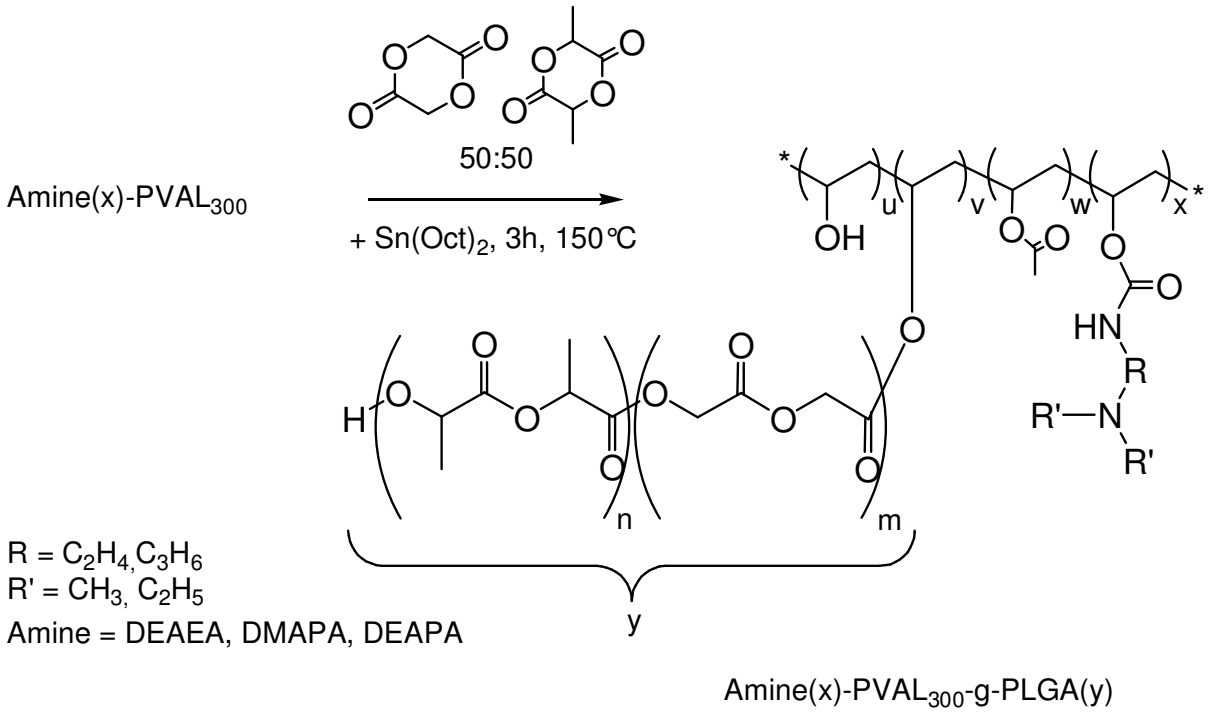

Abbildung 2. *Struktur und Synthese des Pfropfcopolyesters mit Nomenklaturerläuterung*

Zwei Haupttypen wurden synthetisiert, mit deren Hilfe die Eigenschaften der Polymerklasse genauer untersucht wurden. Dies waren zum einen relativ kurzkettige Pfropfpolyester in den stöchiometrischen Ansatzverhältnissen 1:1 und 1:2 (Typ I) und zum anderen Polyester in den Ansatzverhältnissen 1:10 und 1:20 (Typ II).

Als vereinfachende Nomenklatur und Abkürzung wurde die Formel A(x)-y eingeführt (A steht für das Amin und kann lauten P, E oder M (3-Diethylaminopropylamin=DEAPA=P, 2-Diethylaminoethylamin=DEAEA=E und 3-Dimethylaminopropylamin=DMAPA=M); x steht für die durchschnittliche Anzahl aminmodifizierter Monomere pro Kette, während y die theoretisch aus der Ansatzgröße ermittelte Seitenkettenlänge wiedergibt) (Abbildung 2).

Die allgemein amphiphile Struktur dieser Polyester bedingt eine starke Abhängigkeit des Lösungsverhaltens von Seitenkettenlänge und Aminsubstitution. Mit steigender Aminsubstitution werden zunehmend hydrophilere Polymere erhalten, während bei Verlängerung der Polyesterseitenketten ein gegenteiliger Trend zu verzeichnen ist. Die hochsubstituierten, kurzkettigen Polyester (Typ I) sind weitgehend wasserlöslich, während gering substituiert und neutrale, langkettige Polyester nur noch eine Löslichkeit in organischen Lösungsmitteln wie Dichlormethan und Aceton aufweisen.

Die Pfropfstruktur der Polyester wurde mittels NMR-Spektroskopie und GPC-MALLS Untersuchungen nachgewiesen. Die Molekulargewichte wurden mittels statischer Lichtstreuung bestimmt. Die doppelt- logarithmische Auftragung des Gyrationsradiuses gegen die Molmasse bestätigte den hochverzweigten, kompakten Charakter der Polyester.

Neben dem Lösungsverhalten zeigen auch die Glasübergangstemperaturen eine starke Abhängigkeit von den Seitenkettenlängen und eine weniger starke von

der Aminsubstitution. So weisen die ungepfropften PVAs die höchsten Glasübergangstemperaturen (T_g) auf, während die der anderen Polymere entlang einer Parabel angeordnet sind, deren Minimum bei den 1:10-Polyestern zu finden ist. Allgemein lässt sich bei einer Steigerung der Aminsubstitution eine Abnahme von T_g beobachten. Beide Phänomene können durch eine Störung der Mikrostruktur des Polymers, die bei PVA hauptsächlich durch die Fähigkeit zur Bildung von Wasserstoffbrückenbindungen bedingt ist, erklärt werden. Im Falle der 1:10-Polyester ist diese Störung und damit die Kettenbeweglichkeit am Größten. Durch eine Verlängerung der Seitenketten kommt es dann aufgrund der jetzt zu lang gewordenen Ketten zu einer Umkehr des Trends, da diese sich gegenseitig in ihrer Beweglichkeit behindern. Die Erhöhung der Aminsubstitution stört ebenfalls die Bildung von rotationsbehindernden Wasserstoffbrückenbindungen, da tertiäre Aminfunktion weniger zu deren Bildung geeignet sind und es somit zu einer Abnahme von T_g kommt. Alle Typ II Polyester haben T_g unter Körpertemperatur und sind dadurch für einen Einsatz als Mikropartikel oder Implantat gut geeignet.

Die Nanostruktur der Polyester ließ sich mittels TEM Untersuchungen nachweisen. Durch die Anfärbung mit Osmiumtetroxid findet eine Schwärzung der hydrophilen Bereiche des Polymers statt. Dadurch konnte innerhalb der Pfropfcopolyester eine Auftrennung der hydrophilen und lipophilen Polymerbereiche in der Größenordnung um 1-2 nm nachgewiesen werden.

Durch Abbauuntersuchungen an den Polyestern wurde der starke Einfluss der Aminsubstitution gezeigt. Durch Umsatz eines Achtels der freien OH-Gruppen konnte eine extrem kurze Abbauzeit von weniger als zwei Tagen erreicht werden. Weiterhin wurde deutlich, dass es sich bei dem Abbaumechanismus dieser Polyester um eine Mischung aus ‚Bulk Erosion' und Oberflächenabbau handelt. Offensichtlich wirkt die Aminsubstitution durch ihre hydrophilen,

basischen Eigenschaften und ihren teilweise protonierten Zustand stark abbaufördernd.

Zusammenfassend lässt sich sagen, dass eine neue Klasse von Polyestern synthetisiert worden ist, die sich durch Variation der Seitenkette und der Aminsubstitution direkt an das jeweilige Einsatzgebiet anpassen lässt. Ihr struktureller Aufbau konnte durch NMR Spektroskopie und GPC-MALLS (Mehrwinkel-Laser-Lichtstreuung) eindeutig charakterisiert werde. Durch Veränderung von Seitenkettenlänge und Aminsubstitution lässt sich sowohl das Lösungsverhalten als auch das thermische Verhalten einstellen. Mittels gezieltem Amineinbau ist es gelungen die Abbaugeschwindigkeit der Polyester weitestgehend zu steuern. Durch Einbau kurzer Polyesterseitenketten ließ sich der Abbauverhalten von einem reinen „Bulk Erosion"-Mechanismus hin zu einer Mischung aus Oberflächenabbau und ‚Bulk Erosion' verschieben. Dadurch ist es unseres Wissens erstmals, trotz erstaunlich hoher Molekulargewichte, möglich geworden PLGA Polyester mit Abbauzeiten von unter 15 Tagen zu synthetisieren, sowie deren Abbauverhalten durch Änderung von Seitenkettenlänge und Aminsubstitution frei zu variieren. Diese eignen sich aufgrund Ihres Lösungsverhaltens und ihres thermischen Verhaltens für die Herstellung von Nano- und Mikropartikeln wie auch Implantaten.

Kapitel 3: Durch zweidimensionale NMR-Spektroskopie wie ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC und ^{1}H-^{13}C HMQC konnte eine eindeutige Zuordnung der ^{1}H und ^{13}C NMR-Signale des aminmodifizierten PVA erhalten werde. Mikrostrukturen bis hin zu Pentaden lokalisiert bei rr, mr and mm konnten der Triade VOHOHVOH zugeordnet werden, während für die Triade VOHVOHVA nur rr und mr beobachtet werden konnten. Im ^{1}H-^{13}C HMQC zeigte sich, das die Reaktion zwischen aktiviertem Diamin und PVA vornehmlich an isotaktischen Triaden stattfindet. Mittels Long-Range Kopplungen wurde zweifelsfrei die

kovalente Bindung zwischen Aminsubstitution und PVA Rückgrat nachgewiesen. Die Feinstruktur der Aminsubstitution wurde als bestehend aus 54 % VOHVAMVOH, 30 % VAVAMVOH und 16 % VAVAMVA bestimmt. Eine durchschnittliche Blocklänge der VOH Einheiten von 9 wurde ermittelt.

Kapitel 4: Durch kurze Seitenketten konnte eine deutliche Verringerung der Abbauzeiten erreicht werden. P(33)-10 zeigt extrem kurze Abbauzeiten von weniger als 2 Tagen. Durch ^{1}H-NMR spektroskopische Untersuchungen wurde nachgewiesen, dass es im Laufe des Abbaus tatsächlich zu einer Verkürzung der PLGA-Seitenketten kommt. Aus den langkettigen, aminmodifizierten Polyestern (Typ II) lassen sich unter Verwendung von ‚Solvent Displacement' Techniken DNA-beladene Nanopartikel mit Größen um 150 nm herstellen. Mittels TEM und REM Aufnahmen konnte eine oval bis kugelförmige Gestalt dieser Partikel festgestellt werden. Es zeigte sich, dass einige der synthetisierten Polyester außerordentlich gute Transfektionseigenschaften im *in vitro* Experiment an L929 Mausfibroblasten besitzen. Partikel aus dem Polymer P(68)-10 bewiesen ein Transfektionsvermögen, das das von PEI/DNA Komplexen überstieg. Es wurde festgestellt, dass sich durch Erhöhung der Aminsubstitution am Rückgrad bei gleicher Seitenkettenlänge eine Verbesserung der Transfektion erreichen ließ, während eine Verlängerung der Seitenketten bei gleicher Aminsubstitution einen gegenteiligen Effekt zeigte.
Die Synthese eines neuen multifunktionalen, DNA-Trägersystems für die DNA-Vakzinierung kann als gelungen angesehen werden.

Schlussfolgerung: Aus allen Messungen und Beobachtungen geht hervor, dass sich das entwickelte Polymersystem für den Einsatz als maßgeschneidertes Trägersystem für Arzneistoffe eignet. Es besitzt besonders interessante Abbau

und Transfektionseigenschaften. Damit hat es großes Potential für einen Einsatz als Trägersystem für DNA-Impfstoffe und andere hydrophile Arzneistoffe.

5.4 Ausblick

Neben einer genauen toxikologischen Untersuchung der einzelnen Polymere sind auch genauere Untersuchungen des Abbauverhaltens erforderlich. Bisherige Ergebnisse lassen vermuten, dass die Polyester eine gegenüber herkömmlichen Transfektionsreagenzien geringere Toxizität aufweisen. Genaue Untersuchungen mittels MTT-Test und anderer Toxizitätstest müssen eingesetzt werden, um diese Befunde zu bestätigen.

Das Abbauverhalten der Polymere weist sowohl Charakteristiken eines Oberflächenabbaus wie auch einer ‚Bulk-Erosion' auf. Um den Abbaumechanismus genau zu klären, ist eine Abbaustudie an einigen ausgewählten Polyestern notwendig.

Die eingebauten Amingruppen lassen eine Verbesserung der Arzneistoffbeladung eines Trägersystems aus aminmodifizierten PVA-Polyestern gegenüber Trägersystemen aus linearen Polyestern erwarten. Außerdem sollte sich das Freisetzungsverhalten durch die eingebaute Amingruppen verändern. Beladungs- und Freisetzungsverhalten konnten bisher nicht ausreichend untersucht werden und sollten in weiteren Studien aufgeklärt werden.

Neben den bisher untersuchten Parametern wie Seitenkettenlänge, Art der Aminsubstitution und Substitutionsgrad ist die Variation weiterer Parameter denkbar. So kann ebenfalls das Lactid/Glykolid Verhältnis in der Polyesterseitenkette, der Polymerisations- und Verseifungsgrad des PVA Rückgrads sowie die Art der Polyesterseitenkette (z.B. ε-Caprolacton) variiert werden.

Dadurch ist der Anpassungsfähigkeit des Systems keine Grenze gesetzt und den Anforderungen an ein maßgeschneidertes Polymer genüge getan.

Appendix

A.1 Abbreviations

DMAPA :	3-dimethylaminopropylamine = M
DEAPA:	3-diethylaminopropylamine = P
DEAEA:	2-diethylaminoethylamine = E
PVA:	Poly(vinyl alcohol)
PLGA:	Poly(lactide-co-glycolide)
A(x)-y:	A indicates the type of amine substitution [P= DEAPA= 3-diethylaminopropylamine, M= DMAPA= 3-dimethylaminopropylamine, E= DEAEA= 2-diethylaminoethylamine], x is the number of monomers in the backbone carrying amine substitutions, y is the PLGA side chain length calculated from feed)
PEI:	polyethylenimine
NMR:	Nuclear magnetic resonance
GPC:	Gel permeation chromatography
DSC:	Differential scanning calorimetry
TGA:	Thermo-gravimetric analysis
MALLS:	Multi angle laser light scattering
HMBC:	Heteronuclear multiple-bond correlation
HMQC:	Heteronuclear multiple-quantum correlation
HSQC-TOCSY	Heteronuclear single-quantum correlation-total correlation spectroscopy
COSY:	Correlated Spectroscopy
WAXD	Wide angle x-ray diffratometry
TEM:	Transmission electron microscopy
SEM:	Scanning electron microscopy
FT-IR spec.:	Fourier transformation Infrared spectroscopy
DMSO:	Dimethylsulfoxide
pCMV-luc:	plasmid DNA encoding the luciferase gene
DNA:	Desoxyribonucleic acid
mm:	isotactic triad
mr:	heterotactic triad
rr:	syndiotactic triad

A.2 Publications

Fast degrading, high-molecular weight, brush-like branched, amine-modified poly(vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide)s as a platform for parenteral drug delivery systems: Synthesis, characterization and degradation behavior.
Matthias Wittmar, Florian Unger, Andreas K. Schaper, Thomas Kissel
Macromolecules, *2003*, submitted

Effective Biodegradable DNA Nanocarrier Systems of Newly Synthesized Amphiphilic, Amine-Modified Graft Polyesters
Christine G. Oster[§], Matthias Wittmar[§], Florian Unger, Lucian Barbu-Tudoran, Andreas K. Schaper, Thomas Kissel
[§] The authors have equally contributed to this work**.**
Pharmaceutical Research, *2003*, accepted

A two dimensional NMR study of Poly(vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol)
Matthias Wittmar, Xiulan Xie, Thomas Kissel
Macromolecules, in preparation

Nebulization of biodegradable nanoparticles: impact of nebulizertechnology and nanoparticle characteristics on aerosol features
L.A. Dailey, T. Schmehl, T. Gessler, M. Wittmar, F. Grimminger, W. Seeger, T. Kissel
J. Controlled Release, *2002*, **86(1)**, 131-144

Novel Biodegradable Ternary Copolymers *hy*-PEI-*g*-PCL-*b*-PEG: Synthesis, Characterization, and Potential as Efficient Nonviral Gene Delivery Vectors
Xintao Shuai, Thomas Merdan, Florian Unger, Matthias Wittmar, Thomas Kissel
Macromolecules, *2003*, **36(15)**, 5751-5759

Self-assembling nanocomplexes from insulin and water-soluble branched polyesters, poly[(vinyl 3-(diethylamino)-propylcarbamate-co-(vinyl acetate)-co-(vinyl alcohol)]-graft-poly(L-Lactic acid): A novel carrier for transmucosal delivery of peptides.
Simon M., Wittmar M., Bakowsky U., Kissel T.
Bioconjugate Chemistry, *2003*, submitted

Surfactant-free biodegradable Nanoparticles for aerosol therapy based upon DEAPA-PVAL-g-PLGA
L.A. Dailey, E. Kleemann, M. Wittmar, T. Gessler, T. Schmehl, C. Roberts, W. Seeger, T. Kissel
Pharm. Res, *2003*, accepted

A.3 Poster

1998: GPEN Zürich:

New charge-modified poly(vinyl alcohol) and its use in Sn(II)octoate-initiated graft-Polymerization of L-lactide,
M. Wittmar, T. Kissel

2001: Europolymer Congress Eindhoven:

The synthesis of polyesters with amine-modified poly(vinyl alcohol) backbones: A novel class of positively charged biopolymers for drug delivery and DNA vaccination., M. Wittmar, T. Kissel

2000 GPEN Upsalla:

Self-Assembling-Polymer conjugates for Oral/Mucosal delivery preparation and characterization,
M. Simon, M. Wittmar, I. Behrens, T. Kissel

2002 CRS Seoul:

Self-assembling insulin-polymer complexes for peroral delivery: Interaction with Caco-2 cell monolayers, peptide transport and cytotoxicity
I. Behrens, M. Simon, M. Wittmar, T. Kissel,
Proceed. Intern. Symp. Control. Rel. Bioact. Mater., *2002*, **29**, 468-470

Protein delivery systems based on branched biodegradable polyesters
T. Kissel, M. Simon, M. Wittmar
Proceed. Intern. Symp. Control. Rel. Bioact. Mater., *2002*, **29**, 160-162

2003 CRS Glasgow:

Nanoparticulate DNA Delivery System in Novel Amine-Modified Polyesters
C G Oster, M Wittmar, U Bakowsky, T Kissel,
Proceed Intern. Symp. Control. Rel. Bioact. Material, *2003*, **30**, 303-304

Branched Polyesters of the type PVAL-g-PLGA allow design of rapidly degrading parenteral delivery systems
F Unger, M Wittmar, T Kissel,
Proceed Intern. Symp. Control. Rel. Bioact. Material, *2003*, **30**, 499-500

A.4 Curriculum vitae

Persönliche Daten Matthias Wittmar,
geboren am 14.07.1972 in Winterberg/Sauerland,
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Schulausbildung:

1979 – 1983	Grundschule Medebach
1983 – 1989	Gymnasium Medebach
1989 – 1992	Gymnasium Winterberg Abschluss: Allgemeine Hochschulreife (Abitur)

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Hochschulausbildung:

10.1993–09.1998	Philipps - Universität Marburg, (Diplomstudiengang Chemie mit Wahlpflichtfach: Makromolekulare Chemie)
davon 01.1998 – 9.1998	Diplomarbeit bei Prof. Dr. A. Greiner und Prof. Dr. T. Kissel, (FB Pharmazie, Institut für Pharmazeutische Technologie und Biopharmazie) Thema: Neue, kammartig-verzweigte, ladungsmodifizierte Polyester: Aminmodifizierte, wasserlösliche Polyvinylalkohole als Rückgrat gepfropfter Poly-L-lactide
September 1998	Abschluss des Chemiestudiums mit dem akademischen Grad „Diplomchemiker“
seit 12.1998	Doktorand im Arbeitskreis des Prof. Dr. T. Kissel, Anfertigung der vorliegenden Arbeit mit dem Titel: Charge modified, comb-like graft-polyesters for drug delivery and DNA vaccination: Synthesis and Characterisation of Poly[vinyl dialkylaminoalkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide)]s

Praktika /Tätigkeiten:

08.1996 – 10.1996	Praktikum bei der Hoechst AG (6 Wochen)
11.1996 – 12.1996	studentische Hilfskraft am FB Chemie
05.1998–08.1998	studentische Hilfskraft am FB Pharmazie
seit 12.1998	wissenschaftlicher Mitarbeiter im Fachbereich Pharmazie, Institut für Pharmazeutische Technologie & Biopharmazie