Comb polymers for biomedical application obtained by grafting biodegradable polyester chains onto hydrophilic polyol backbones

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Contents

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- A. Breitenbach, T. Jung, W. Kamm, T. Kissel, Neue pharmazeutische Zubereitung, enthaltend kolloidale Polymer-Wirkstoff-Assoziate, insbesondere auch für mucosale Wirkstoffverabreichung, German Patent Application AZ 19839515.9 (1998).
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Chapter 1

1.1 Background and Scope

Ongoing advances in the areas of biotechnology, biochemistry and recombinant DNA technology have led to a growing number of potent and highly specific hydrophilic macromolecular drugs, such as peptides, proteins and oligo-nucleotides, targeting poorly controlled diseases [1,2]. In most cases clinical application of these drugs without suitable carrier systems is limited or even impossible due to a variety of problems, such as unwanted side effects or drug instability. Therefore, intensive research is focussed on polymeric carrier systems, such as e.g. drug loaded microspheres and implants (parenteral depot systems), nanoparticles and other colloidal systems (for mucosal as well as parenteral application), hydrogels, polymer modified (pro-)drugs etc. [3-8].

In the complicated relation of drug, technology and carrier polymer, often described as magic triangle, especially the polymers dominate the characteristics of the resulting delivery devices. The minimal requirements for a suitable polymeric carrier are processability, biocompatibility, biodegradability and drug compatibility.

Among others the most frequently used polymers are based on poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) because of their well-documented biocompatibility and safety record [3,9]. These polyesters degrade by simple hydrolysis back to the nontoxic monomeric acids and, therefore, act as prometabolites in the Krebs cycle.

Drug release from parenteral depot systems (PDS) is generally controlled by both drug diffusion and polymer erosion (mass loss of the device). Despite considerable efforts, protein release rates from PDS prepared from linear polyesters often deviate from continuous or 'infusion like' profiles [4]. Long

diffusional pathways cause an entrapment of acidic polymer breakdown products inside the polymeric matrix catalytically accelerating hydrolytic degradation. Thereby, encapsulated proteins are exposed to an environment of increasing acidity. Moreover, this type of bulk erosion from inside out of the polyester matrix causes undesired drug release lag phases [10,11].

While the release properties of microspheres can be influenced to a limited extent by formulation parameters [12,13], polymer modifications provide a broader spectrum of possibilities. Two different strategies have been proposed to modify polyesters with regard to parenteral protein delivery: For one, increased hydrophilicity results in faster water uptake and swelling of the polymeric matrix, promoting drug release by pore diffusion [14,15]. Secondly, grafting short PLGA chains onto small hydrophilic backbone molecules should accelerate erosion, because the degradation products become water-soluble after fewer cleavage steps, which could possibly shorten undesired lag phases [16].

Surprisingly little is known about the combination of both strategies in a single polymer [17]. In an ideal case it will allow to synchronize polymer degradation and device erosion and possibly lead to a change from undesired bulk erosion to more preferable surface erosion types, yet unknown for PLGA.

In this approach the three-dimensional polymer architecture, by grafting a large number of PLGA chains onto a hydrophilic macromolecular polyol, should provide new degrees of freedom of polymer-property adjustment, as outlined in figure 1.

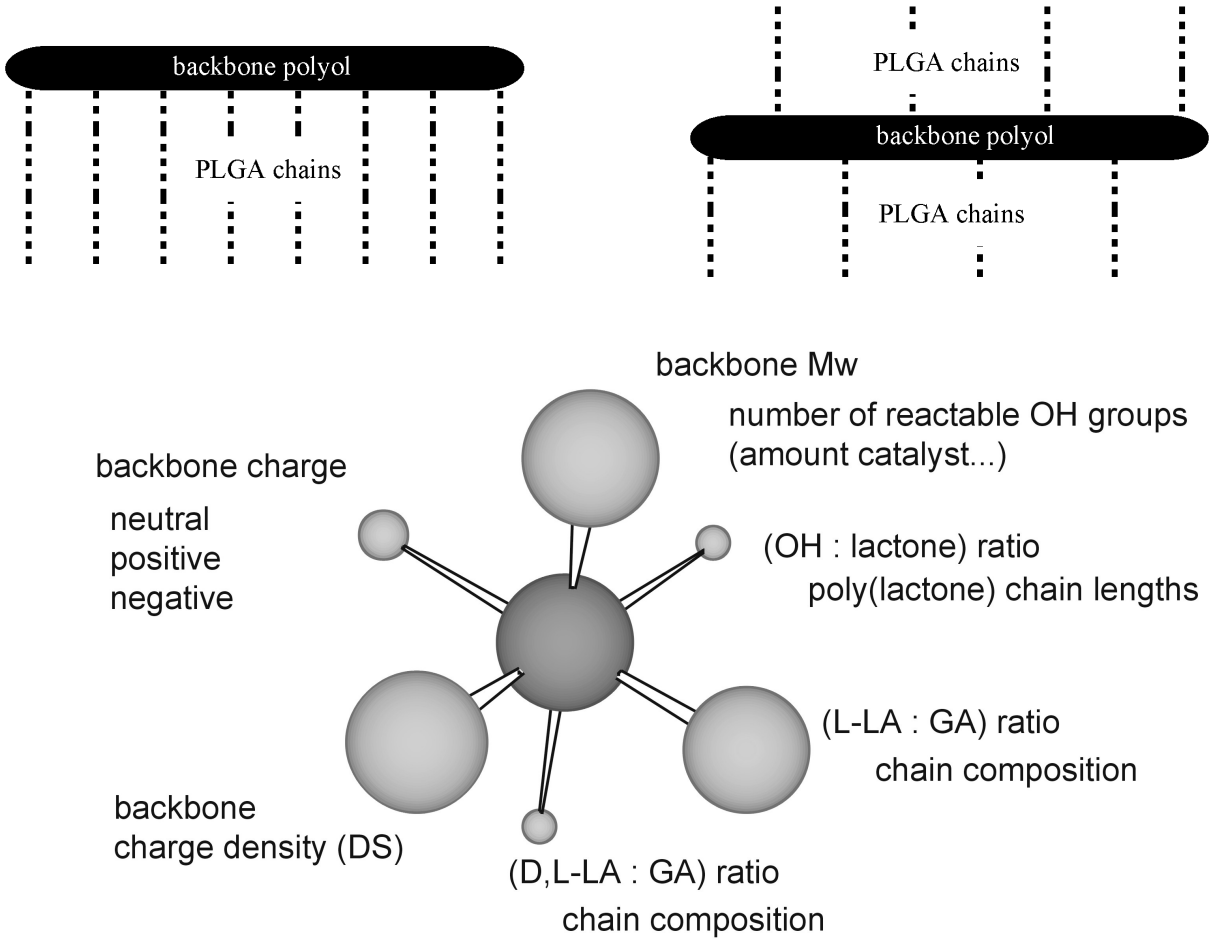

Figure 1: Schematic representation of the possible polymer modifications

While the properties of linear polyesters can be manipulated only to a limited extent, e.g. by their molecular weight (Mw), the properties of the Mw modulator and polymer composition, the molecular weight of comb or brush-like grafted polyesters can be adjusted over a much broader range.

Besides the choice of backbone, for one the chain composition, the ratio of lactide (L,L-LA, D,L-LA) and/or glycolide (GA) will influence polymer hydrophilicity, crystallinity and degradability. Furthermore, the ratio (polyol hydroxyl groups to lactones) should define the PLA or PLGA chain lengths and could be a versatile tool to manipulate polymer crystallinity (L-LA only) as well as to study the influence of the chain lengths on biodegradation and microsphere

drug release. Moreover, the molecular weight of the polyol backbone and the amount of esterified hydroxyl groups should have a comparable influence on polymer hydrophilic/hydrophobic balance and, therefore, should be of importance for biodegradation and drug release, too. An additional factor is the introduction of charged groups into the polyol backbone. Not only the influence of charge on biodegradation, drug stability and drug release can be investigated but also the potential of these polymers for other application routes.

Although numerous authors demonstrated polymeric (nano)particle uptake from the gastrointestinal tract (GIT) over the past two decades, transmucosal delivery of hydrophilic macromolecules similarly remains a major challenge [18,19]. The GIT provides a variety of morphological (e.g. mucus, epithelial cells) and physiological (e.g. pH, enzymes) barriers to absorption. Due to its limited capacity, mucosal vaccination has become more important in recent years [20]. Amongst convenient application of oral or nasal formulation in combination with improved patient compliance and the possibility of frequent boosting, mucosal vaccination is a very effective way to induce a protective immune response. Many viral or bacterial pathogens invade the organism from mucosal surface; therefore, mucosal immunity is an important protective barrier.

Especially the nature of the polymers used for nanoparticle (NP) preparation was found to be a key factor for successful protein delivery [21]. It is widely accepted that NP properties, such as size, surface charge and hydrophilic/hydrophobic balance affect intestinal absorption [22]. These properties cannot be achieved with linear PLGA. Therefore, novel amphiphilic polyesters facilitating the preparation of nanoparticles with defined surface structure and mucoadhesive properties would be most preferable.

Besides encapsulation of the hydrophilic macromolecules into microspheres and implants or adsorptive drug loading onto the surface of nanoparticles another strategy gained increasing attention, lately: the macromolecular self-assembly of polymer-drug conjugates [6]. Biofunctions such as gene information and antigen-antibody reactions are based on the complexation of biopolymers such as proteins, polysaccharides and nucleic acids. Therefore, the concept of self-assembling colloidal systems might be a promising strategy for oral as well as parenteral protein delivery, since it is already under investigation for non-viral gene transfer [23-25].

An ideal delivery system would be based on biocompatible (and biodegradable) polyelectrolytes, whose structure and properties can be easily adjusted to the special needs of such a system, such as type of charge, charge density and hydrophilic/hydrophobic balance. These polymers should be able to protect the protein from self-aggregation as well as from unwanted interactions with the application environment, for instance gastric enzymes in case of an oral delivery.

Therefore, the aim of the studies presented in this thesis was to experimentally assess a novel comb polymer class for biomedical application by grafting biodegradable polyester chains (PLA and PLGA) onto hydrophilic backbone polyols. Furthermore, the physico-chemical characterization of these polymers as well as the employment of an appropriate property optimization strategy were subjects of this work. Finally, the investigation of the structure-property-application function by means of microspheres, nanoparticles and self-assembled polymer-protein conjugates is issued.

In the following summary the detailed structure of this work and description of the results are given.

1.2 Summary

This work is concerned with the development of a novel polyester class by combining the advantages of linear poly(lactic acid), PLA, and poly(lactic-co-glycolic acid), PLGA, such as biodegradability, biocompatibility and low toxicity, with a three-dimensional polymer architecture and increased polymer hydrophilicity. Special emphasis is placed on the potential use and optimization of these polymers for biomedical application, namely parenteral and oral protein delivery by means of (1) microspheres, (2) nanoparticles and (3) colloidal polymer-protein complexes.

Therefore, this work is divided into several parts, depending on the obtained polymer-property relationships. The first part (1) describes the general evaluation of the synthesis and the physico-chemical characterization of PLGA chains brush-like grafted onto unmodified hydrophilic poly(vinyl alcohol) (PVA) backbones. The utility of these polymers in parenteral drug delivery by means of biodegradation, microsphere preparation and protein release is investigated. In a second part (2), the synthesis of charged polymers by grafting PLGA chains onto charge modified PVA backbones, poly(2-sulfobutyl-vinyl alcohol) and poly(diethylaminoethyl-vinyl alcohol), is evaluated. The potential of these polymers as nanoparticulate (NP) protein carrier for mucosal delivery by means of NP surface characterization is investigated. Finally (3), the synthesis of stimuli-sensitive, water-soluble polyesters, charged as well as uncharged, for protein complexation by a major reduction of the PLGA chain lengths is exploited.

These parts have been subdivided into several chapters:

The second chapter covers the evaluation and characterization of a synthetic pathway to create a novel polyester class. Bulk melt polymerization could

successfully be utilized for the preparation of brush-like grafted polyesters of L-lactide, D,L-lactide and their random copolymers with glycolide containing different poly(vinyl alcohol)s as backbones using stannous octoate as catalyst. The absence of linear homopolymers and the incorporation of the hydrophilic backbone into graft polymers could be confirmed be various spectroscopic and analytical methods. The new degree of freedom to manipulate physico-chemical properties, such as structure, molecular weight, crystallinity, glass transition temperature, hydrophilic/hydrophobic balance etc., over a broad range by adjusting side-chain lengths, number and composition, type and MW of the backbone make this type of polymerization a versatile tool to create novel polymeric candidates for the preparation of drug delivery systems.

Chapter 3 gives a short overview about the general advantages of the combined approach, increased hydrophilicity and introduction of a three-dimensional architecture into PLGA. Results on improved degradation and protein drug release from microspheres prepared from polyesters containing either two-, four- and eight-arm poly(ethylene oxide) (PEO) or poly(saccharide) backbones are reported. During the in-vitro degradation of star-block copolymers hydrophilic PEO was retained longer in the matrix, due to more connecting bonds between PLGA and PEO blocks. This led to a preservation of the physico-chemical stability of the polymeric matrix for a longer time. The acceleration of the degradation after about 3 weeks offers a fast elimination of the polymeric matrix after drug exhaustion. Comb-like PLGAs containing charged polysaccharide backbones equally showed improved degradation rates compared to linear polyesters. The mechanism was strongly influenced by the used backbone polyol and could be adjusted from random to nonrandom hydrolysis of the polyester chains. The faster degradation rates, accompanied by a high water content and a porous structure of the delivery devices allowed the preparation of model protein delivery systems with almost zero order kinetics for a time period

of about 2 to 3 weeks. Since both types of polymers are limited to some extent regarding polymer property adjustment as well as drug compatibility, the investigations in the next chapters are focussed on polyesters containing more flexible PVA based backbones.

In the fourth chapter a detailed description of the degradation and erosion properties of PVA-g-PLGA as a function of polymer structure and composition is reported. Moreover, initial results on the relationship of polymer properties, erosion mechanism and release of hydrophilic macromolecules from microspheres (MS) are described. It was possible to change and adjust the degradation and erosion profiles in a systematic manner by parameters, such as PLGA chain lengths and composition as well as PVA molecular weight. The degradation mechanism could be switched from bulk to a more surface front-like erosion behavior. The transition from bulk to surface erosion seems to be mainly influenced by the PLGA chain lengths. Grafting water-insoluble side chains onto PVA resulted in high molecular weight polymers exhibiting bulk erosion, while polymers bearing water-soluble PLGA chains seem to erode by a different mechanism. The release of hydrophilic macromolecules from microspheres was significantly improved compared to linear polyesters. Release was dominated by the same relationships found for degradation and erosion. The shorter the PLGA chains, the faster the drug release rates. Moreover, an increasing molecular weight of the PVA backbone shifted release from a degradation controlled mechanism to pore diffusion.

In chapter 5 the results with hydrophilic macromolecules, described in chapter 4, are transferred to model proteins and the influence of polymer properties on drug encapsulation and release are investigated. Independent of the properties of the encapsulated substances, such as molecular weight or point of isocharge, the same relationships were found. A reduction of PLGA chain lengths resulted in

increasing drug release. A shift to pore diffusion release was achieved by a MW increase of the PVA backbone. The ability to change MS characteristics by polymer property adjustment from a low to a high burst, from fast to slower release rates, equally covering short to long duration, in combination with negligible polymer cytotoxicity, makes PVA-g-PLGA potent candidates for protein delivery.

Since most phase separation techniques for MS preparation are based on the extensive use of organic solvents, a novel technology utilizing the extraction properties of supercritical carbon dioxide (aerosol solvent extraction system, ASES) for MS preparation is investigated by means of finding process polymer relationships in chapter 6. PVA-g-PLA and PVA-g-PLGA were applied to monitor the influence of several factors, such as structure modification by variation of the side-chain lengths, of the side-chain number and of the use of different chain compositions. The key parameter of microsphere production by ASES could be related to polymer crystallinity, either present or induced. Moreover, the spectrum of processable biodegradable polymers could be expanded to comb polyesters, which have already demonstrated their beneficial utility in controlled drug delivery, especially for protein and peptides.

In chapter 7 the new polymeric concept was extended by the introduction of charged groups into the polymer backbones. Poly(2-sulfobutyl-vinyl alcohol) P(SB-VA) and poly(diethylaminoethyl-vinyl alcohol) P(DEAE-VA) were synthesized and used as backbone for graft PLGA. Amongst initial data on accelerated biodegradation these amphiphilic polymers facilitated the preparation of nanoparticles with narrow size distributions and defined surface structure. The blocked polymer structure, caused by the high number of lipophilic PLGA chains grafted onto the hydrophilic charged backbones, allowed to design NP surface properties. Especially in case of negatively

charged polyesters a core-corona like NP structure with an inner polyester core and an outer hydrophilic, charged groups containing coating was obtained. The possibility to tailor the NP surface characteristics by choice of the polyester backbone and PLGA chain lengths will be a major advantage in adsorptive protein drug loading and bioadhesive force. Therefore, this polyester class is of particular interest for the preparation of colloidal mucosal carrier systems.

Based on above findings the synthesis of comb PLGA is utilized to prepare water-soluble PVA-g-PLGA and P(SB-VA)-g-PLGA by a major reduction of PLGA chain lengths as described in chapter 8. Increasing backbone contents to about 50 weight% and more resulted in polymers exhibiting a lower critical solution temperature. The preparation of protein drug delivery systems was possible either by this temperature-induced precipitation or by ionic interaction, leading to polyelectrolyte complexation. Spontaneous reversible formation of complexes with a number of proteins could be achieved. While colloid sizes, ranging from ca. 100 nm to several µm, were controllable by adjustment of concentrations, solution pH and ionic strengths, the release rates of the complexed proteins could be equally manipulated by pH. Preserved bioadhesion and initial oral vaccination data in a mice model indicate the considerable potential of this system in controlled drug delivery.

In conclusion, comb polyesters with PVA-based backbones could be successfully prepared and their adjustable structure and composition allowed property adjustment to the needs of protein drug delivery. A complete choice of possible applications, microspheres, nanoparticles as well as colloidal complexes, could be covered with a single polymer class. These results confirm the need for 'intelligent', tailor-made macromolecules.

1.3 Einleitung und Zielsetzung

Große Fortschritte auf dem Gebiet der Biotechnologie, Biochemie und rekombinanten DNS Technologie haben zu einer immer größer werdenden Zahl an hochwirksamen und spezifischen Wirkstoffen geführt [1,2]. Diese Wirkstoffe sind insbesondere dadurch gekennzeichnet, daß es sich bei ihnen um hydrophile makromolekulare Substanzen handelt, die auf Indikationsgebiete zielen, bei denen bisher effektive Therapeutika fehlen.

In den meisten Fällen ist die klinische Anwendung dieser Substanzen aber durch eine Vielzahl an Problemen, wie zum Beispiel unerwünschte Nebenwirkungen oder unzureichende Wirkstoffstabilität, ohne Einsatz geeigneter Trägersysteme stark erschwert beziehungsweise zum Teil nicht möglich. Daher wurden und werden intensive Anstrengungen unternommen, geeignete polymere Trägersysteme ausfindig zu machen. Darunter sind z.B. zu verstehen, wirkstoffbeladenene Mikrosphären (MS) und Implantate (als parenterale Depotsysteme), Nanopartikel und andere kolloidale Systeme (für parenteralen wie auch mukosalen Einsatz), Hydrogele, polymermodifizierte Prodrugs, usw. [3-8].

In dem komplexen Zusammenspiel von Wirkstoff, Technologie und Trägerpolymer, oft als Magisches Dreieck bezeichnet, dominiert besonders das eingesetzte Polymer die Eigenschaften des resultierenden Systems. Ein geeigneter polymerer Träger sollte wenigsten folgende Eigenschaften besitzen: Prozessierbarkeit, Biokompatibilität, Bioabbaubarkeit und Wirkstoffkompatibilität.

Neben einigen anderen werden bisher besonders Polymere auf der Basis von Poly(milchsäure) und Poly(milch-co-glycolsäure) zur Evaluierung derartige Trägersysteme eingesetzt, da ihre Biokompatibilität und Sicherheit umfassend dokumentiert werden konnte [3,9]. Diese Polyester bauen im Wäßrigen unter

einfacher Hydrolyse zu den monomeren, ungiftigen Säuren ab, die wiederum im Krebs-Zyklus verstoffwechselt werden.

Die Freisetzung eines Wirkstoffes aus Parenteralen Depotsystemen wird sowohl durch Wirkstoffdiffusion als auch Polymererosion (Massenverlust der polymeren Matrix) kontrolliert. Trotz großer Anstrengungen weichen die Freisetzungsprofile von hydrophilen Makromolekülen aus Polyestermatrizen häufig von kontinuierlichen, infusionsähnlichen Raten ab [4]. Einschluß von sauren Abbauprodukten im Matrixinneren durch zu große Diffusionswege erzeugt dort eine Beschleunigung des Abbaus. Dies führt zum einen zu einer für Proteine schädlichen Übersäuerung des Inneren. Außerdem erzeugt diese sogenannte Bulkerosion vom Inneren der Matrix nach außen unerwünschte Phasen ohne Wirkstoffliberation [10,11].

Während man derartige Freisetzungen nur in begrenztem Maße durch Herstellungsparameter beeinflussen kann [12,13], bietet der Ansatz über bessere Polymere bzw. geeignete Polymermodifikationen eine höhere Aussicht auf Erfolg. Im wesentlichen wurden bisher zwei Strategien untersucht, um die Eigenschaften von Polyestern im Hinblick auf ihre Verwendung als Träger-material zu verbessern.

Zum einen resultiert aus einer deutlichen Steigerung der Polymerhydrophilie eine schnellere Wasseraufnahme und Quellung des Trägersystems am Applikationsort, was zu einer Verbesserung der Wirkstoffliberation durch Diffusion führt [14,15]. Eine Strukturmodifikation, z.B. durch Anknüpfen von kurzen PLGA-Ketten an ein niedermolekulares Rückgrat bewirkt eine Beschleunigung des Polymerabbaus, da weniger Hydrolyseschritte nötig sind, bis die Abbauprodukte wasserlöslich werden [16]. Dieses wiederum sollte Phasen ohne Wirkstoffliberation verkürzen.

Erstaunlich wenig hingegen ist über die gleichzeitige Anwendung beider Strategien in einem einzigen Polymer bekannt [17]. Im Idealfall sollten sich dadurch Polymerabbau und Matrixerosion synchronisieren und somit die Freisetzung des Wirkstoffes linearisieren lassen. Dieses Verhalten entspräche der bisher für lineare Polyester unbekannten Oberflächenerosion.

Für diesen kombinierten Ansatz werden an ein makromolekulares Polyol eine Vielzahl bioabbaubarer Polyesterketten geknüpft. Durch die daraus resultierende dreidimensionale Polymerstruktur ergeben sich neue Freiheitsgrade zur gezielten Einstellung der Polymereigenschaften, wie in Abbildung 1 darstellt.

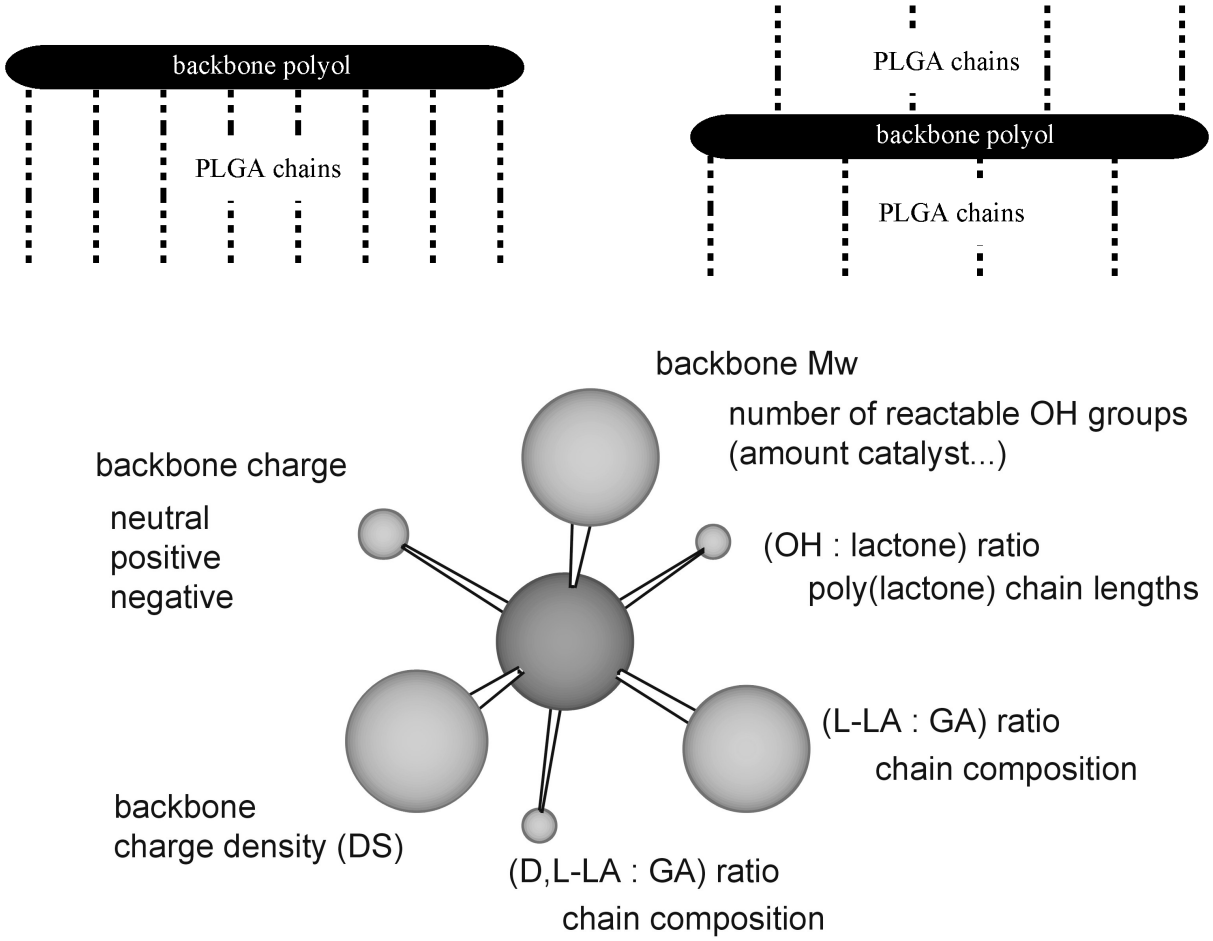

Abbildung 1: Schematische Darstellung der Polymermodifikationsmöglichkeiten

Während die Eigenschaften von linearen Polyestern nur begrenzt variiert werden können, im wesentlichen durch das Molekulargewicht, die Eigenschaften der Molekulargewichtsmoderatoren sowie die Polymerzusammensetzung, kann bei den kamm- oder buschartig verzweigten Polyestern bereits das Molekulargewicht über einen viel größeren Bereich eingestellt werden.

Neben der generellen Auswahlmöglichkeit des eingesetzten Rückgratpolyols läßt desweiteren wie bei linearen Polyestern auch die Zusammensetzung der PLGA-Ketten einen Einfluß auf Polymer-Hydrophilie, -Teilkristallinität und -Abbaubarkeit erwarten. Zudem sollte sich die PLGA-Kettenlänge durch geeignete Syntheseführung (Verhältnis Polyolhydroxylgruppen zu Monomeren) ebenso einstellen lassen wie die Zahl der PLGA-Ketten pro Rückgrat durch Zahl und Abstand der Hydroxylgruppen im Rückgrat (also indirekt durch dessen Molekulargewicht). Somit kann dadurch die Bedeutung dieser beiden Faktoren auf Bioabbau und Proteinfreisetzung untersuchen werden.

Eine weitere interessante Möglichkeit ergibt sich durch Integration von ladungstragende Gruppen in diese Polymere durch entsprechenden Modifikationen des Rückgrates. Dabei sind nicht nur deren Einfluß auf Abbau, Wirkstoffstabilität und -freisetzung von großer Bedeutung, sondern auch die Anwendung dieser Polymere für andere Arten von Trägersystemen.

Denn, obwohl seit langem literaturbekannt ist, daß kleine polymere Nanopartikel das Vermögen besitzen, im Gastrointestinaltrakt (GIT) aufgenommen zu werden, fehlen auch hier geeignete Polymere, die den transmukosalen Transport von hydrophilen Makromolekülen ermöglichen [18,19]. Im GIT existieren eine Reihe von morphologischen (Mukus, Epithelzellen) und physiologischen (pH-Werte, Enzyme) Absorptionsbarrieren. Bedingt durch die geringe Kapazität des GIT erweckt daneben auch die mukosale Vakzinierung

wachsendes Interesse [20]. Abgesehen von den generelle Vorteilen derartiger Applikationsrouten, wie bequeme nasale oder orale Anwendung, große Akzeptanz bei Patienten und der Möglichkeit effizient und häufig zu Boostern, erscheint mukosale Vakzinierung ein effektiver Weg, um Immunisierung zu erreichen. Viele virale oder bakterielle Pathogene infizieren den Organismus über mukosale Oberflächen, daher wäre mukosale Immunität eine wirksame Schutzbarriere.

Es wurde bereits gefunden, daß besonders die Natur der Polymere, mit denen Nanopartikel hergestellt werden, einen signifikanten Einfluß auf die mukosale Partikelresorption hat [21]. Neben der Teilchengröße sind besonders die Oberflächenladung und die Ausgewogenheit von hydrophilen und hydrophoben Bereichen bei Nanopartikeln von großer Bedeutung [22]. Eigenschaften, die mit herkömmlichen linearen Polyestern nicht erreicht werden können. Daher sind auch für diese Applikationsart neue Polymere erforderlich, die durch ihren amphiphilen Charakter die Herstellung von mukoadhäsiven Nanopartikeln mit definierter Oberflächenstruktur und -ladung ermöglichen.

Neben den bisher beschriebenen Arten des möglichen Proteintransportes erscheint eine weitere Strategie besonders vielversprechend. Makromolekulare Selbstassoziate aus hydrophilem makromolekularem Wirkstoff und wasserlöslichen Polymeren [6]. Da auch Biofunktionen, wie z.B. Antigen-Antikörper-Reaktionen, prinzipiell nach einem vergleichbaren Prinzip ablaufen, der Komplexierung eines Biopolymers (Proteine, Polysaccharide, Nukleinsäuren), scheint dieses Konzept, das bereits Einzug in die Untersuchungen des nichtviralen Gentransfers gefunden hat [23-25], auch für generellere mukosale oder parenterale Anwendung geeignet.

Ein ideales Trägersystem würde auf biokompatiblen und bioabbaubaren Polyestern basieren, deren Struktur und Eigenschaften, wie z.B. Art der Ladung, Ladungsdichte, Hydrophile-Hydrophobe-Balance, an die speziellen Anforderungen derartiger Anwendungen angepaßt werden können. Weiterhin müßten die Polymere die Stabilität der Wirkstoffe verbessern und ungewollte Interaktionen mit der Umgebung des Applikationsortes bzw. -zieles verhindern.

Aus den oben ausgeführten Gründen ist das Ziel der vorliegenden Arbeit, eine neue Klasse von verzweigten Polyestern für biomedizinische Anwendungen durch das Anknüpfen von bioabbaubaren Polyesterketten an hydrophile Rückgratpolyole zu kreieren. Schwerpunkt der experimentellen Arbeit stellt neben der Polymersynthese auch die physikochemische Charakterisierung der Polymere dar. Zusätzlich sind Auswahl und Einsatz geeigneter Optimierungsstrategien im Hinblick auf die oben genannten Anforderungsprofile der Trägersysteme (Mikrosphären, Nanopartikel, kolloidale Protein-Polymer-Komplexe) Bestandteil dieser Arbeit, um zu den erforderlichen Struktur-Eigenschafts-Funktions-Beziehungen zu gelangen.

In der nachfolgenden Zusammenfassung werden die generelle Gliederung dieser Arbeit und die Ergebnisse der einzelnen Kapitel aufgeführt.

1.4 Zusammenfassung

Die vorliegende Arbeit befaßt sich mit der Entwicklung einer neuen Klasse von bioabbaubaren Polyestern mit dem Ziel, die literaturbekannten Vorteile von Poly(milchsäure), PLA, und Poly(milch-co-glycolsäure), PLGA, wie Biokompatibilität und Ungiftigkeit, mit einer dreidimensionalen Polymerarchitektur und gesteigerter Polymerhydrophilie zu kombinieren. Besonders im Vordergrund stehen dabei der mögliche Einsatz und die geeignete Optimierung dieser Polymere als Ausgangsmaterial für biomedizinische Anwendungen. Parenterales und orales Delivery von hydrophilen Makromolekülen mittels (1) wirkstoffbeladenen Mikrosphären (MS), (2) Nanopartikeln (MP) und (3) kolloidalen Polymer-Protein-Komplexen werden schwerpunktmäßig beschrieben.

Diese Arbeit ist daher in Abhängigkeit der erhaltenen Polymereigenschaften in drei Blöcke unterteilt.

Der erste Teil (1) umfaßt die generelle Evaluierung der Polymersynthese und die physikochemische Charakterisierung der neuen Polyester, hergestellt durch die Anknüpfung einer großen Zahl von PLA- bzw. PLGA-Ketten an verschiedene Poly(vinylalkohol)-Rückgrate. Die Verwendbarkeit dieser Polymere als Ausgangsmaterial für Parenterale Depotsysteme wird durch Untersuchung ihrer Bioabbaubarkeit, der Herstellung von mit Modellsubstanzen beladenenen Mikrosphären (MS) und der Freisetzung dieser Substanzen aus den MS untersucht.

Im zweiten Teil (2) erfolgt eine gezielte Eigenschaftsoptimierung durch Einführung von ladungstragenden Gruppen in die Rückgratpolymere. Die so erhaltenen Poly(2-sulfobutyl-vinylalkohol)-graft-PLGA und Poly(diethylaminoethyl-vinylalkohol)-graft-PLGA werden im Hinblick auf ihre Verwendung als

mukosale Träger in Form von Nanopartikeln mit definierter Oberflächenstruktur und -ladung untersucht.

Abschließend (3) wird die Synthese und Charakterisierung von wasserlöslichen, stimuli-sensitiven Polyestern beschrieben, erhalten durch eine deutliche Verkürzung der PLGA-Seitenketten. Ihre spontane, reversible Reaktion mit Proteinen zu kolloidalen Komplexen macht diese Polymere zu interessanten Kandidaten für parenteralen wie auch mukosalen Wirkstofftransport.

Im Einzelnen gliedert sich die vorliegende Arbeit in folgende Kapitel:
Das zweite Kapitel beschreibt die Evaluierung und Charakterisierung eines Syntheseweges, der Zugang zu einer neuen Polyesterklasse schafften soll. Durch Substanzpolymerisation, ausgehend von einer Monomerschmelze in der die gewünschten Rückgrat-Polyole gelöst sind, konnten unter Verwendung von Zinnoktoat als Katalysator kammartig verzweigte Polyester erfolgreich hergestellt werden. Die Abwesenheit von unerwünschten linearen Homopolymeren wurde durch eine Vielzahl an spektroskopischen und analytischen Methoden ebenso belegt, wie der Einbau der Rückgrate in die Polymere. Durch die dreidimensionale Polymerstruktur ergeben sich neue Freiheitsgrade, um Eigenschaften, wie Molekulargewicht, Teilkristallinität, Hydrophile-Hydrophobe-Balance etc., gezielt zu beeinflussen. Die Möglichkeiten, die sich durch Manipulation der Polyesterseitenkettenlänge, -zahl und -zusammensetzung, sowie der Art und Größe des Rückgrates ergaben, machen diese Art der Synthese zu einem potenten Werkzeug zur Herstellung neuer Trägerpolymere für Drug Delivery Systeme.

Kapitel 3 gibt einen kurzen Überblick über die generelle Vorteile der neuen Strategie, vergrößerte Hydrophilie und dreidimensionale Architektur in einem Polymer zu realisieren. Ergebnisse über verbesserte Bioabbaubarkeit und

Proteinfreisetzung aus Mikrosphären werden berichtet. Während des in-vitro Abbaus von Sternpolyestern mit Zwei-, Vier- und Acht-Arm-Poly(ethylenoxid)-Rückgraten wurde beobachtet, daß mit zunehmender Anzahl der Seitenketten das PEO-Rückgrat länger in der Matrix verweit und somit die Matrixstabilität länger gewährleistet ist. Nach etwa dreiwöchiger Inkubationszeit erfolgte eine deutliche Beschleunigung des Abbaus, was nach Beendigung der Wirkstoff-freisetzung eine schnelle Eliminierung der polymeren Matrix garantiert. Auch für buschartig verzweigte Polyester mit ladungstragenden Polysaccharid-Rückgraten konnte in Abhängigkeit des eingesetzten Rückgrates eine deutliche Verbessung des Abbauverhaltens im Vergleich zu linearen Polyestern beobachtet werden. Der Abbaumechnismus konnte von zufällig auf bevorzugt an der Verbindungsstelle Rückgrat-Seitenkette verbessert werden. Die schnelleren Abbauraten in Verbindung mit einem hohen Wasseraufnahmever-mögen dieser Polymere ermöglichten für Modellproteine die Herstellung von PDS mit nahezu linearer Wirkstoffliberation über einen Zeitraum von zwei bis drei Wochen. Da aber beide Polymertypen nur bedingt eine weitergehende Eigenschaftsoptimierung ermöglichen, erfolgten die nachfolgenden Studien mit Polyestern, die PVA basierte Rückgrate enthielten.

Im vierten Kapitel wird eine umfassende Beschreibung des Polymerabbaus und der Polymererosion von PVA-g-PLGA in Abhängigkeit der Polymerstruktur und -zusammensetzung berichtet. Es war möglich, einen Zusammenhang zwischen den Faktoren Seitenkettenlänge / -zahl und Polymerabbau / Freisetzung von hydrophilen Modellmakromolekülen aus Mikrosphären herzustellen. Der Polymerabbau konnte von bekannter Bulk-Erosion bis hin zu bisher nicht für Polyester bekannter Oberflächenfront-Erosion verbessert werden. Dies wurde insbesondere durch die Variation der Länge der Polyesterseiten möglich. Lange, wasserunlösliche PLGA-Ketten, an die Rückrate geknüpft, dominierten mit ihren Eigenschaften das Verhalten der Polymere. Somit erhielt man die für

Polymere mit hohen Molekulargewichten typische Bulk-Erosion. Kammpolyester, deren Seitenketten so kurz waren, daß sie alleine wasserlöslich wären, zeigten hingegen ein anderes Verhalten, da alle Abbauprodukte sofort aufgrund ihrer Löslichkeit aus der Polymermatrix gewaschen wurden. Diese Zusammenhänge konnten auch für die Wirkstoffliberation erreicht werden, je kürzer die Seitenketten, desto schneller die Freisetzung. Desweiteren wurde über die Vergrößerung des Molekulargewichtes der Rückgratpolyole eine Möglichkeit gefunden, die Wirkstoffliberation von einem eher abbaukontrollierten hin zu einem porendiffusionskontrollierten Mechanismus zu verschieben.

Die Übertragung der Ergebnisse für hydrophile Makromoleküle aus dem vorangegangenen Kapitel auf Modellproteine ist Thema des fünften Kapitels. Mit verschiedenen Proteinen beladene Mikrosphären werden hier im Hinblick auf die Trägerpolymereigenschaften untersucht. Unabhängig von den Eigenschaften der verkapselten Substanz (Molekulargewicht, Isoelektrische Punkt) wurden mit Kapitel 4 vergleichbare Ergebnisse erzielt. Eine Verkürzung der Polyesterseitenketten bewirkte eine Steigerung der Freisetzungsrate, während die Vergrößerung des Rückgrates den Freisetzungsmechanismus von Abbaukontrolle zu Porendiffusion verschob. Die vielfältigen Möglichkeiten, die Eigenschaften der beladenen Mikrosphären einzustellen, von langsamer zu schneller Freisetzung, von niedrigem zu hohem Burst, von zweiwöchiger zu dreimonatiger Freisetzung, machen diese Polymerklasse, in Kombination mit in Zellkultur gefundener vernachlässigbar geringer Zytotoxizität, zu potenten Kandidaten für die Verkapselung von therapeutisch relevanten Proteinen, Peptiden und Oligonukleotiden.

Da die meisten Technologien zur Herstellung von Mikrosphären den Einsatz großer Mengen organischer Lösungsmittel erfordern, wird in Kapitel 6 ein neuartiges Verfahren untersucht. Das Aerosol Solvent Extraction System

(ASES) macht sich die Lösungsmittelextraktionseigenschaften von überkritischem Kohlendioxid zunutze. Die neuartige Struktur der kammartig verzweigten Polyester ermöglichte erstmals, ein pharmazeutisches Herstellungsverfahren auf Zusammenhänge zwischen gezielt einstellbaren Polymereigenschaften und Prozeßparametern zur erfolgreichen Herstellung von Mikrosphären zu untersuchen. Dabei wurde als ein Schlüsselfaktor für ASES Polymerteilkristallinität, entweder vorhanden oder über Seitenkettenorientierung induziert, identifiziert. Desweiteren konnte durch diese Polyester das Spektrum der mit ASES prozessierbaren Polymere erweitert werden.

In Kapitel 7 wird der Einfluß von ladungstragenden Gruppen, zusätzlich in die Rückgrate der kammartig verzweigten Polyester eingebracht, untersucht. Dazu wurden die Rückgrate zu Poly(2-sulfobutyl-vinylalkohol), P(SB-VA), und Poly(diethylaminoethyl-vinylalkohol), P(DEAE-VA), derivatisiert und anschließend wie zuvor mit einer Vielzahl an PLGA-Ketten verknüpft. Die resultierenden amphiphilen Polymere konnten - neben initialen Ergebnissen zu beschleunigtem Bioabbau - erfolgreich für die Herstellung von Nanopartikeln mit definierter Oberflächenstruktur und -ladung herangezogen werden. Die blockartige Polymerstruktur - hydrophiles, geladenes Rückgrat einerseits und hydrophobere Polyesterseitenketten andererseits - scheinen die Ausbildung einer Core-Corona-Struktur der Nanopartikel mit einem Polyester-Kern und einer geladenen, hydrophilen Oberflächenstruktur zu bewirken. Die Möglickeit, die Oberflächeneigenschaften von Nanopartikel durch Faktoren, wie Ladungsart und -dichte des Rückgrates sowie PLGA-Seitenkettenlänge zu steuern, stellt einen deutlichen Fortschritt im Hinblick auf Proteinadsorption und Bioadhäsion dar. Daher eignen sich diese Polymere besonders als Trägermaterial für kolloidale mukosale Systeme.

Basierend auf diesen Ergebnissen wurde im Kapitel 8 die Synthese von wasserlöslichen Polyestern untersucht. Dieses wurde durch eine deutliche Seitenkettenlängenverkürzung der bisher untersuchten Polymere erreicht. PVA-g-PLGA und P(SB-VA)-g-PLGA, die mehr als 50 Gewichtsprozent Rückgrat enthielten, zeigten thermoreversible Präzipitation im Wäßrigen (Untere Kritische Entmischungstemperatur) und konnten entweder durch dieses Temperaturverhalten oder durch geeigneten pH-Wert einstellbare Ionische Wechselwirkungen zur Beladung mit einer Reihe von Proteinen herangezogen werden. Im Falle der Polyelektrolyt-Komplexe zwischen den negativen P(SB-VA)-g-PLGA und Proteinen konnten Teilchengrößen von etwa 100 nm bis hin zu mehreren Mikrometern, sowie Beladungsgrade von bis zu 200% (w/w) systematisch durch Faktoren wie Konzentration, pH-Wert und Ionenstärke eingestellt werden. Dabei war die Komplexierung reversibel, so daß durch Veränderung des Umgebungs-pH-Wertes die Freisetzung der Proteine erzwungen werden konnte. Initiale Ergebnisse zur Bioadhäsion derartiger Assoziate in Zellkultur, sowie erste erfolgversprechende Versuche zu oraler Vakzinierung mit Tetanus Toxoid in einem Mäusemodell deuteten das mögliche Potential der wasserlöslichen Polyester als kolloidales Trägersystem für hydrophile makromolekulare Therapeutika an.

Besonders hervorzuheben ist, daß in der vorliegend Arbeit mit nur einer Polymerklasse aufgrund der dreidimensionalen Polymerstruktur ein großes Spektrum an möglichen Anwendungen, von Mikrosphären über Nanopartikel bis hin zu Polymer-Protein-Komplexen, abgedeckt werden konnte. Dies stützt die Vorstellung, daß ein wachsender Bedarf an 'intelligenten', maßgeschneiderten Makromolekülen besteht.

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Chapter 2

Biodegradable comb polyesters: part I
Synthesis, characterization and structural analysis of poly(lactic acid) and poly(lactic-co-glycolic acid) grafted onto water-soluble poly(vinyl alcohol) as backbone

2.1 ABSTRACT

To overcome discontinuous or polyphasic drug release rates of parenteral delivery systems (PDS) for peptides and proteins, typical for linear polyesters, novel biodegradable brush-like poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) grafted onto water-soluble poly(vinyl alcohol) (PVA) as backbone were investigated.

These polymers were synthesized by ring opening melt polymerization using stannous octoate (SnOct) as catalyst. The branched PVA-g-PLGAs were characterized by 1D and 2D NMR spectroscopy and other methods such as IR, SEC, DSC, and static light scattering. The incorporation of the backbone and the comb structure were demonstrated by ^{1}H- and ^{13}C-NMR spectra as well as light scattering studies.

The physico-chemical properties, such as molecular composition and architecture, molecular weight, degree of crystallinity, melting point, glass transition point, could be systematically adjusted to the requirements of drug delivery. Therefore, this new class of biodegradable polymers has considerable potential for PDS.

2.2 INTRODUCTION

For more than three decades aliphatic polyesters on the basis of lactic and glycolic acid have been extensively used as biomaterials and carriers for drug delivery systems. Due to their low toxicity, excellent biocompatibility and well documented biodegradation to nontoxic cleavage products, they have received approval by regulatory authorities [1]. These biopolymers are used for parenteral delivery systems (PDS), such as microparticles [2] or implants [3], as well as for surgical sutures [4] and implants for bone fixation [5].

Especially for the controlled delivery of bioactive agents it is necessary to carefully adjust both drug release rates and polymer degradation properties to achieve desired formulation properties. In the case of linear polyesters consisting of lactic and/or glycolic acid this is partially achieved by copolymerization or adjustment of molecular weight [6]. But in many cases drug release of peptides and proteins from linear polyesters is not satisfactorily controlled, leading to undesired discontinuous or polyphasic release patterns [7,8].

To overcome these discontinuous drug release profiles, both under in vitro and in vivo conditions, two major modifications of the polymer properties have been proposed: (1) On one hand increasing the hydrophilicity of the polymers will result in a faster water uptake and swelling of the polymer matrix, causing a faster and more prolonged drug release during initial pore diffusion phase. (2) On the other hand, accelerating the degradation rate of linear polyesters by branching the PLGA might be of general interest. This modification will generate many short PLGA chains, reaching more rapidly the threshold of water-solubility, thus promoting the polymer erosion.

An example for the first concept are linear ABA triblock copolymers consisting of PLGA A blocks and a central hydrophilic poly(ethylene oxide) (PEO) B

block, which showed a more rapid swelling, accelerated degradation rates and a continuous release of hydrophobic macromolecular agents from microspheres [9-11]. The in vitro release profiles of proteins were found to approach constant release rates by synchronizing both the pore diffusion and the polymer erosion phase. Unfortunately, some proteins are sensitive to PEO induced aggregation [12] and, therefore, hydrophilic backbones compatible with sensitive proteins are of particular interest for parenteral depot systems.

As for the second concept, branched polyesters consisting of ε-caprolactone or δ-valerolactone grafted onto glycerol [13] were described by Pitt and coworkers, but protein release was not studied. Kissel and coworkers were the first to study the properties of brush-like grafted PLGA [14], used for a PDS of Bromocripitin [15]. Star branched PLAs and PLGAs with low molecular multifunctional alcohols, like glycerol [16,17], pentaerythritol [18-21], mannitol/sorbitol [22,23] or star shaped PEGs [24] have subsequently been described. Multifunctional polyols, such as sugar alcohols, will affect the molecular architecture, but the changes in the polymer degradation rates are insufficient to provide continuous release profiles for proteins.

Surprisingly little is known about the synthesis and the properties of brush-like branched polyesters using water-soluble polymers as backbone materials [14,15,25,26]. Polyols offer the possibility of integrating and manipulating different physicochemical properties in graft polyesters by the type and amount of backbone material used. The increased hydrophilicity will lead to more rapid initial water uptake, promoting the degradation rates of these PVA-g-PLGA.

We report here a detailed investigation of new brush-like grafted polyesters consisting of a hydrophilic polymer backbone, poly(vinyl alcohol) (PVA), to which hydrophobic PLA and PLGA are chemically bound by ring-opening

polymerization of L-lactide or D,L-lactide and glycolide in bulk, using stannous octoate as catalyst. These polymers could be of interest for parenteral protein delivery systems.

2.3 EXPERIMENTAL SECTION

Materials

The following designation for poly(vinyl alcohol) (PVA) will be used to specify the different type of polymer: the first two digits designate the molecular weight of the polymer in kg/mol, the following two its original degree of hydrolysis in mol%. These PVAs were obtained by the following suppliers: Fluka: PVA(15.88), PVA(49.88), PVA(100.88), Hoechst AG: PVA(20.74), PVA(08.80), PVA(24.80), Sigma-Aldrich: PVA(10.80) and Polysciences: PVA(06.80). All samples were rigorously dried at 80 °C in vacuo until constant weight was obtained, and stored in a desiccator under vacuum at room temperature over P_2O_5.

D,L-lactide (D,L-LA), L-lactide (L-LA) and glycolide (GA) [Boehringer Ingelheim, S-grade] were recrystallized twice from dry ethyl acetate (refluxed over calcium hydride) and dried for 48 hours in vacuo directly before use. The melting points were 125-126 °C, 95-96°C and 82-83 °C, respectively.

Stannous octoate (SnOct), dichloromethane (DCM) [Aldrich], benzoyl chloride [Merck] and all other materials were of analytical grade and used as received.

Bulk polymerization of the graft lactones

Under nitrogen the lactones and PVA were charged into a rigorously dried 100 ml nitrogen flask which then was degassed at 50 - 55°C in a vacuum line for 1 hour, purging three times with dry nitrogen. The flask was then immersed into a preheated oil bath (T = 130°C) for about 15 minutes to obtain a clear melt of

monomers and backbone material. Then the catalyst was injected under nitrogen and the reaction was allowed to proceed for three hours at 130°C. After cooling to room temperature, using a water bath, the products were dissolved in 50 ml of DCM, washed twice with 50 ml of water for 30 minutes to remove unreacted PVA and precipitated in 500 ml of cold ethanol. The polymers were collected by filtration, washed with ethanol, and dried at 35°C in vacuo for at least 48 hours until constant weight was obtained. The polymerization of linear polyesters was carried out accordingly without addition of PVA (table 1).

Modification of poly(vinyl alcohol) with benzoyl chloride

PVA was modified by an interfacial Schotten-Baumann reaction with benzoyl chloride as recently reported by Gimenez et al. [27] leading to a vinyl alcohol-vinyl benzoate copolymer (PVB).

In a specific example of this synthesis 2.2 g (0.05 mol) PVA(15.88) were dissolved in 50 ml of water, 50 ml aqueous sodium hydroxide solution (5 mol/l) were added at room temperature and then cooled down to 0°C. At this temperature a solution of 0.0375 mol benzoyl chloride in a mixture of 60 ml MEK and 15 ml toluene was added dropwise. Under stirring the polyol was allowed to react for additional three hours at this temperature. The two solvent layers were separated and the copolymer was isolated from the organic phase by solvent evaporation. PVB was purified twice by dissolving in MEK and precipitating in petroleum ether. The yield was 65% and the degree of substitution from ^{1}H NMR analysis was found to be 60%, which is in excellent agreement with the results reported previously [27].

Characterization

Size Exclusion Chromatography (SEC) analysis was carried out relative to poly(styrene) reference materials [Merck]. 0.5% (w/v) polymer solutions in

DCM were injected into a Merck-Hitachi SEC system consisting of a L-6000 pump, two size exclusion columns (Lichrogel PS mix and Lichrogel PS 40, 10 μm) and a differential refractometer (RI 71) as detector. DCM was degassed and pumped at a flow rate of 1 ml/min. Molecular weights were calculated by a 3rd order universal calibration method with Millennium Chromatography Manager software (Waters, Eschborn, Germany).

Differential Scanning Calorimetry (DSC) was conducted with a differential scanning calorimeter (Perkin Elmer DSC 7) in sealed aluminum pans in a nitrogen atmosphere, relative to indium and gallium standards. Thermograms covered a range of 0°C to 200°C with heating and cooling rates of 10°C/min. Glass transition temperatures (Tg) were determined from the second run.

Intrinsic viscosities were determined using an Ubbelohde viscosimeter (Schott Geräte, Germany) from solutions in THF at 30 °C with at least four different concentrations.

400 MHz ^{1}H- and 100 MHz ^{13}C-NMR as well as ^{13}C-APT (attached proton test) spectra were recorded at 35 °C with a Jeol GX400 Delta N FT spectrometer using 6% (w/v) solutions of the polymers in different deuterated solvents, like d-chloroform, d_6-DMSO, D_2O and d_6-acetone. 500 MHz ^{1}H- and 125 MHz ^{13}C- as well as 2D-NMR spectra were recorded using a Jeol LA500 eclipse+ Delta FT spectrometer. Chemical shifts were calculated relative to tetramethylsilane (TMS) as internal standard with the NMR Data processing program WinNuts 2D (Acorn NMR).

Infrared spectroscopy was conducted with a Nicolet 510 P FT-IR spectrometer and Nicolet PC/IR v. 3.20 software with films cast from DCM solutions on NaCl plates and with KBr disks.

Combined SEC and light scattering analysis was carried out with an equipment from Wyatt Technology Corporation (Santa Barbara, USA), consisting of a SEC column (SDV linearcolumn, 300×8 mm, 10 μm), a K5 cell, an Optilab 309 differential refractometer and a MiniDawn Light Scattering detector operating at a laser wavelength of 690 nm (20 mW) and three detecting angles (45°, 90° and 135°). Degassed THF at 25°C with a flow rate of 0,7 ml/min was used as eluent. The system was calibrated relative to poly(styrene) (Merck) and the data were processed with Astra for Windows 4.1 software (Wyatt Technology Corp., Santa Barbara, USA).

Table 1: Physico-chemical properties of the polymers

No	**Polymer**	**OH : dimer : cat** [mol : mol : mol]	**Yield** [1)] [%]	**Mw** [2)] [kg/mol]	**D** [2)]	**Tg** [°C]	**Tm** [°C]	**ΔHm** [J/g]
1	linear L-PLA	0 : 100 : 0.2	87	105	2.0	57	174	52
2	PVA(15.88)-g-L-PLA	1 : 100 : 0.2	93	274	2.5	57.6	164	52
3	- ” -	2 : 100 : 0.2	85	277	2.7	54.3	155	44
4	- ” -	4 : 100 : 0.2	79	166	2.8	52.0	134	32
5	- ” -	11 : 100 : 0.2	76	125	1.7	43.3	-	-
6	- ” -	29 : 100 : 0.2	74	98	1.8	37.3	-	-
7	PVA(10.80)-g-L-PLA	1 : 100 : 0.2	86	261	3.0	54.9	167	44
8	- ” -	6 : 100 : 0.3	82	101	3.4	52.4	130	20
9	- ” -	11 : 100 : 0.7	80	86.6	2.4	50.4	-	-
10	- ” -	26 : 100 : 1. 6	76	77.2	2.2	43.8	-	-
11	PVA(06.80)-g-L-PLA	26 : 100 : 1. 6	76	76.3	1.5	45.2	-	-
12	linear D,L-PLA	0 : 100 : 0.2	87	134	1.5	53.1	-	-
13	PVA(15.88)-g-D,L-PLA	1 : 100 : 0.2	82	260	2.5	44.3	-	-
14	- ” -	4 : 100 : 0.2	81	180	2.5	43.9	-	-
15	- ” -	11 : 100 : 0.2	71	125	1.8	37.7	-	-
16	PVA(24.80)-g-L-PLA	26 : 100 : 1.6	76	174	1.6	45.6	-	-
17	PVA(20.74)-g-L-PLA	1 : 100 : 0.2	86	122	2.4	61.7	177	51

1) 3 hours at 130 °C

2) determined by SEC

2.4 RESULTS AND DISCUSSION

It is well known that graft polyesters have different physico-chemical properties compared to their linear counterparts due to their molecular architecture. Long poly(L-lactide) chains grafted onto smaller central core molecules will result in polymers whose physico-chemical properties are comparable to those of linear ones. But these polymers offer the possibility to specifically adjust the degree of crystallinity by variation of the chain length and number. The lower melting points and melt viscosities will be an major advantage for melt processing of polylactides such as for sutures and bone fixation in surgery. On the other hand, grafting short hydrophobic PLGA chains to a hydrophilic backbone will generate polyesters with a more rapid water uptake and faster biodegradation rates.

To investigate above hypotheses, we prepared various PVA containing polyesters, brush-like grafted L-PLAs, D,L-PLAs and D,L-PLGAs, using stannous octoate as catalyst. The properties of the resulting polymers are summarized in table 1.

Synthesis and SEC analysis

The polymerization was carried out in the presence and absence of the polyol. Using PVA, polymers with much higher molecular weights (Mw) were obtained. Such high molecular weights could not be reached with an initiation by neat tin octoate of lactide in our studies. The resulting molecular weights were directly related to the amount of backbone incorporated, as outlined in fig. 1. The more PVA-OH groups present during the polymerization, the lower the Mw of the products, indicating the role of the PVA hydroxyl groups as effective propagation centers. An increasing Mw of the PVAs used as backbone caused a proportional increase in the Mw of the resulting graft polymers (fig.1).

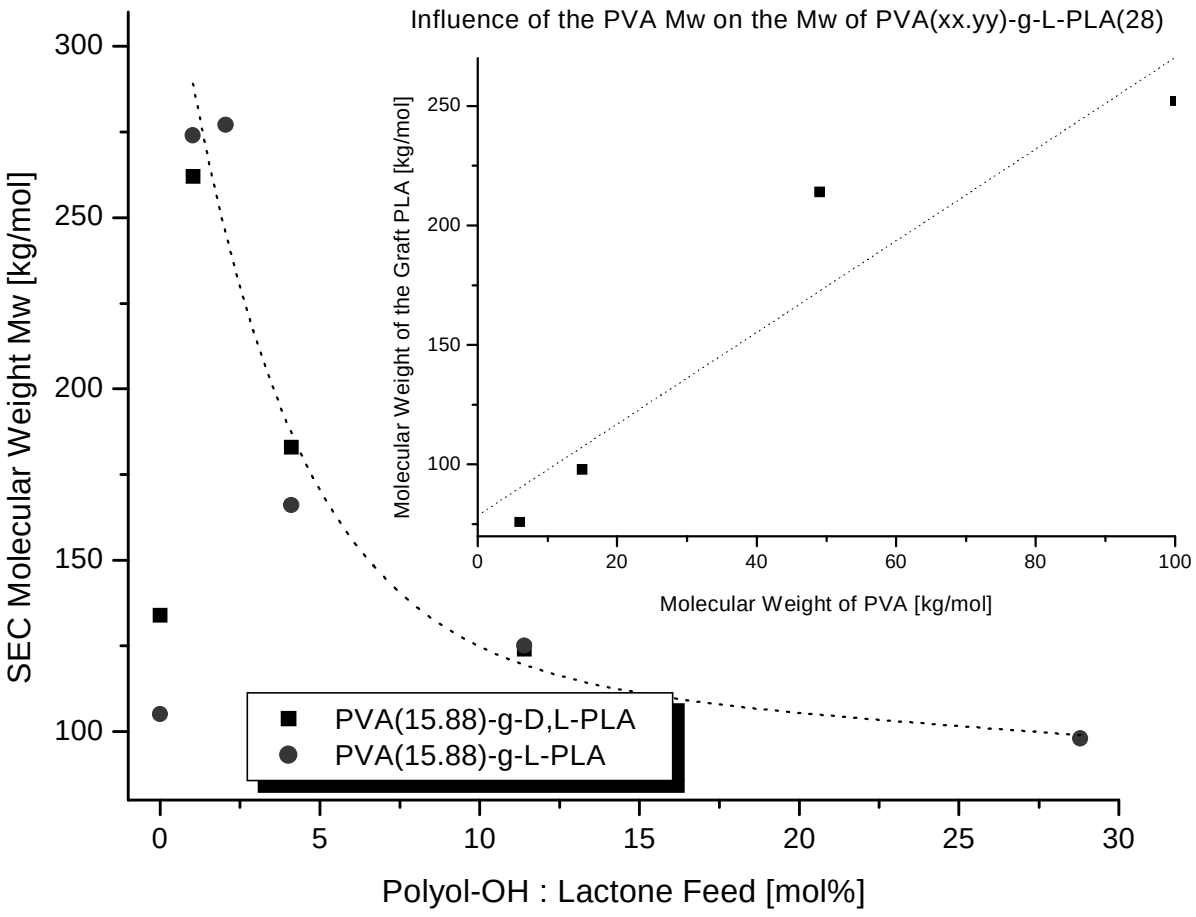


Figure 1: Influence of the backbone on the Mw of graft PLA

To investigate the influence of the reaction conditions a series of PVA-g-D,L-PLGAs was prepared under variation of reaction time and temperature (fig. 2). All polymers were synthesized under rigorously anhydrous conditions. PVAs were carefully dried, to avoid an initiation by water, which would lead to a mixture of linear and grafted products. We found 130°C and 3 hours to be most suitable reaction conditions. At lower temperatures the solubility of PVA in the melt of the monomers was insufficient. At higher temperatures discoloration of

the reaction products, accompanied by increased polydispersity and only partial solubility in DCM were observed.

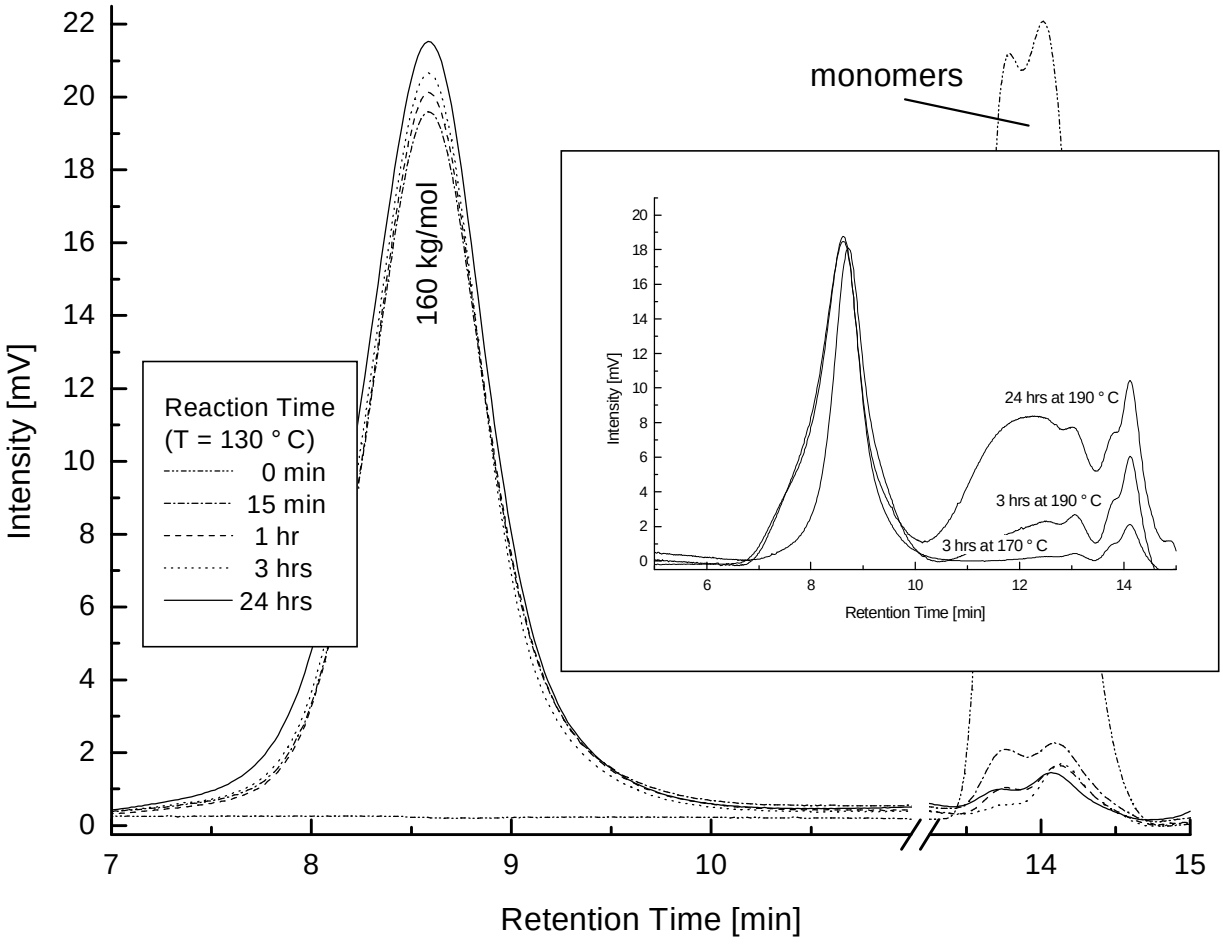
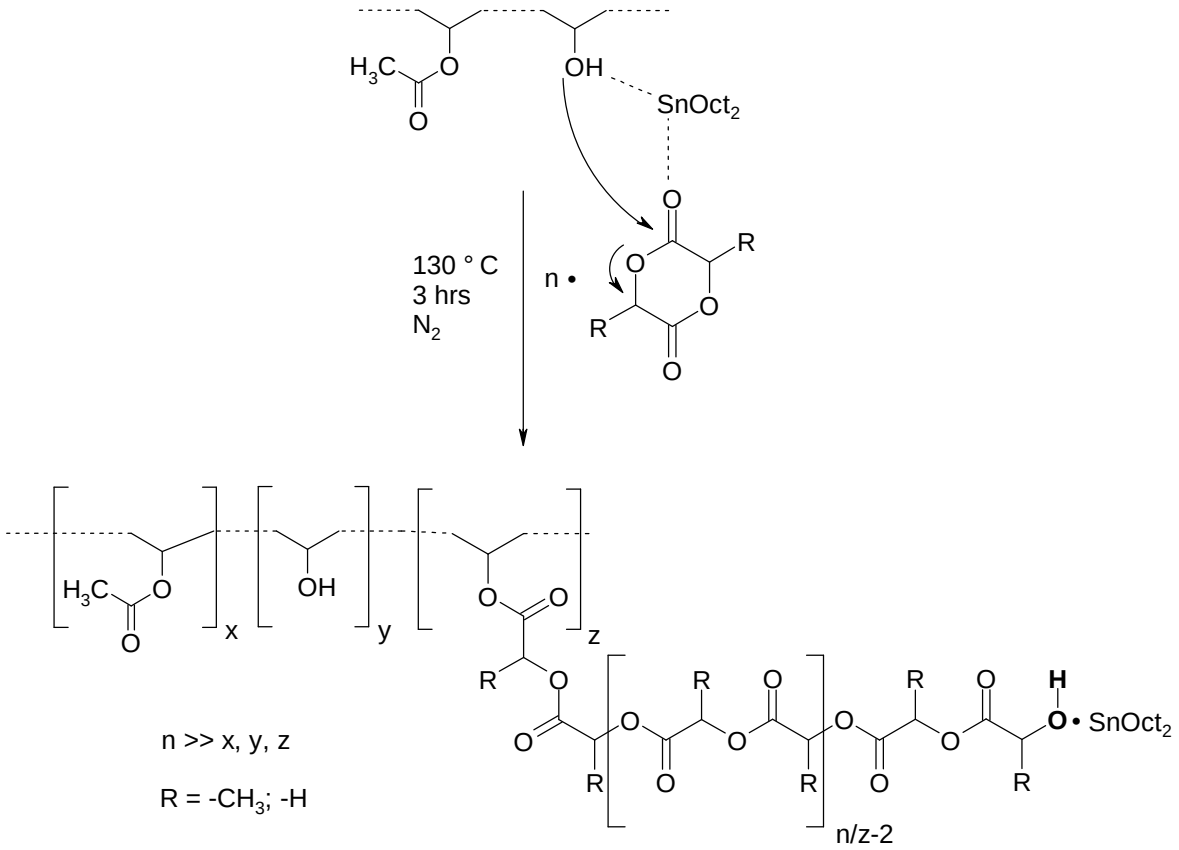


Figure 2: Influence of Reaction Time and Temperature (SEC traces, DCM as eluent) (D,L-LA + GA) : OH groups [PVA(06.80)] = (50 + 50) : 5.6 [mol%]

Their SEC analysis revealed a massive increase of low molecular by-products due to transesterification and thermal degradation. At 130°C yields of ca. 90 % and complete conversion even after 15 min reaction time were noted (fig. 2). The SEC traces of the graft polymers were symmetrical and monomodal, suggesting that no mixture of graft and linear polymers was formed. After ca. 15 min at the selected reaction temperature a clear colorless low viscosity melt of

the monomers and the backbone was formed. At that time point no polymerization could be detected by SEC. After injection of the catalyst a massive increase in viscosity was observed after a lag period of 5 to 10 minutes.

The ratio catalyst to polyol influenced the molecular weight of the PVA-g-PLGA as expected (data not shown): At PVA-OH/catalyst ratios in the range from 0 to ca. 100 mol% a constant increase of the resulting Mw of the polyesters was observed. Obviously SnOct produced more active sites per single PVA molecule, resulting in the growth of more and longer branches per molecule. At higher ratios the Mw starts to decrease, probably due to transesterification, as reported for linear polymer syntheses [22]. The results presented above, polydispersities in the range of about 2 to 3 and decreasing yields with increasing polyol in-feed, typical for step reactions, can best be explained by a reaction mechanism outlined in scheme 1.



Scheme 1: Schematic representation of the postulated polymerization mechanism

Tin octoate seems to activate the lactone carboxylic function as well as the OH groups of the polyol, possibly with its unoccupied d-orbitals. A coordination insertion polymerization mechanism is therefore a likely explanation for these results. The addition of tin octoate to a solution of PVA in DMSO lead to the formation of a white precipitate, which was accompanied by a decrease of the intensities of the PVA-OH signals in NMR, indicating the complexation by the catalyst, even leading to the formation of DMSO insoluble salts.

SEC analysis is not the method of choice to determine molecular weights, since it always underestimates the Mw of the grafted polymers due to their smaller hydrodynamic volume in solution compared to linear poly(styrene) reference material. Therefore, some selected comb polymers were analyzed by a combination of SEC and Static Light Scattering, to characterize their effective molecular weights and hydrodynamic volumes in solution.

Structure of the graft polymers

Spectroscopic Analysis. The branched structure of comb polyesters is characterized by an increase in the number of terminal OH groups and a decrease of carbonylic end groups. This is clearly demonstrated by NMR and IR. Fig. 3 shows the structure of PVA-g-L-PLAs with an increasing branch number and decreasing branch length. Among the signals typical for linear L-PLA in DMSO-d6, δ = 1.45 ppm (C<u>H</u>$_3$) and δ = 5.16 ppm (C<u>H</u>), several new signals appear in the spectra: δ = 1.97 ppm (PVA: -**C<u>H</u>$_2$**-CH-OCO) and δ = 5.35 (PVA: -CH$_2$-**C<u>H</u>**-OCO), which is in agreement with data reported previously for modified PVA [27], δ = 2.8 ppm (lactide: terminal -O<u>H</u>, only visible in $CDCl_3$ as solvent), δ = 4.2 ppm (lactide: terminal -**C<u>H</u>**(CH$_3$)OH) and δ = 1.28 ppm (lactide: terminal -CH(**C<u>H</u>$_3$**)OH). The assignment of the hydroxyl terminated lactide units is in excellent agreement with literature data [28,29]. It is worth

noting that signals of the methine protons of carboxylated lactyl end units (4.9 - 5.0 ppm) and free lactic acid (4.0 ppm) cannot be detected in the spectra, indicating that under the reaction conditions used no or less than 5 % homopolymerization of L-lactide occurred.

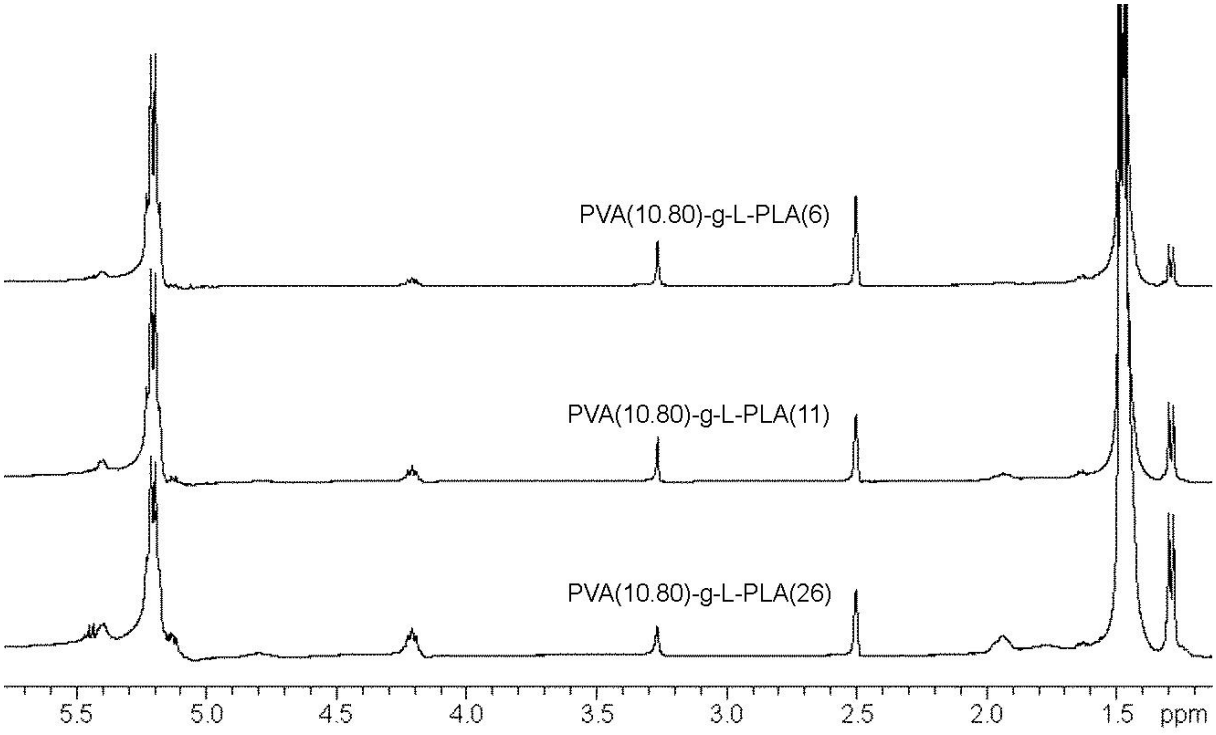


Figure 3: 400 MHz ^{1}H-NMR of graft L-PLA in DMSO-d_6 with increasing PVA incorporation

Fig. 4 confirms the signal assignments discussed above by the cross signals in the 2D COSY ^{1}H-^{1}H spectrum, the lactide chain coupling (1.46 ppm/5.16 ppm) can be seen as clearly as the coupling of the lactide end groups (1.28 ppm/4.2 ppm).

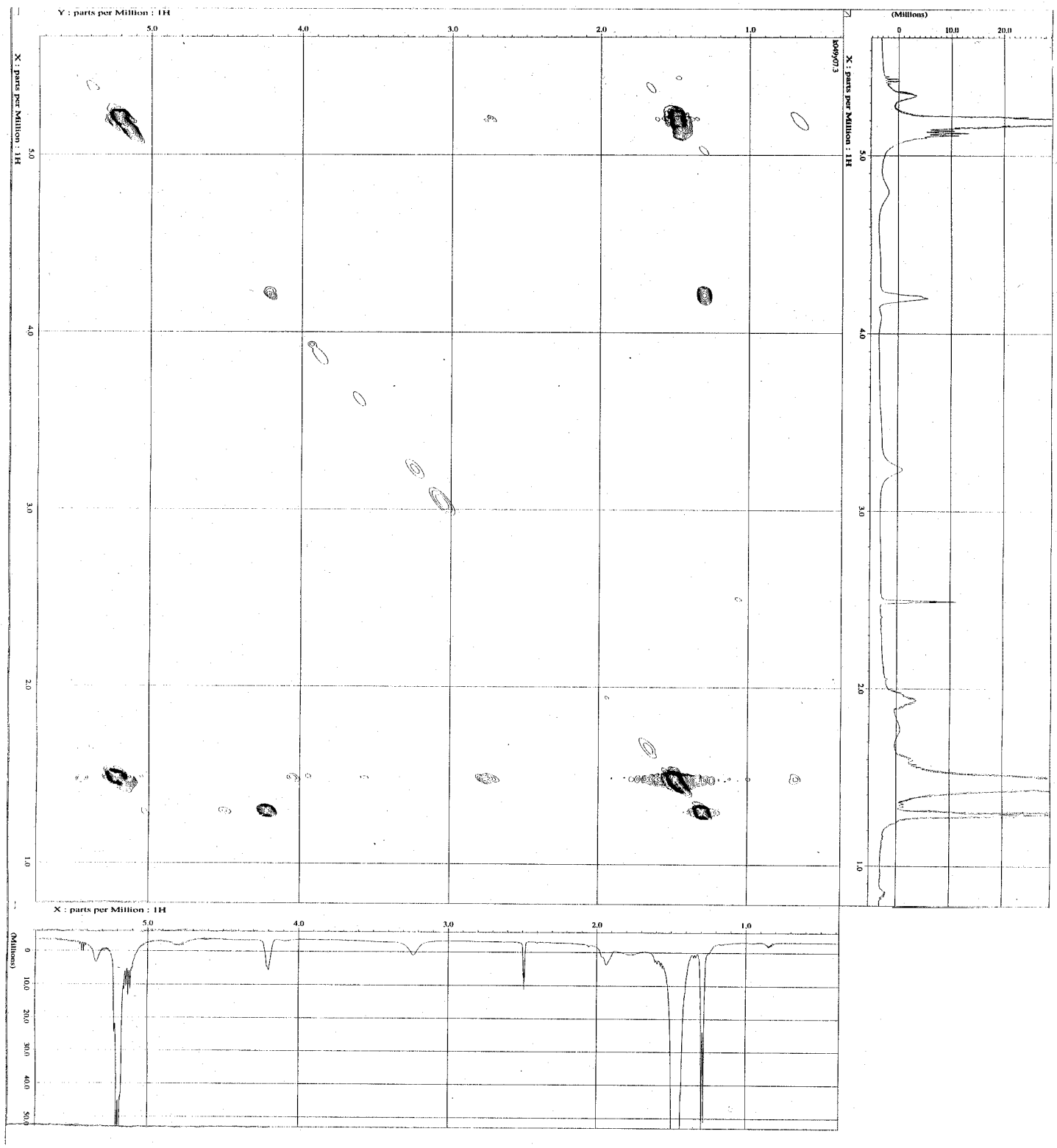


Figure 4: 2D COSY 500 MHz ^{1}H-^{1}H-NMR of graft PVA-g-L-PLA in DMSO-d_6

The signals of the terminal lactide unit resonate in ^{13}C spectra (compare fig. 6) at 20.38 ppm ($\underline{C}H_3$), 66.74 ppm ($\underline{C}H$) and 175.05 ppm ($\underline{C}O$), respectively. The usual signals for the lactide chains appeared at 169.6 ppm ($\underline{C}O$), 69.04 ppm ($\underline{C}H$) and 16.72 ($\underline{C}H_3$). The connecting ester bond was found at about 170 ppm and the PVA-**$\underline{C}$H**-OCO at about 70 ppm.

To quantitate the amount of PVA incorporated into the graft polymers an aromatic derivative of PVA was used, which could be easily detected by NMR. Therefore, a (vinyl alcohol-vinyl benzoate(40:60)) copolymer (PVB) was prepared, whose aromatic signals are found at ca. 7.2 to 7.6 ppm (^1H) and 128 to 135 ppm (^{13}C) in the final grafted PVB-g-L-PLA (fig. 5).

Polymer	**OH : dimer : cat** [mol : mol : mol]	**Yield**[1)] [%]	**Mw** [kg/mol]	**D**[2)]	**Tg**[2)] [° C]
PVA(15.88)	-	-	15	3.5	44.5
PVB(40 : 60)	-	65.0[3)]	33.5	2.7	63.7
PVB-g-L-PLA	13.1 : 100 : 0.46	88.2	163	2.1	58.5

[1)] 3 hours at 130 ° C
[2)] determined by SEC
[3)] 3 hours at 0 °C

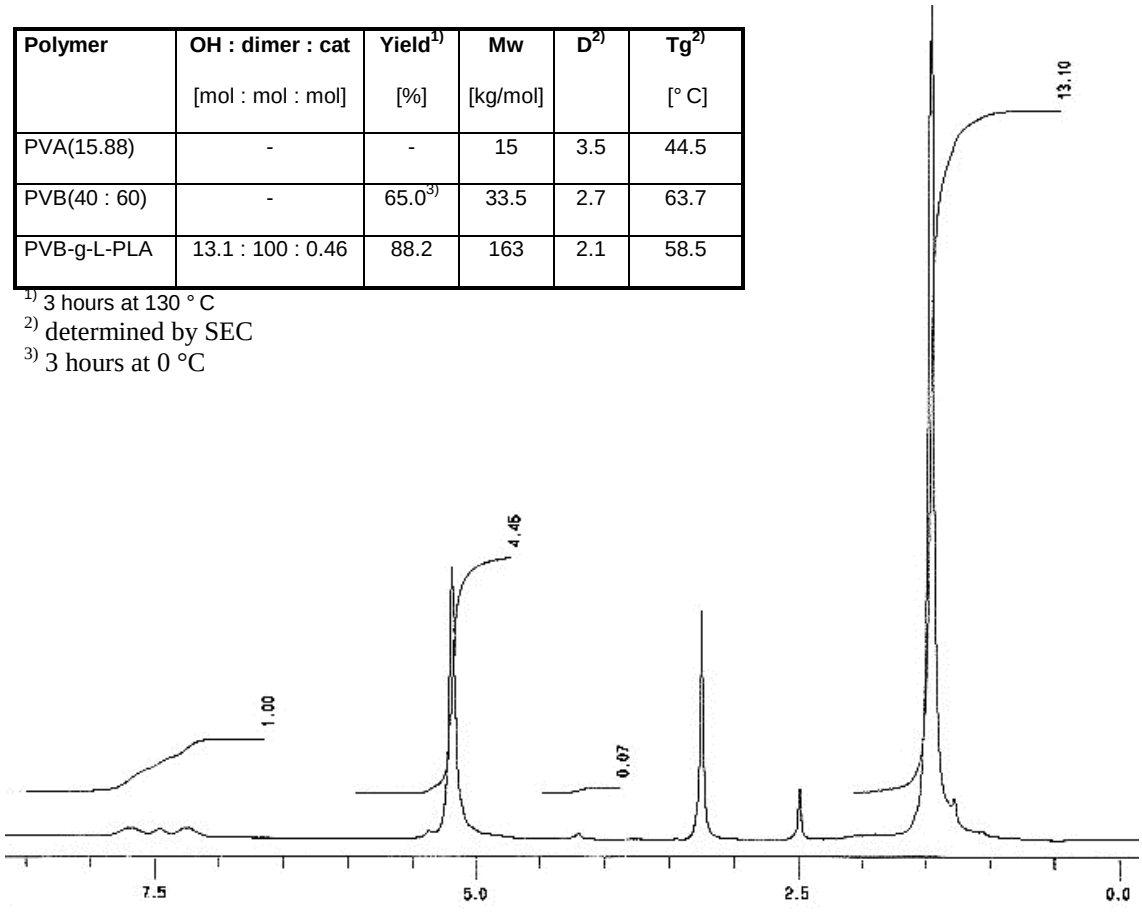


Figure 5: 400 MHz ^1H-NMR of PVB-g-L-PLA in DMSO-d_6

The integration of the intensities suggests quantitative incorporation of the polyol backbone in the polyester.

The chain number and length derived from NMR analysis by comparison of the intensities of the lactide end units and the lactide chains followed the polyol infeed ratio. The more PVA-OH groups present, the shorter the PLA chains, leading to polymers where nearly all free OH groups had reacted. A chain length of only 8 lactyl units per propagation center was observed (theoretical 6.9). The complete results are summarized in table 2.

When PVA is used as coinitiator the molecular weight is decreasing with the polyol/lactide ratio. The NMR examination revealed the presence of PVA ester units but the absence of octoate end groups. These results indicate that the energy of activation of an initiation involving polyols is obviously lower than that of neat tin octoate. Kricheldorf and coworkers [30] came to the same conclusions for the polymerization of L-lactide with benzyl alcohol as coinitiator. In agreement with their results, we found $CH(CH_3)$-OH end groups in addition to the PVA ester units in nearly identical quantities which is compatible with a polymerization mechanism as outlined in scheme 1.

Table 2: Chain length and branching number derived from NMR analysis

Polymer No [1]	**Chain number / length** [from NMR]	**Chain number / length** [from feed]	**Mn(NMR)** [2] [kg/mol]	**Mn(LS)** [3] [kg/mol]	**Mn(LS) / Mn(SEC)**
4	2.7 : 100 / 37.6	2.05 : 100 / 48.8	828	773	13.04
5	4.6 : 100 / 21.7	5.7 : 100 / 17.5	470	406	5.52
6	11.1 : 100 / 9.1	14.4 : 100 / 6.9	211	234	4.30
11	10.3 : 100 / 9.7	13.1 : 100 / 7.6	82.3	165	3.24
10	12.5 : 100 / 8.0	13.1 : 100 / 7.6	115	149	4.25
9	6.9 : 100 / 14.5	5.6 :100 / 17.8	200	n.d.	-
8	3.7 : 100 / 27.0	2.8 : 100 / 35.7	364	n.d.	-
16	10.4 : 100 / 9.6	13.1 : 100 / 7.6	302	n.d.	-

1) Numbers of polymers referring to Table 1

2) Number average molecular weight calculated from NMR assuming complete conversion of PVA-OH

3) Number average molecular weight determined by combined SEC and Light Scattering analysis

n.d. = not determined

NMR calculations by signal intensity comparison of the PLA chain and end groups

Attached proton test technique (APT) was employed to assign the low intensive and very broad signals of PVA OCO-CH-**C**H_2-CH-OCO. Fig. 6 shows a ^{13}C-APT spectrum in chloroform-d with the inverted methylene signal in the range of 37.5 to 40.5 ppm, while nearly no remaining PVA **C**H_2-CH-OH was detectable. The appearance of this signal can be taken as evidence for the incorporation of PVA in the comb polyesters. In this spectrum even weak signals of the connecting ester bond at about 170 ppm and of the PVA-**C**H-OCO at about 70 ppm were visible.

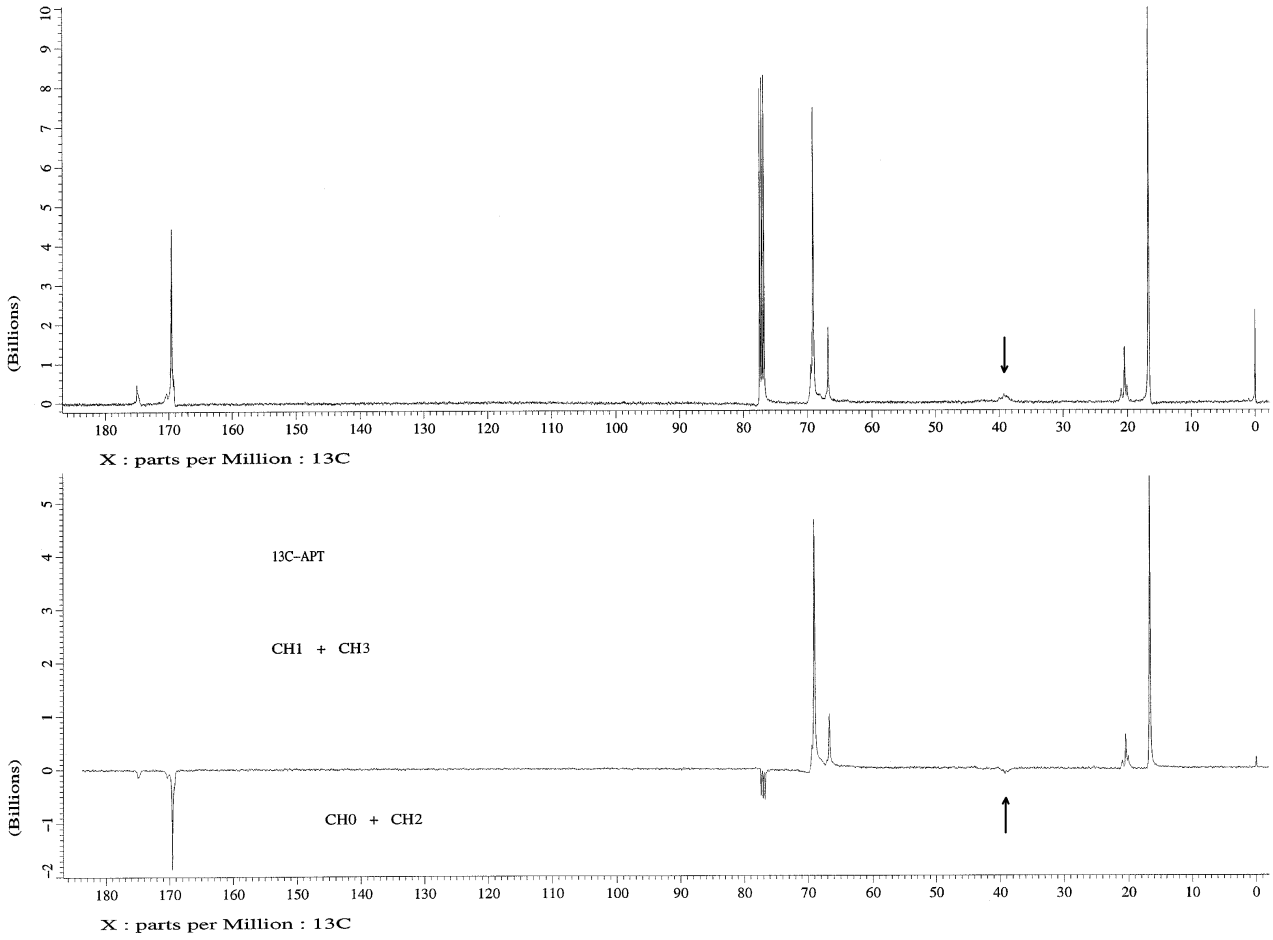


Figure 6: 100 MHz ^{13}C-APT NMR of graft PVA(20.74)-g-L-PLA(48) in $CDCl_3$

Fig. 7 shows the increase of terminal hydroxyl groups with an increase of the PVA amount in the polymer by the increase of the intensity of the OH vibration in the IR spectra. These IR spectra alone of course cannot be taken as evidence for the polymer structure, since this increase could also be caused by residual water even after intensive drying of the samples.

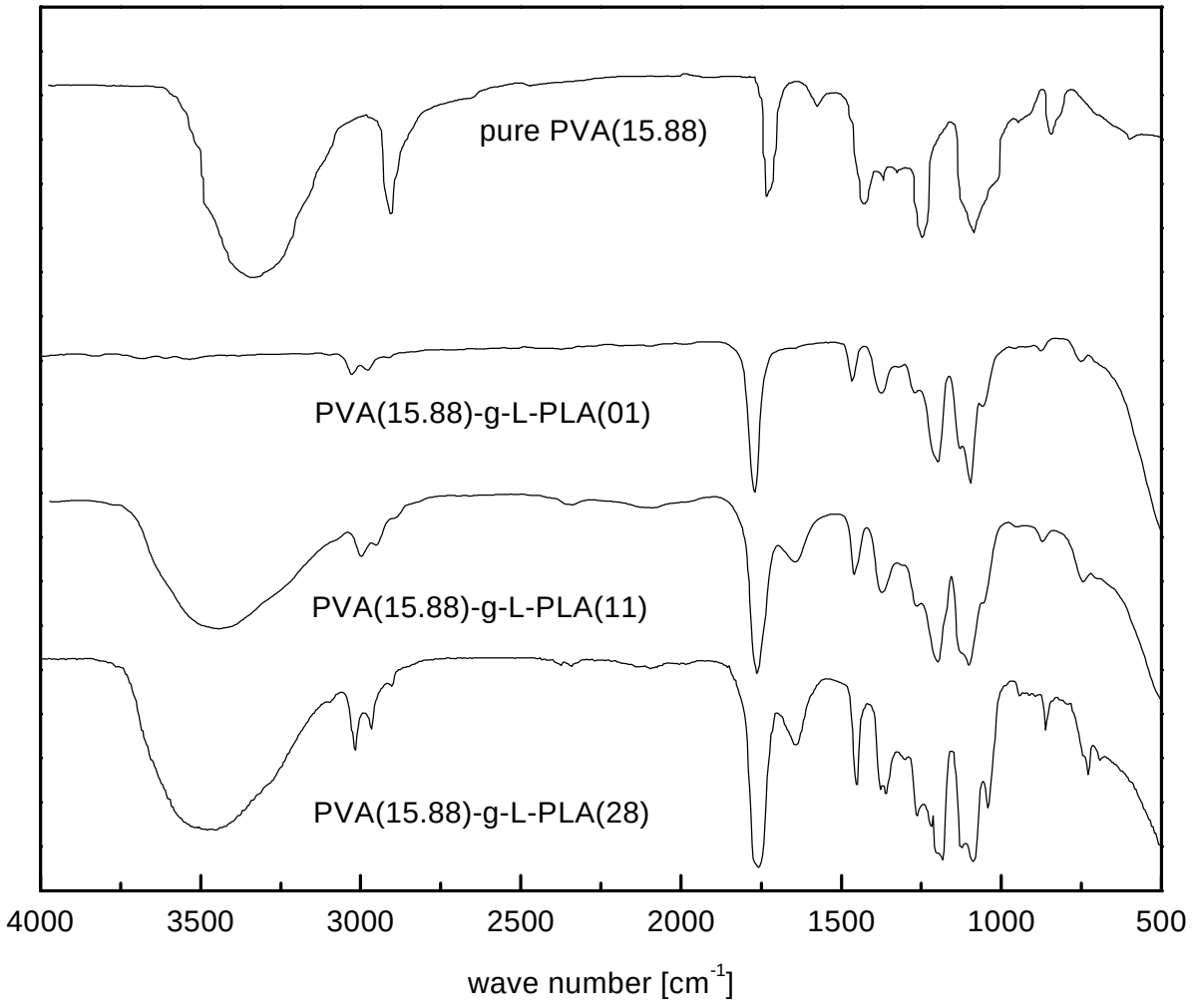


Figure 7: IR spectra of graft L-PLA with increasing PVA incorporation

Solution Properties. Static Light Scattering (LS) and the determination of the intrinsic viscosities are effective methods to investigate and prove the molecular structure of polymers. Therefore, linear and graft PLAs were characterized by both methods. The weight average molecular weights and the root mean square

radii of gyration (Rg) were determined by LS using Zimm's method and the results are summarized in table 3.

Table 3: Light Scattering Analysis and Intrinsic Viscosities

Polymer	**Rg(LS) / α(LS)** [nm] / [-]	**Mw(LS)** [kg/mol]	**Intr. Viscosity** [dl/g]
L-PLA	20.2 / 0.52	105	1.14
- " -	n.d.	91	1.06
- " -	27.3 / 0.22	27.7	0.43
D,L-PLA	23.5 / 0.59	140	n.d.
PVA(15.88)-g-L-PLA(28)	23.7 / 0.14	445	0.26
PVA(15.88)-g-L-PLA(11)	27.5 / 0.25	1,010	0.31
PVA(15.88)-g-L-PLA(4)	29.3 / 0.35	1'877	0.37
PVA(15.88)-g-L-PLA(1)	37.5 / 0.53	4'570	0.53
PVA(06.80)-g-L-PLA(26.6)	12.3 / 0.55	292	n.d.
PVA(10.80)-g-L-PLA(26.6)	17.7 / 0.54	215	n.d.

n.d. = not determined

Rg is a physical property only depending on molecular architecture and molecular weight, following the equation: $Rg = A * Mw^{\alpha}$, in which A is a constant and α is correlated to the polymeric structure. Stiff macromolecular chains show a more rod like structure, leading to larger radii of gyration and α values. A random coil structure leads to smaller Rg as well as α, but still larger than the values of molecules with a spherical structure in solution. This difference between linear and graft PLA is demonstrated in fig. 8.

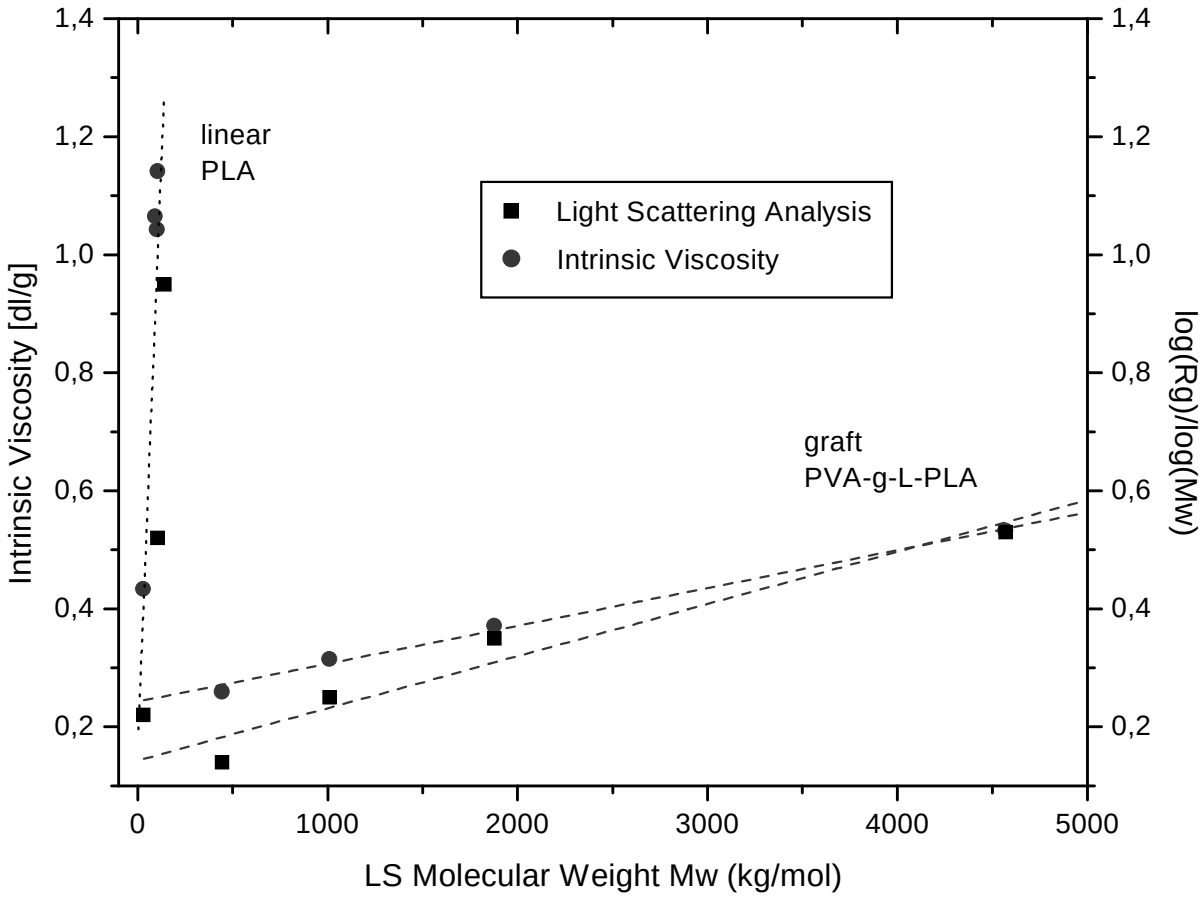


Figure 8: Light Scattering analysis and intrinsic viscosities

Linear PLAs behave more rod like as indicated by their higher values of Rg and α. Graft PLAs exhibit smaller hydrodynamic volume in solution, which is a further evidence for their comb structure. Increasing molar ratios of PVA incorporated into the comb polyesters lead to shorter PLA branches and, therefore, lower the values for Rg and α. The final evidence for the molecular structure can be seen in the dependency of α with the molecular weight. It is much lower for the graft polymers than for the linear ones. Because of the comb structure an increase in chain length or chain number will not affect the size of the molecules as significantly as in the case of linear polymers.

Table 2 describes a comparison between the number average molecular weights as calculated from the NMR data assuming quantitative esterification of PVA hydroxyl groups with the actual values determined by light scattering. Since it is unlikely that all PVA-OH groups will react due to steric hindrance, the calculated theoretical values will always exceed the actual LS results. Polymers 4 to 6 showed an interesting trend: with less PVA incorporated, a more pronounced deviation of the theoretical values from the experimental ones is observed. This suggests that at lower polyol concentrations more PVA hydroxyl groups remain unreacted. These results are compatible with the polymerization mechanism discussed above: After the first ring-opening insertion of a lactone, tin octoate seems to move with the terminal hydroxyl group of the growing chain, respectively. Due to an increasing viscosity of the reaction mixture and a greater chain length it is unable to reach free OH-groups of the backbone at later stages of the reaction, when only low amounts of the polyol are present during the polymerization. Fig. 8 also demonstrates the same trends for the intrinsic viscosities. PVA-g-PLAs had a significant lower viscosity, although their Mw was much higher than that of the linear ones, confirming their smaller hydrodynamic volume in solution as a consequence of the grafted structure.

Thermal Properties. Differential Scanning Calorimetry (DSC) was used to determine the thermal properties of the polymers. The expected decreases in the glass transition temperatures (Tg), melting points (Tm) and melting enthalpies (degrees of crystallinity) could be observed. The decreases were proportional to the PVA infeed ratio. All DSC traces showed only one Tg (and Tm). Therefore, both components are totally miscible and do not lead to phase separation (fig. 9). Both Tg and Tm decrease with increasing PVA/PLA ratio due to higher chain mobility.

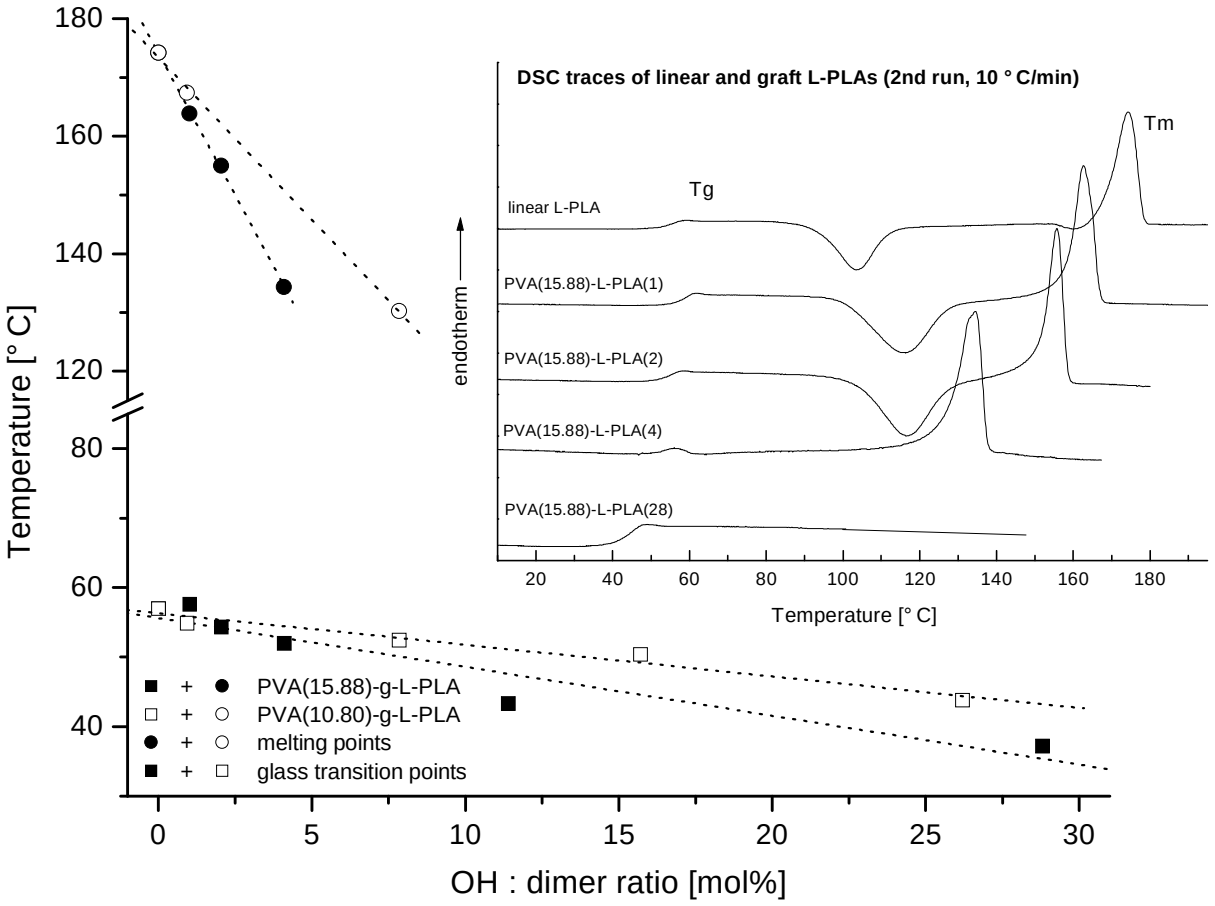


Figure 9: Thermal properties of graft PLA

Taking all results in account, we present the possibility not only to manipulate the molecular weight of the PLGA by grafting onto a hydrophilic core molecule in an effective way, but also to synthesize polymers with specific thermo-mechanical properties. Since aliphatic polyesters are thought to degrade by a random hydrolytic cleavage of the ester bonds, crystallinity and water uptake are the key factors determining the rate of polymer degradation, which can be manipulated specifically with comb-like polyesters. The hydrophilic backbone introduced into the comb-like polyester and the adjustable degree of amorphous segments make these polymers promising candidates for encapsulation of drugs such as proteins and peptides. In vitro degradation studies as well as analysis of water uptake and protein release profiles are currently under investigation [31].

2.5 CONCLUSIONS

The bulk polymerization of brush-like grafted polyesters of L-lactide, D,L-lactide and their random copolymers with glycolide containing different poly(vinyl alcohol)s as backbones using stannous octoate as catalyst was established. The presence of linear homopolymers was ruled out on the basis of SEC, light scattering and NMR analysis.

The comb polymers were characterized by NMR, IR, SEC, LS, intrinsic viscosity measurements and DSC. The incorporation of the hydrophilic backbone was demonstrated most clearly by NMR spectroscopy, light scattering and the relation of the polymer properties and the reaction conditions. Light scattering analysis and intrinsic viscosity measurements confirmed their smaller hydrodynamic volumes in solution with a lower dependency on Mw. Molecular weights were proportional to the polyol/lactone ratio. NMR analysis revealed hydroxyl terminated end units confirming a different reaction mechanism. Tgs of the grafted PLGA were lowered by ca. 5 to 10 °C due to their nonlinear architecture. The same trend was observed for the melting points of grafted L-PLA.

Biodegradable graft PLGAs with polymeric polyols as backbone may be interesting new materials for biodegradable drug delivery systems.

2.6 ACKNOWLEDGEMENTS

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Chapter 3

Branched biodegradable polyesters for parenteral drug delivery systems

3.1 ABSTRACT

Continuous, “infusion-like” drug release profiles from biodegradable parenteral delivery systems are difficult to achieve for proteins and other hydrophilic macromolecular drugs with commonly used linear polyesters from lactic acid (PLA) and its random copolymers with glycolic acid (PLGA). Drug release rates can be modified either by increasing the hydrophilicity of polyesters or by manipulating the polymer architecture to adjust polymer degradation rates and thus drug release.

Therefore, we investigated different branching concepts for biodegradable polyesters of PLA and PLGA. For one 4- and 8-arm poly(ethylene oxide)s (PEO) were grafted with shorter polyester chains leading to star-branched structures. Secondly we obtained comb-like polyesters using both charged and uncharged dextrans or poly(vinyl alcohol)s (PVA) as hydrophilic backbones. The star-shaped and brush-like grafted polymers were intensively characterized by methods, such as NMR, IR, SEC-SLS, DSC and viscosity measurements. Tailor-made properties make these novel biodegradable polyesters promising candidates for parenteral protein delivery systems.

While the star-branched polyesters have shown some interesting properties with respect to their degradation behavior, retaining the PEO blocks longer than ABA triblock copolymers, their release properties need further optimization. Brush-like branched polyesters on the other hand seem to possess both degradation and release properties meriting further investigations for parenteral protein delivery systems.

3.2 INTRODUCTION

New biomaterials have provided significant opportunities and progress for therapeutic and diagnostic systems [1]. Established applications range from contact lenses to wound dressings and emerging new fields like tissue engineering may provide additional challenges to create biomaterials with specific properties allowing cells or tissues to grow most commonly in polymer matrices [2,3]. New biomaterials for drug delivery are of particular interest for the parenteral administration of peptides and proteins. Both hydrogels as well as biodegradable microspheres have been studied extensively [4-6] and several peptide delivery systems have become commercially available.

Commonly used linear biodegradable polyesters, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and especially their copolymers poly(lactic-co-glycolic acid) (PLGA), are playing a prominent role in drug delivery [7]. For parenteral delivery systems it is necessary to carefully adjust drug release and polymer degradation rates.

Drug release from polyesters, such as PLA and PLGA, is generally controlled by both drug diffusion and polymer erosion. But, despite considerable efforts, it often deviates from an ideal "infusion like" profile. Especially hydrophilic drugs with molecular weights above 0.5 kg/mol, such as peptides and proteins, have shown in vivo plasma-level/time curves with clear discontinuities [8]. In an initial phase release occurs predominantly by pore-diffusion through an interconnecting network formed by the dissolving drug substance itself. The second release phase is governed by polymer degradation.

Polyphasic drug release profiles can be overcome either by formulation approaches or by modification of the biodegradable polymers. While the release properties of biodegradable microspheres can be modified only in a limited

sense, polymer modifications provide a broader spectrum of possibilities. For one, increasing the hydrophilicity of PLGA leads to accelerated polymer degradation rates. Increased hydrophilicity of PLGA causes a faster swelling of the polymeric matrix and, thereby, promotes both drug diffusion and polymer erosion. This modification of polymer-polymer phase behavior [9] seems to allow the manipulation of release properties [10-13].

The molecular architecture of biodegradable polymers can be exploited in another way to adjust polymer degradation and erosion. By incorporation of multifunctional polyols into the polyester chain, branched structures are obtained in suitable solvents ranging from star-branched [14-17] to brush-like structures [14,18-23]. In the respective delivery systems this architecture does not compromise the thermo-mechanical properties and accelerates the polymer degradation rate by providing preformed break-point in the polymer chain with short chain length, after swelling of the device. The star- or brush-like geometry should, therefore, promote erosion controlled drug release. Polymer brushes [24] and star-burst dendrimers [25] could serve as models for more complex structures of biodegradable polymers. Grafting of biodegradable polymer chains, such as PLGA onto these backbone may provide new biomaterials with interesting properties.

Here we report some results of star- and brush-like grafted PLGA using either hydrophilic multi-arm PEOs or charged, hydrophilic polyelectrolyte backbones. Both types of biodegradable polyesters seem to be promising candidates for parenteral protein delivery systems, allowing a synchronization of both pore diffusion and polymer erosion for the controlled release of hydrophilic drug candidates.

3.3 MATERIALS AND METHODS

Syntheses of PEO-PLGA and PEO-PLA

The polyesters were synthesized by ring-opening polymerization of the lactones, L-lactide, D,L-lactide and glycolide in the presence of the hydrophilic backbones. All reactions were carried out with purified and rigorously dried core molecules and monomers in anhydrous nitrogen atmosphere. PEO-L-PLA_n and PEO-L-PLG_n (n = 2, 4, 8) were polymerized in toluene solution at 70°C for 72 to 96 hours, after activation of the backbones with aluminum triethylene for 30 min at 25°C [23,24].

Syntheses of brush-like grafted PLGA and PLA

Comb polyesters were obtained by ring-opening bulk polymerization of the lactones in the presence of the hydrophilic core molecules using stannous octoate as catalyst. The reactions were carried out in a dry nitrogen atmosphere at 110°C to 190°C for 3 to 24 hours, depending on the used polyol [21,25-30].

Preparation of films and in-vitro degradation studies

Films, cast from 5 % (w/v) dichloromethane (DCM) solutions on Teflon® coated plates, were allowed to dry for 24 hours. Residual solvent was removed in vacuo at room temperature and finally at 40°C in the course of 2 days until constant weight was obtained. Films were cut into 20*10 mm^2 samples with a thickness of about 0,150 mm. The specimen with known weight (ca. 50 mg, n = 3) were immersed in 5 ml of an isotonic phosphate buffer saline solution (PBS, pH 7.4) and stirred in a rotating thermostat (Rotatherm, Liebisch) at 30 rpm and 37°C. At preset time intervals, the samples were recovered and freeze dried at -20°C for 48 hours, followed by drying in vacuo at room temperature to constant weight.

Microsphere preparation

Microspheres were prepared by a modified triple-emulsion-technique [8]. Briefly, 800 µl of an aqueous FITC-Dextran (40 kg/mol, Sigma) or bovine serum albumin (BSA, Sigma) solution were emulsified in 2.33 ml of a polymer solution (10 % (w/w) in DCM using an Ultraturrax homogenizer Type18/10 (Janke & Kunkel, Staufen) at 20000 rpm for 2 x 30 s. The resulting emulsion was injected with a syringe (18 G needle) in 400 ml of an aqueous solution (0.4 % (w/w)) of poly(vinyl alcohol) and emulsified with a rotor/stator-homogenizer (Ultraturrax T25, Janke & Kunkel, Staufen) at 8000 rpm for one minute. The system was maintained at room temperature under magnetic stirring for 3 hours to allow solvent evaporation. Microspheres were collected by centrifugation, washed with distilled water, freeze-dried and stored at 4°C.

Microsphere characterization and in-vitro release profiles

Surface morphology and sizes were investigated by scanning electronic microscopy (SEM, Hitachi S 510, Tokyo, Japan) and laser diffractometry (Mastersizer X, Malvern Instruments, Malvern, UK, as described previously [26]. A known amount of microspheres (ca. 50 mg, n = 3), loaded with FITC-dextran or BSA (drug loading: 4.3 - 4.7 % (w/w), average sizes: 30 - 60 µm) [26] was immersed in isotonic phosphate buffer saline solution (pH 7.4) at 37°C under stirring for drug release studies.

Characterization of polymer samples

Size Exclusion Chromatography (SEC) was conducted from 0.5 % (w/v) polymer solutions in dichloromethane (DCM) with two size exclusion columns (Lichrogel PS mix and Lichrogel PS 40, 10 µm) and a differential refractometer (RI 71) as detector [Merck-Hitachi SEC system]. Degassed DCM was pumped at a flow rate of 1 ml/min. Molecular weights were calculated by 3rd order universal calibration relative to poly(styrene) reference materials [Merck].

Differential Scanning Calorimetry (DSC): Glass transition temperatures (Tg) (from the second run) and melting temperatures (Tm) were obtained with a differential scanning calorimeter [Perkin Elmer DSC 7] (0°C to 200°C, heating and cooling rates: 10°C/min). Intrinsic viscosities were determined using an Ubbelohde viscosimeter [Schott Geräte] from THF solutions at 30°C with at least four different concentrations. Nuclear Magnetic Resonance (NMR): All spectra were recorded at 35°C from 6 % (w/v) solutions of the polymers in different deuterated solvents, like d-chloroform and d_6-DMSO [400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectra: Jeol GX400 Delta N FT spectrometer, 500 MHz ^{1}H- and 125 MHz ^{13}C-NMR spectra: Jeol LA500 eclipse+ Delta FT spectrometer]. Combined SEC and static light scattering analysis (SLS) was carried out with a SDV linear column (300×8 mm, 10 μm), an Optilab 309 differential refractometer and a Mini Dawn Light Scattering detector [Wyatt Technology Corporation] (K 5 cell, laser wavelength of 690 nm, 20 mW, detecting angles: 45°, 90° and 135°). Degassed THF at 25°C with a flow rate of 0,7 ml/min was used as eluent.

3.4 RESULTS AND DISCUSSION

Syntheses

ABA block copolymers as well as 4- and 8-arm PEO-PLA and -PLGA were synthesized by solution polymerization in toluene, using pre-activation of the PEOs with aluminum triethylene as catalyst [24]. High yields and no detectable homopolymers of lactide and glycolide demonstrate the versatility of this polymerization reaction. First evidence for the non-linear structure of the star-branched polyesters was demonstrated by NMR analysis, confirming the incorporation of the star-branched PEO backbone. While the ^{1}H NMR signals of the L-PLA chains were found at 1.55 ppm (CH_3) and 5.1 ppm (CH), their hydroxyl terminated end groups resonated at 1.45 ppm (CH_3) and 4.15 ppm

(CH). Moreover, among the signals of the PEO chains at 3.6 ppm, the connecting PEO units were found at 3.75 ppm and 4.3 ppm (CH_2-CH_2-OCO-).

The results are in agreement with the combined SEC and static light scattering analysis as outlined in fig. 1. While linear polymers behave more rod-like in solution, significantly smaller radii of gyration were found for the star-type polyesters although their molecular weights increased according to the number of polyester chains connected to a single backbone.

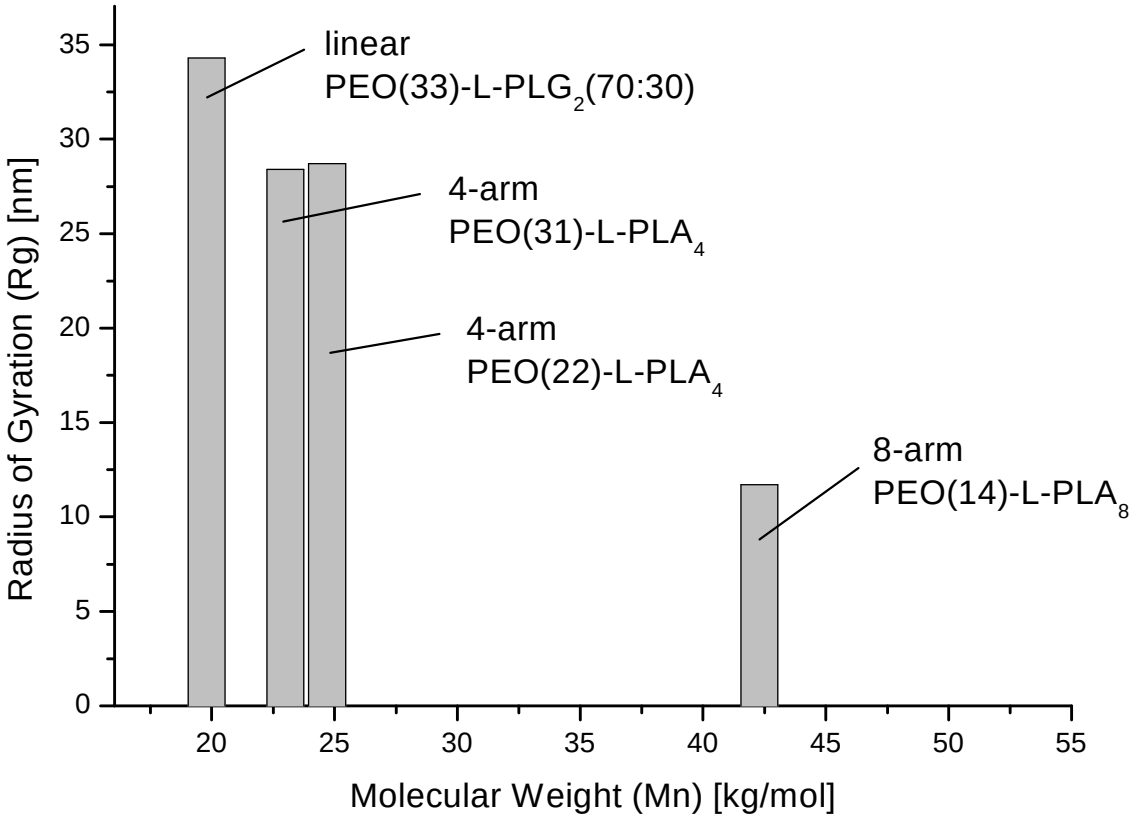

Figure 1: Static Light Scattering Analysis of linear and star-type PEO-PLA

The reduction of interactions between the molecules due to their steric architecture lowered glass transition temperatures (Tg) and melting points (Tm) compared to linear ABA block copolymers as well as linear polyesters in a similar manner. A significant decrease in the crystallinity of L-PLA blocks was also observed. The physico-chemical properties of these polyesters are summarized in table 1.

Table 1: Physico-chemical properties of linear and star-type PEO-PLA

Nr.	**Polymer**	**PEO**	**Mn(PEO)** [kg/mol] [1)]	**L-LA : GA : EO** (NMR) [wt%]	**L-LA : GA** (NMR) [mol%]	**Mn** (NMR) [kg/mol]	**Mn** (SLS) [kg/mol]	**Rg** (SLS) [nm]	**D**	**Tg** [°C]	**Tm** [°C]
1	PEO-L-PLG_2(7:3)	linear	6.0	50 : 17 : 33	70 : 30	18	19.8	34.3	1.9	42	-
2	PEO-L-PLA_4	4-arm	5.9	78 : 0 : 22	100 : 0	26.9	24.7	28.7	1.3	45	165
3	PEO-L-PLA_4	4-arm	5.9	69 : 0 : 31	100 : 0	19.1	23.0	28.4	1.3	42	163
4	PEO-L-PLG_4(8:2)	4-arm	5.9	57 : 8 : 35	85 : 15	16.9	19.8	-	1.5	38	149
5	PEO-L-PLG_4(7:3)	4-arm	5.9	54 : 18 : 28	71 : 29	21.1	21.7	-	1.6	34	-
6	PEO-L-PLA_8	8-arm	7.1	86 : 0 : 14	100 : 0	51.1	42.3	11.7	1.5	35	154
7	PEO-L-PLG_8(7:3)	8-arm	7.1	55 : 15 : 30	75 : 25	23.7	21.2	-	1.7	24	-

1) from combined SEC and static light scattering (SLS) analysis

Branched PLGAs were synthesized by bulk melt polymerization of the lactones in the presence of the polyols, poly(vinyl alcohol) PVA, dextran, dextran sulfate sodium (DSS) and di-ethyl-amino-ethyl dextran (DEAED) using stannous octoate as catalyst. High yields and conversion could be reached in each case. The presence of linear lactone based homopolymers was ruled out on the basis of SEC and NMR analysis. The incorporation of the hydrophilic backbones was demonstrated by NMR spectroscopy, static light scattering and the relation of the polymer properties and the reaction conditions.

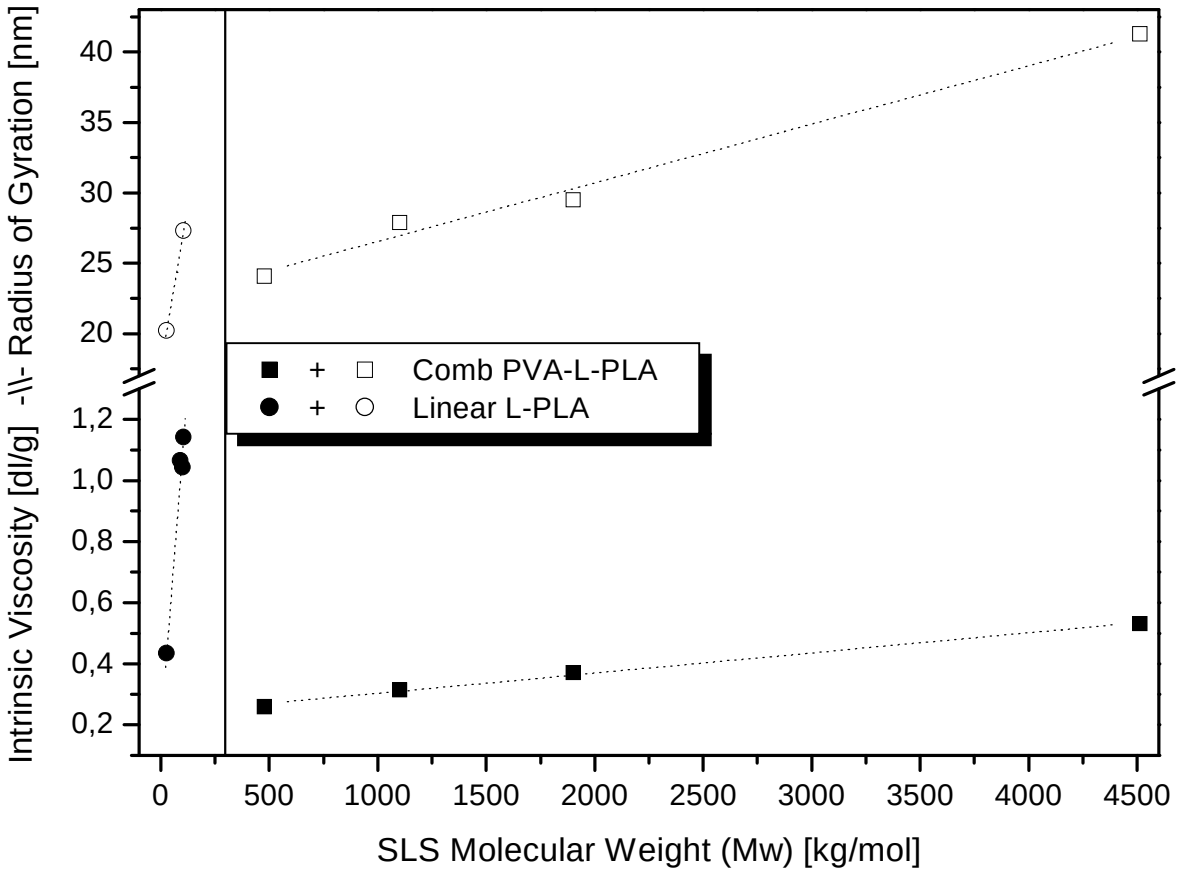

Figure 2: Intrinsic Viscosities and Radii of Gyration of linear PLA and comb PVA-g-PLA vs. Absolute Molecular Weight as investigated by SLS analysis

Light scattering analysis and intrinsic viscosity measurements confirmed the smaller hydrodynamic volumes in solution showing different slopes for linear and branched polymers as a function Mw (fig. 2). Molecular weights, glass transition temperatures and all other properties of the branched polyesters were proportional to the polyol/lactone ratio and, therefore, could be systematically designed. NMR analysis (328 kg/mol PVA(10)-g-L-PLA) demonstrated hydroxyl terminated end units and the absence of carboxylated end units, as outlined in fig. 3, compatible their branched structure. These and other properties are summarized in table 2.

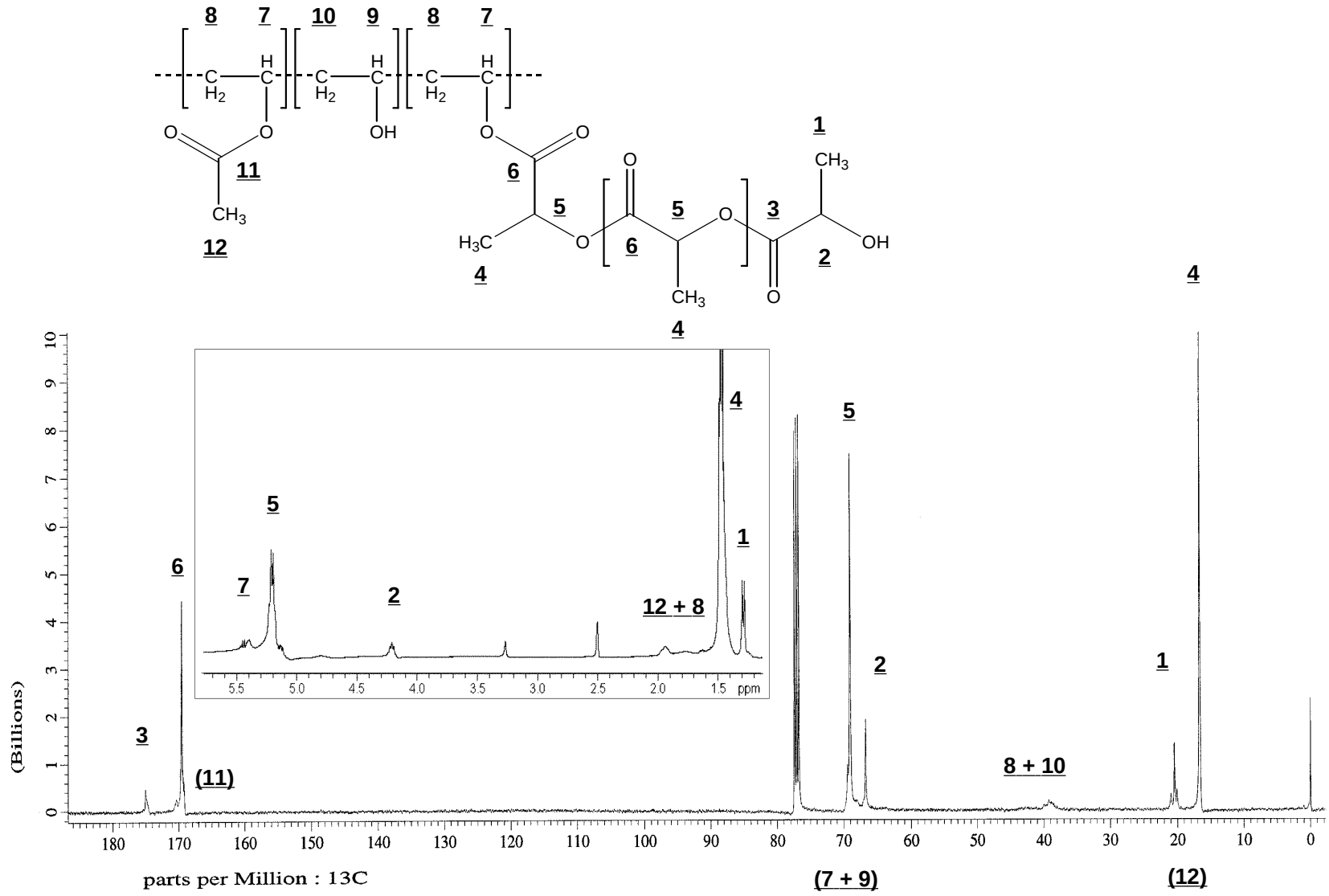

Figure 3: Structural analysis of comb PVA-g-L-PLA by ^{13}C- and ^{1}H-NMR spectroscopy

Table 2: Physico-chemical properties of linear PLA and comb PVA-g-PLA

No	**Polymer**	**OH : dimer : cat** [mol : mol : mol]	**Mw** (SEC) [kg/mol]	**D** (SEC)	**Mn** (SLS) [kg/mol]	**Mn** (NMR)[1] [kg/mol]	**Rg** (SLS) [nm]	**Intr. Visc.** [dl/g]	**Tg** [°C]	**Tm** [°C]	**ΔHm** [J/g]
1	L-PLA	0 : 100 : 0.16	105	2.0	81.7	n.d.	20.2	1.14	57	174.2	52
2	PVA(15)-g-L-PLA	1 : 100 : 0.16	274	2.5	3324	n.d.	37.5	0.53	57.6	163.8	52.3
3	- " -	4 : 100 : 0.16	166	2.8	773	828	29.3	0.37	52.0	134.3	31.7
4	- " -	11 : 100 : 0.16	125	1.7	406	470	27.5	0.31	43.3	-	-
5	- " -	29 : 100 : 0.16	98	1.8	234	211	23.7	0.26	37.3	-	-
6	PVA(10)-g-L-PLA	26 : 100 : 1.56	77.2	2.2	149	115	17.7	n.d.	43.8	-	-

1 = calculated from 1H-NMR by signal intensity comparison of PLA chain and end groups assuming complete conversion of PVA-OH

n.d. = not determined

Degradation and drug release

It is known, that linear ABA block copolymers show a degradation behavior different from the random copolymers of lactide and glycolide [30]. The hydrophilic PEO block promotes rapid hydrolysis, resulting in accelerated degradation rates. The erosion of these polyesters is accompanied by a constant elimination of PEO from the matrix as outlined in fig. 4a.

This fast elimination of the hydrophilic core could be a disadvantage in controlled drug release by means of microparticles because the hydrophilicity of the matrix is decreasing accordingly over the time. Especially hydrophilic macromolecular drugs will be confronted with a more and more hydrophobic environment. Star-branched block-copolymers on the other hand possess comparable swelling properties in water, which will be beneficial for the release of proteins and other hydrophilic macromolecules. The rate of molecular weight and mass loss in the initial 2 to 3 weeks, on the other hand is significantly slower as shown in fig. 4b-c.

After 3 weeks a distinct acceleration of the degradation rate is observed. This degradation pattern can be explained as follows: in the initial degradation phase, PEO is not released from the polymeric matrix and the multi-arm PEO core is retained for a longer period of time compared to linear ABA polymers. This leads initially to slower degradation rates of the polymer, which is in swollen state. Since water soluble breakdown products of the PLGA segments are attained after few cleavage steps, due to the shorter lactone chains, erosion of the matrix is accelerated after this initial phase. This biphasic degradation pattern offers an uniform environment for encapsulated drugs with nearly constant hydrophilic environment during release and a fast elimination of the polymeric matrix after drug exhaustion.

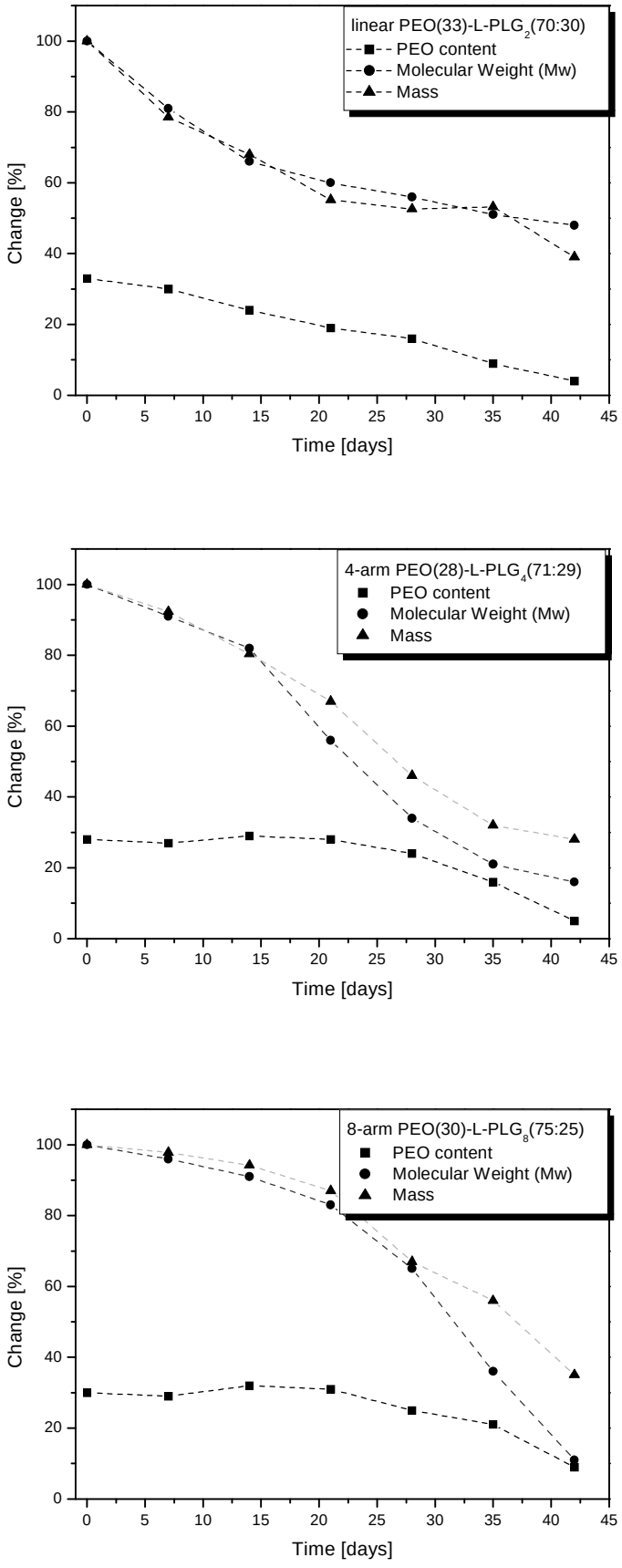

Figure 4: Degradation and erosion of linear and star-type PEO-L-PLGA

Fig. 5 outlines the improvement of hydrophilic model drug release from microspheres. Although the polymers are not completely comparable as far as their PLGA composition is concerned, a general trend can be seen. Due to the PEO block retained the initial release phase, characterized by diffusion control, is prolonged. Therefore, FITC-dextran (40 kg/mol) is released continuously in the degradation controlled phase with almost constant release rates. Similar results of protein release from star-branched PEO-PLGA block-copolymers were also obtained for microspheres containing recombinant human Erythropoietin [27].

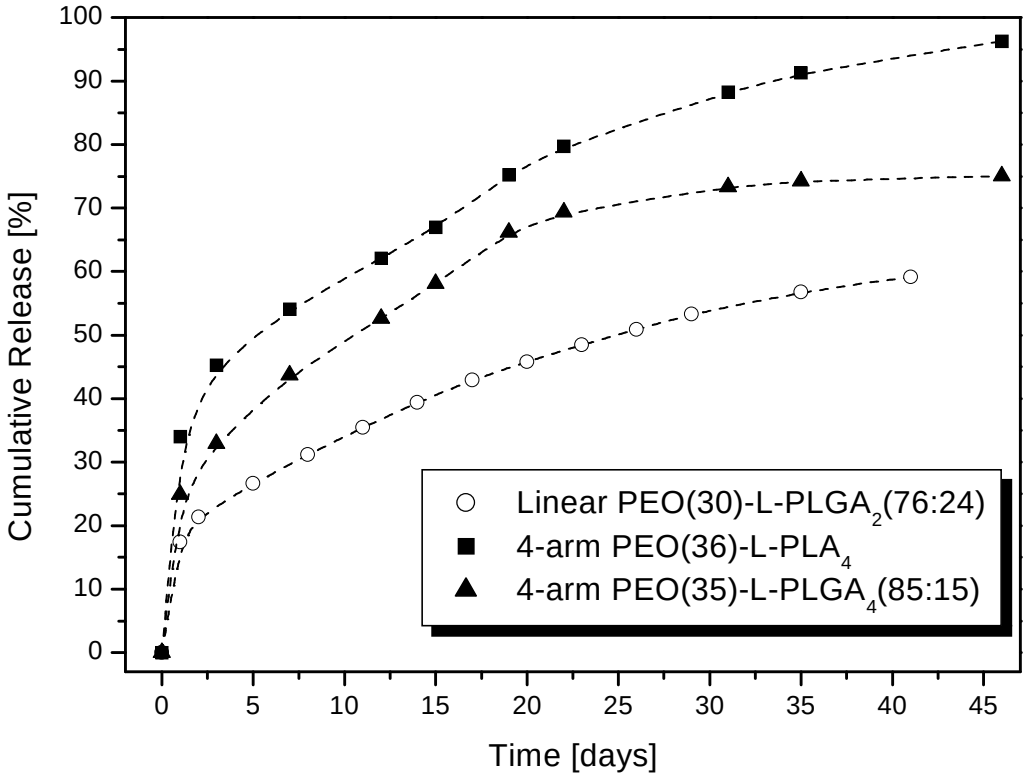

Figure 5: Release of FITC-dextran (40 kg/mol) from microspheres prepared from linear and star-type PEO-L-PLGA

The degradation of the polysaccharide backbone-containing branched PLGAs (table 3) is also accelerated by their nonlinear structure as shown in figure 6.

Table 3: Physico-chemical properties of polysaccharide containing PLGAs

No	**Polymer**	**sugar : dimer : cat** [mol : mol : mol]	**Mw** (SEC) [kg/mol]	**D** (SEC)	**Tg** [°C]
1	linear D,L-PLGA(1:1)	-	42	2.8	44
2	DSS-D,L-PLGA(1:1)	0.44 : 50+50 : 0.085	54	2.2	37
3	DEAED-D,L-PLGA(1:1)	1.2 : 50+50 : 0.18	43	2.8	37
4	Dex-D,L-PLGA(1:1)	1.2 : 50+50 : 0.18	43	2.8	37

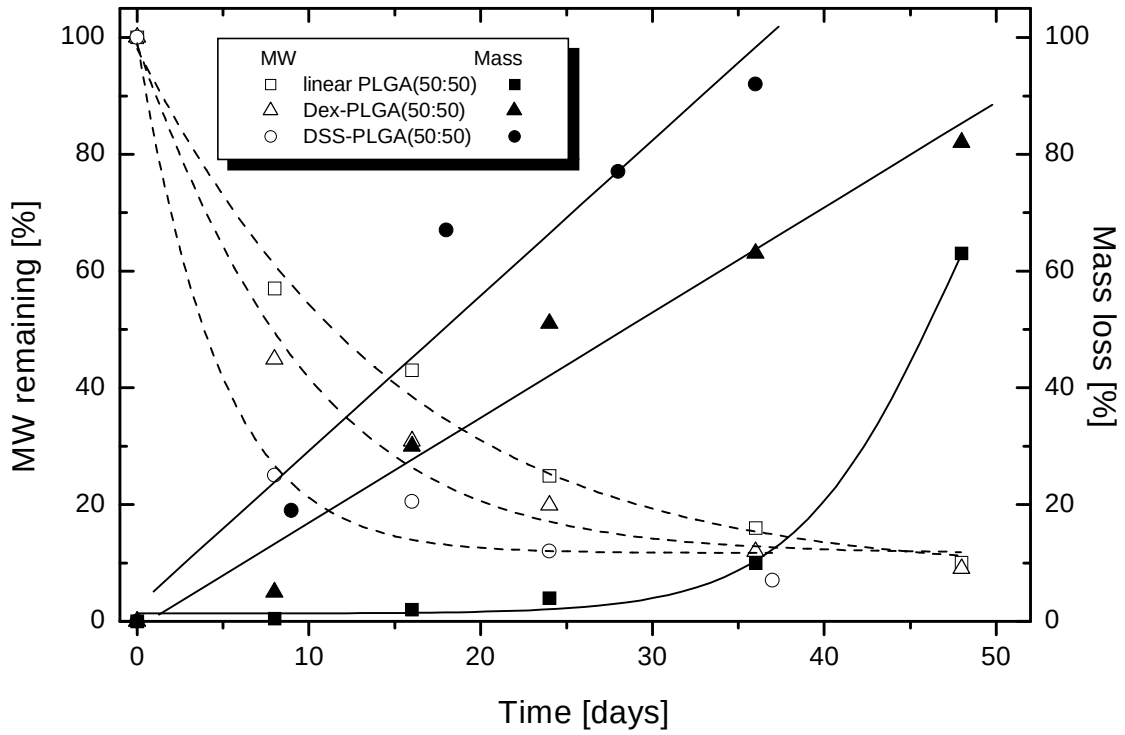

Figure 6: Degradation and Erosion of linear and comb D,L-PLGA

The effect of branching leads to a faster formation of water-soluble degradation products and consequently a slightly faster mass loss under in vitro conditions. Introduction of positive charges in the hydrophilic backbone of dextran even

accelerates the molecular weight decay, but increases the polymer erosion rate only slightly. The branched polyesters contain many short biodegradable branches attached to a hydrophilic macromolecular backbone, which is retained in the polymeric matrix for a longer period of time similar to the star-type polymers described above. Due to the large number of short PLGA chains, matrix stability as well as hydrophilicity is maintained during the first weeks.

The mechanism of the polymer degradation is influenced by the backbone properties. In the case of DEAED as backbone, the predominant chain scission of the graft polyesters seems to occur in a random hydrolytic ester cleavage, similar to linear PLGA. In contrast, a nonrandom chain scission in the vicinity of the branching points of the backbone was found for DSS-PLGA, as can be seen e.g. in the fast nearly zero order mass loss. By manipulating the composition and structure of graft PLGA even the well-known heterogeneous degradation mechanism of linear PLGA could be rendered homogeneous, which will be an important aspect for parenteral delivery systems containing peptides and proteins [23].

The release of bovine serum albumin (BSA), from microspheres prepared from these brush-like grafted PLGA showed promising profiles (fig. 7). After a small initial burst, almost continuous release rates were obtained for both DSS-PLGA(50:50) and DEAED-PLGA(50:50) for about three weeks. The effect of charged groups on the backbone significantly influenced the in vitro release, compared to uncharged Dextran-PLGA.

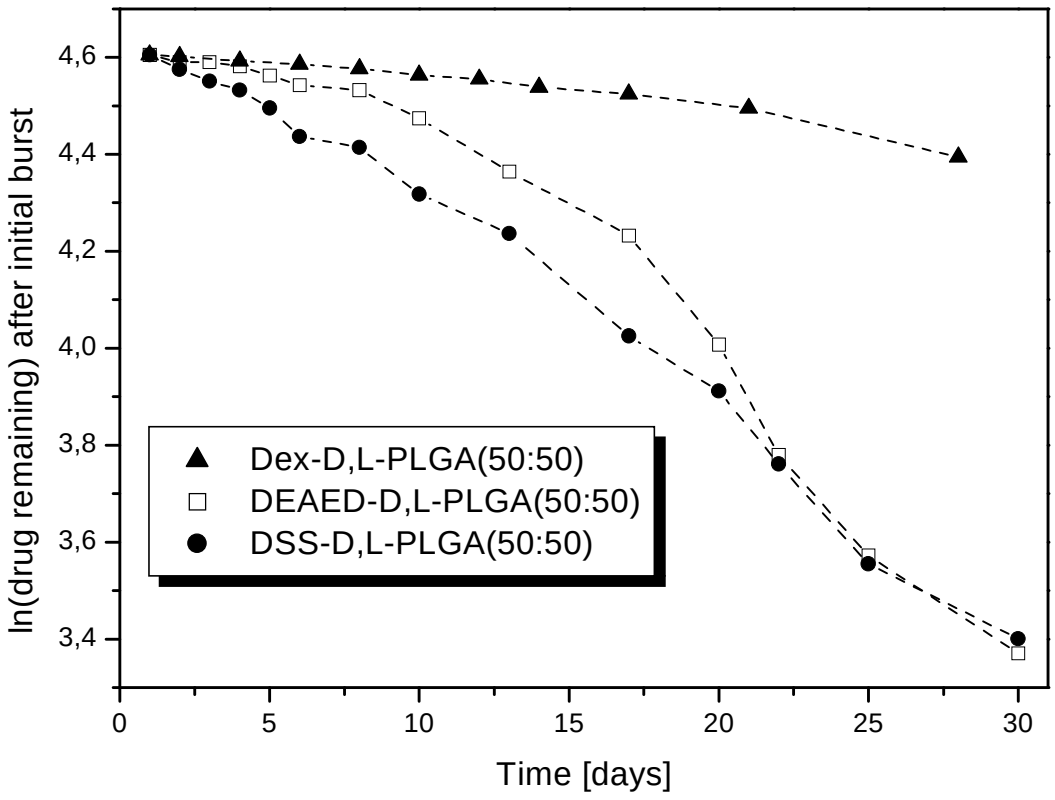

Figure 7: Release of BSA from microspheres prepared from comb D,L-PLGA

The more rapid degradation of the graft PLGA especially, DSS-PLGA(50:50) and DEAED-PLGA(50:50), lead to changes in the morphology of the microspheres: The formation of a porous, water filled structure, facilitating both drug release by diffusion as well as degradation of the microspheres, lead to nearly zero order release profiles. One limitation of this polymeric carrier system can be seen in strong interaction between PLGA segments, leading to hydrophobic structures with reduced swelling properties. Another problem can be seen in interactions between protein drugs and the charged backbone groups, leading to degradation of these sensitive molecules. Both phenomena are influenced by the polymer composition, such as e.g. branching number and lengths as well as the balance of hydrophilic and hydrophobic parts. Presently studies are under way to further characterize these properties of branched polyesters.

3.5 CONCLUSIONS

The combined approaches, introducing hydrophilicity as well as a three-dimensional architecture to polyesters, resulted in biomaterials with promising properties, especially for the controlled delivery of proteins and peptides.

The in-vitro degradation of the star-block copolymers shows a different behavior compared to linear ABA block copolymers. A longer retention of PEO, owing to more connecting bonds between PLGA and PEO blocks, leads to a slower change in the physico-chemical properties of polymeric matrix. The acceleration of the degradation after about 3 weeks offers a fast elimination of the polymeric matrix after drug exhaustion.

Biodegradable comb-like PLGAs containing polyelectrolyte backbones are equally potential biomaterials for the controlled release of hydrophilic macromolecular drugs, like proteins and peptides. The mechanism of the degradation of these polymers is strongly influenced by the used polyol. It is adjustable from random to nonrandom hydrolysis of the polyester chains. The faster degradation rates, accompanied by a high water content and a porous structure of the delivery devices allow the design of protein delivery systems with almost zero order kinetics for a time period of about 2 to 3 weeks.

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Chapter 4

Biodegradable comb polyesters part II: Erosion and release properties of poly(vinyl alcohol)-g-poly(lactic-co-glycolic acid).

4.1 ABSTRACT

Poly(lactic acid) (PLA) and its random copolymers with glycolide (PLGA) were modified by grafting onto hydrophilic macromolecular backbones, such as poly(vinyl alcohol) (PVA), to increase both hydrophilicity and to manipulate the polymer structure. The resulting branched PVA-g-PLGA offer the possibility to manipulate physico-chemical properties, such as molecular weight and glass transition temperature. The degradation and erosion rates required for continuous release of hydrophilic macromolecules differ significantly from linear PLGA. Microspheres were prepared to investigate the release of hydrophilic dextran as a function of polymer structure.

A reduction of the poly(lactic-co glycolic acid) chain lengths in PVA-g-PLGA caused a change in erosion profiles from bulk erosion to a surface front erosion mechanism, when the molecular weight of the PLGA side chains was below 1'000 g/mol, which is equal to water-solubility when cleaved from the backbone. Drug release rates from microspheres were significantly influenced by the polymer structure. A reduction of the PLGA chain lengths led to increasing erosion controlled release rates, while an increase of the molecular weight of the core PVA resulted in a more diffusion controlled release mechanism. Release profiles could be adjusted over a broad range from ca. 14 days to three months.

In combination with the possibility to avoid accumulation of acidic breakdown products in the delivery device, PVA-g-PLGA are of particular interest for parenteral delivery systems containing proteins, peptides or oligonucleotides.

3.2 INTRODUCTION

Aliphatic polyesters, such as poly(lactic acid) and poly(glycolic acid) (PLA, PGA) and their random copolymers (PLGA) are widely used for parenteral drug delivery systems [1,2]. While drug delivery systems for parenteral administration of peptides have become commercially available, hydrophilic macromolecular drugs, such as proteins, still present a formidable challenge for continuous or infusion-like drug release both under in-vitro and in-vivo conditions. Possible interactions between proteins and hydrophobic matrix polymers can lead to deactivation and denaturation of these sensitive molecules. Therefore, the search for new biomaterials allowing protein, antisense, oligonucleotide or gene delivery remains an ambitious goal [3,4].

Successful delivery of these hydrophilic, macromolecular drugs from microspheres or implants strongly depends on the properties of the polymers used for encapsulation, affecting water uptake, thermo-mechanical properties, rates of biodegradation (cleavage of chemical bonds leading to a reduction of molecular weight) and erosion (mass loss) [5-8]. The erosion mechanism for linear PLA and PLGA is controversially discussed in the literature [8-11]. Autocatalysis by acidic degradation products, which are retained in the polymer matrix, is thought to accelerate degradation of PLGA chains inside the delivery device. Consequently, the protein in the polymeric matrix is exposed to a microenvironment of increasing acidity. In combination with elevated temperatures and hydrophobic surfaces, sensitive proteins are known to become inactivated [12].

In spite of considerable efforts, drug release rates often deviate from an ideal "infusion like" profile, generated by zero order release kinetics. Polyphasic drug release profiles can be modified either by formulation approaches or by selection of more appropriate biodegradable polymers. While the release properties of biodegradable microspheres can be influenced to a limited extent by formulation parameters, polymer modifications provide a broader spectrum of control during the erosion phase.

Two strategies have been proposed for modifications of polyesters with regard to parenteral protein drug delivery [5,13]: For one, increasing polyester hydrophilicity should result in faster water uptake and swelling of the polymer matrix, affecting protein release during pore-diffusion phase [14,15]. Or secondly, grafting short PLGA chains onto hydrophilic backbone molecules should lead to graft polymers with accelerated erosion behavior, because the degradation products become soluble in water after few cleavage steps [16,17].

The aim of this study was to investigate the effect of grafting hydrophobic PLGA onto PVA with respect to both physico-chemical properties of the branched PVA-g-PLGA, as well as functional properties as biodegradable carriers, namely degradation and release properties [5,18-22].

3.3 EXPERIMENTAL

Polymer Synthesis

The following designation will be used to specify different PVA-g-PLGAs:
aa-bb-cc: the first digits (a) designate the molecular weight of the PVA backbone in g/mol*1000, followed by the degree of hydrolysis (b) and (c) the relative amount of backbone hydroxyl groups per carboxylic acid repeating unit in mol%. The synthesis and characterization of PVA-g-PLGA was reported earlier [22]. Briefly, ring-opening polymerization of lactide and glycolide in the presence of the backbone PVA was carried out in melt, using stannous octoate as catalyst. All reactions were performed under anhydrous conditions under a dry nitrogen atmosphere. Complete monomer conversion was achieved after a polymerization time of three hours at 130 °C. Properties of the purified PVA-g-PLGAs are summarized in table 1.

For comparison, D,L-PLGA (RG503) with a molar lactide/glycolide ratio of 1:1 and a weight average molecular weight (Mw) of 40'000 g/mol was obtained from Boehringer Ingelheim (Germany)

Analytical Methods

Size Exclusion Chromatography (SEC): 0.5 % (w/v) polymer solutions were injected into a thermostated (35 °C) Merck-Hitachi system (columns: Lichrogel PS mix and Lichrogel PS 40, 10 μm) with a differential refractometer (RI 71) as detector. Chromatograms were obtained with dichloromethane (DCM) in case of high molecular weight polymers, HMW-PVA-g-PLGA, and acetone for lower molecular weight polymers, LMW-PVA-g-PLGA, at a flow rate of 1 ml/min. Molecular weights were estimated using 3rd order universal calibration relative to poly(styrene) reference materials [Merck].

For combined SEC and *Static Light Scattering (SLS.)* analysis a MiniDawn light scattering detector [Wyatt Technology Corporation] (100 µl K5 cell, laser wavelength 690 nm, laser power 30 mW, three angles of detection (45°, 90° and 135°)) was added to above system.

Differential Scanning Calorimetry (DSC) was conducted in nitrogen atmosphere using a DSC7 calorimeter [Perkin Elmer] in sealed aluminum pans, relative to indium and gallium standards. Thermograms covered a range of -20°C to 200°C with heating and cooling rates of 10°C/min. Glass transition temperatures (Tg) were determined from the second run.

Nuclear magnetic resonance spectroscopy (NMR) was performed at 35°C with 6 % (w/v) polymer solutions in different deuterated solvents, like d-chloroform, d_6-DMSO and d_6-acetone. 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectra were recorded with a Jeol GX400 Delta N FT spectrometer, 500 MHz ^{1}H- and 125 MHz ^{13}C-NMR spectra as well as 2D COSY (two dimensional **co**rrelation **s**pectroscop**y**) ^{1}H-, ^{1}H- and inverse gated decoupling ^{13}C-NMR experiments (to suppress Nuclear Overhauser Effect) with a Jeol LA500 Eclipse+ Delta FT spectrometer.

Scanning Electron Microscopy (SEM) was performed on gold sputter-coated samples using a SEM 501S [Hitachi].

In-vitro degradation of polymer films

Polymer films cast from 5 % (w/v) DCM or acetone solutions on Teflon® plates were allowed to dry for 72 hours. Residual solvents were then removed in vacuum at room temperature, at 30°C and finally 40°C during 2 days until constant weights were obtained. Films were cut into 50*10 mm^2 slabs with a thickness of 50 to 100 µm. Samples of known weight (ca. 50 mg, n=2) were

immersed in 5 ml of isotonic phosphate buffered saline solution (PBS, pH 7.4, 0.15 M, periodically replaced during incubation experiments) in sealed glass test tubes and stirred in a rotating thermostat [Rotatherm, Liebisch] at 30 rpm and 37°C. At preset intervals samples were recovered and frozen at -20°C for 4 hours, then freeze-dried in vacuum (0.4 mbar) for 48 hours followed by secondary drying at room temperature in vacuum for one week. Molecular weights (GPC, GPC-SLS), mass loss (gravimetry), film morphology (SEM), thermal properties (DSC) and structure (NMR) were investigated. Water uptake was determined gravimetrically on film specimens after the water on the surface was removed by blotting. It was calculated by the following equation: Water Uptake [%] = 100 - (Mass(t)wet / Mass(t)dry * 100).

Microsphere Preparation and Marker Release

Microspheres were prepared at 4°C using a modified W/O/W double-emulsion-technique [12]. Briefly, 250 µl of an aqueous FITC-Dextran (40’000 g/mol, Sigma) solution (1 % w/v) were emulsified in 1.5 ml of a DCM solution containing 500 mg of the different polymers using an Ultraturrax homogenizer Type18/10 (Janke & Kunkel, Staufen) at 20000 rpm for 30 s. The resulting emulsion was injected with a syringe (18 G needle) in 200 ml of an aqueous solution (0.5 % (w/w)) of poly(vinyl alcohol) (Mowiol 18-88, Hoechst AG), emulsified with a high-speed homogenizer (Ultraturrax T25, Janke & Kunkel, Staufen) at 8000 rpm for 30 s and then stirred for 2.5 hours at 200 rpm to allow solvent evaporation. Microspheres were collected by centrifugation (1.5 min, 1000 rpm, RC-5B from Sorvall-Du Pont Instruments, Bad Homburg) washed three times with 150 ml of distilled water, freeze-dried and stored at 4°C. For release studies a known amount of microspheres (ca. 40 mg, n = 3) was immersed in phosphate buffer saline solution (PBS, pH 7.4, 0.15 M) in sealed glass test tubes at 37°C. Loading and release were assayed by fluorescence

spectroscopy using a LS 50 B Luminescence Spectrometer (Perkin Elmer, Ueberlingen) at 493/515 nm.

Sizes and size distributions of the microspheres were analyzed by dispersing ca. 10 mg of the samples in an aqueous solution of Tween®20 (0.1% w/v). The measurements were carried out in triplicate by laser light scattering using a Malvern Mastersizer X (Malvern Instruments, UK).

3.4 RESULTS AND DISCUSSION

The branched structure of PVA-g-PLGA can be adjusted by variation of feed composition and reaction conditions [22]. Using poly(vinyl alcohol) (PVA) as backbone, polymers with higher molecular weights are obtained (e.g. polymers No 1 and 6, table 1), than those accessible with linear poly(lactide-co-glycolide) (PLGA) under similar reaction conditions [22]. Low content of PVA in the feed, corresponding to few polymerization propagation centers, yielded PVA-g-PLGA with very high Mw (HMW-PVA-g-PLGA). In this case up to ca. 300 PLGA chains are connected to each PVA molecule on average, depending on PVA degree of polymerization.

The theoretical number average molecular weight (Mn), calculated from feed composition, e.g. 1'517'000 g/mol for polymer No 1 (table 1), assuming complete conversion of the PVA hydroxyl groups (PVA-OH), and the Mn determined from NMR analysis by comparison of the signal intensities of the PLGA chain and end groups, 1'254'000 g/mol, were in reasonable agreement.

Table 1: Physico-chemical properties of the PVA-g-PLGA

No	**Polymer**	**Mw PVA** [kg/mol]	**PVA-OH : lactone** (feed) [mol : mol]	**LA : GA** (NMR) [mol%]	**Mw** (SEC) [kg/mol] [a)]	**end : chain** (NMR) [%] [b)]	**Mn** [kg/mol] (feed) [c)]	**Mn** [kg/mol] (NMR) [c)]	**Mn** [kg/mol] (SLS) [d)]	**Tg** [°C]	**OH** [mol%] [e)]
1	HMW158801	15	2.6 : 100	50.8 : 49.2	155	1.60	1517	1254	1563	39.7	-
2	MMW158805	15	13.0 : 100	51.2 : 48.8	84.1	5.99	307.7	360.3	-	37	-
3	LMW158810	15	26.0 : 100	50.0 : 50.0	51.9	9.60	171.1	237.8	182.8	34	53
4	LMW158830	15	78.5 : 100	50.0 : 50.0	-	19.68	64.99	134.4	140.4	34.2	46
5	LMW068010	6	23.6 : 100	50.0 : 50.0	71.5	8.20	62.77	83.57	-	37	68
6	HMW207401	20	2.19 : 100	51.8 : 48.2	229	2.0	2011	1135	-	41.1	-
7	LMW207410	20	21.9 : 100	52.0 : 48.0	70.4	10.07	216.9	308.7	-	35	75
8	Linear PLGA*	-	0 : 100	50.0 : 50.0	40.0	-	-	-	-	45	-

a) = DCM as eluent

b) = PLGA (end : chain) groups ratio, calculated from ^{1}H NMR

c) = calculated assuming complete conversion of PVA-OH

d) = values obtained by combined SEC static light scattering (SLS) analysis using acetone as eluent

e) = unesterified PVA-OH groups, calculated from inverse gated decoupling ^{13}C NMR

* = RG503, supplied by Boehringer Ingelheim, Germany

These data suggest that steric hindrance inhibits a complete conversion of all PVA-OH groups. Thus, a single polyester chain connected to a PVA-OH group consisted of ca. 64 repeating units, equivalent to a chain Mn of ca. 4'000 g/mol. This value was found to be in good agreement with light scattering data.

The resulting molecular weights of the branched PLGA were directly related to the amount of backbone incorporated, e.g. polymers 1–4 in table 1. More PVA-OH groups present during the polymerization resulted on average in shorter PLGA chains grafted onto PVA, designated as LMW-PVA-g-PLGA. A hydrophilic PVA backbone onto which up to ca. 300 short PLGA chains are grafted characterizes the structure of these polymers.

For polymer No 3 (table 1) the number average molecular weight calculated from feed composition was 171’000 g/mol, whereas NMR analysis (237’000 g/mol) and combined SEC and static light scattering (SLS) analysis (182’000 ± 50'000 g/mol) yielded slightly higher values, indicating an incomplete conversion of PVA, possibly due to limited solubility in the melt of the monomers. An average PLGA side chain consisted of ca. 6 dimers, equivalent to a chain Mn < 1'000 g/mol. Moreover, increasing molecular weights of the PVA backbone caused a proportional increase in Mw of the resulting graft polymers due to an increase of the PLGA chain number per molecule (polymers No 5, 3 and 7).

SEC analysis calibrated with linear poly(styrene) standards allows a relative estimate for the Mw of the branched PVA-g-PLGA. Their three-dimensional architecture yields smaller hydrodynamic volumes in solution [22] and caused a significant underestimation of absolute molecular weights. Therefore, a combination of SEC and static light scattering analysis (SLS) as well as NMR spectroscopy were used for polymer characterization.

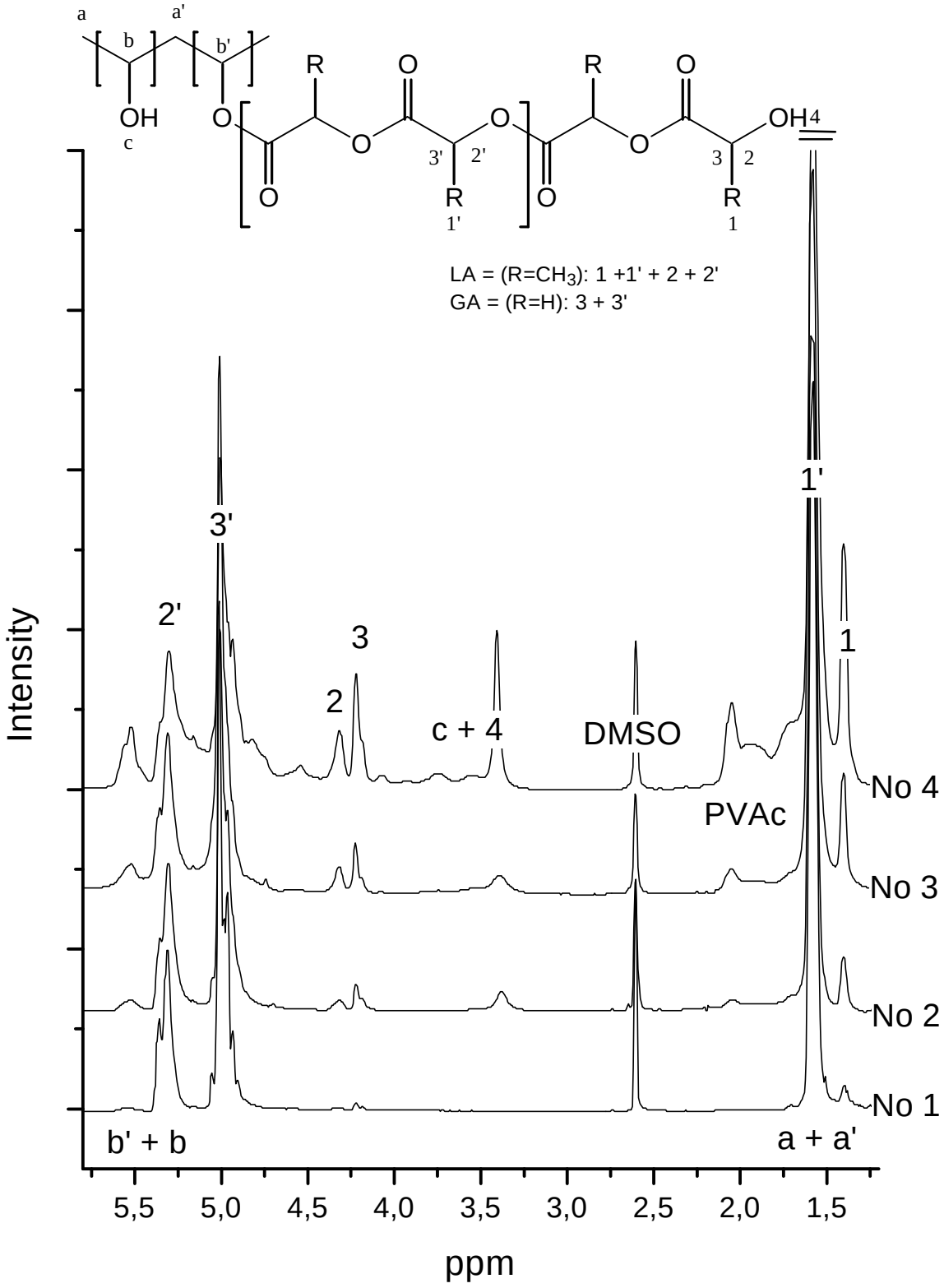


Figure 1a: ^{1}H NMR spectra of PVA-g-PLGAs in DMSO-d_6 with different side chain lengths (polymers 1 to 4, table 1)

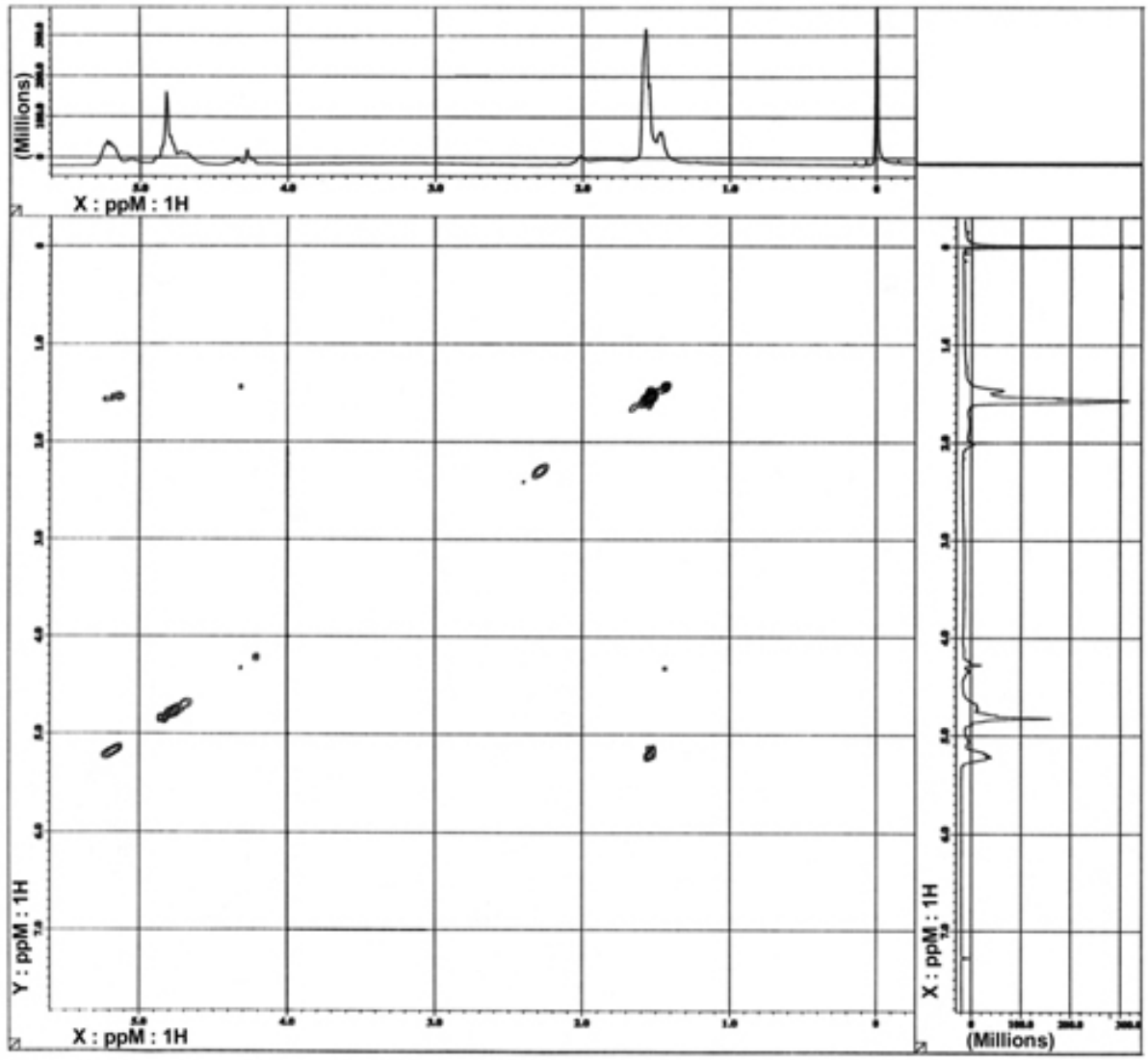


Figure 1b: 2D COSY ^{1}H-^{1}H NMR spectrum of LMW-PVA-g-PLGA (polymer No 3, table 1) in $CDCl_3$

Fig. 1a gives a typical example of the polymer structure determined by ^{1}H NMR spectroscopy as a function of PLGA chain lengths. The signals of the PLGA chain groups resonate at δ = 4.85 ppm (methylene groups of the glycolic acid residues), δ = 5.2 ppm (lactyl C<u>H</u>) and δ = 1.55 ppm (lactyl C<u>H</u>$_3$). In contrast to spectra of linear polyesters, new signals of the corresponding hydroxyl terminated end groups were found at δ = 4.25 ppm (methylene end groups of the glycolic acid residues), δ = 4.35 ppm (lactyl end C<u>H</u>) and δ = 1.45 ppm (lactyl

end C$\underline{H}_3$). Their signal intensity was a function of PLGA chain lengths. Shorter chains caused a corresponding increase in intensity. The assignment of the hydroxyl terminated lactyl units is in excellent agreement with literature data [23-25] and could be confirmed by the cross signals in 2D COSY ^{1}H-^{1}H spectrum (Fig. 1b). The lactic acid chain coupling (1.55 ppm/5.2 ppm) can be seen as clearly as the coupling of their corresponding end groups (1.45 ppm/4.35 ppm).

Degradation and Erosion properties of PVA-g-PLGA.

Factors contributing to degradation properties of PLGA and PLA by random hydrolytic ester bond cleavage are molecular weight, copolymer composition, and crystallinity [20]. Branched PVA-g-PLGA offer an additional possibility to manipulate the hydrophilicity and hence the water penetration by incorporation of the hydrophilic backbone PVA. Using PVA backbones with different degrees of polymerization (5, 3 and 7), leads to PVA-g-PLGA with a different number of PLGA chains grafted per backbone molecule.

Secondly, by variation of the feed composition polymers with relatively short PLGA chains attached to the hydrophilic PVA backbone can be synthesized. These short PLGA chains should be more rapidly cleaved and transported from degrading device due to their water-solubility. Therefore, the molecular weight of the PVA-g-PLGA was varied with respect to the PLGA chain lengths (e.g. polymers No 1 to 4, table 1). As described above, the physico-chemical properties of HMW-PVA-g-PLGA are dominated by the long PLGA chains and should be comparable to linear PLGA of high molecular weight. Consequently, erosion and degradation profiles of HMW-PVA-g-PLGAs were comparable to those, typically observed for bulk erosion of linear PLGA as demonstrated in fig. 2a.

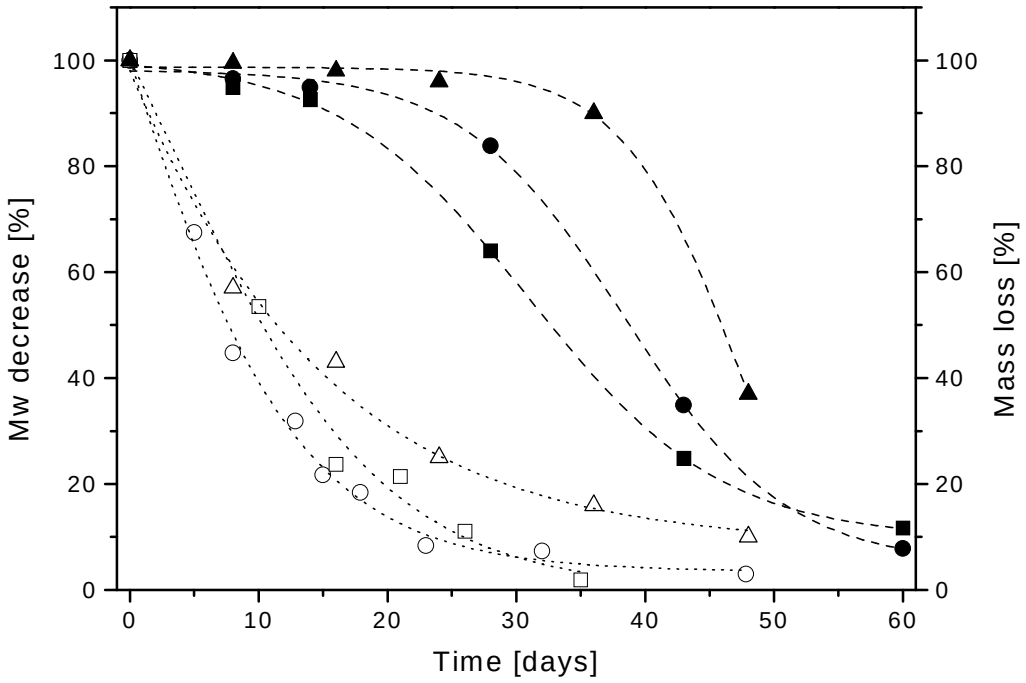


Figure 2a: Mass loss (erosion, full symbols) and loss of molecular weight (degradation, open symbols) of HMW-PVA-g-PLGAs compared to a linear PLGA, polymer No 1 (table 1) circle, polymer No 6 (table 1) square, polymer No 8 (table 1) up triangle

The mass loss of HMW-PVA-g-PLGA was characterized by an initial lag phase without detectable erosion, followed by a sigmoid decay of the polymeric matrix. It seems that the very high weight average molecular weight (Mw) of the HMW-PVA-g-PLGA does not affect the rates of hydrolytic bond cleavage in a significant manner, graft polymer and PLGA show similar degradation rates in spite of large differences in Mw. The duration of the lag phase, however, was found to be influenced both by the molecular weight of the graft polymer and the molecular weight of the PVA backbone. Shorter lag times seen with HMG-PVA-g-PLGA suggest that PVA increases polyester hydrophilicity. The PLGA not containing a PVA backbone yielded a half-time of erosion ($t_{1/2}$) of ca. 46 d, whereas branched PVA-g-PLGA with a 15’000 g/mol PVA backbone reached a $t_{1/2}$ of 39 d and with a 20’000 g/mol backbone a $t_{1/2}$ of 33 d was obtained. This

change in polymer hydrophilicity affected the initial water penetration rate of as depicted in fig. 2c.

Figure 2b: SEC traces of HMW-PVA-g-PLGA (No 1, tab. 1) over the time

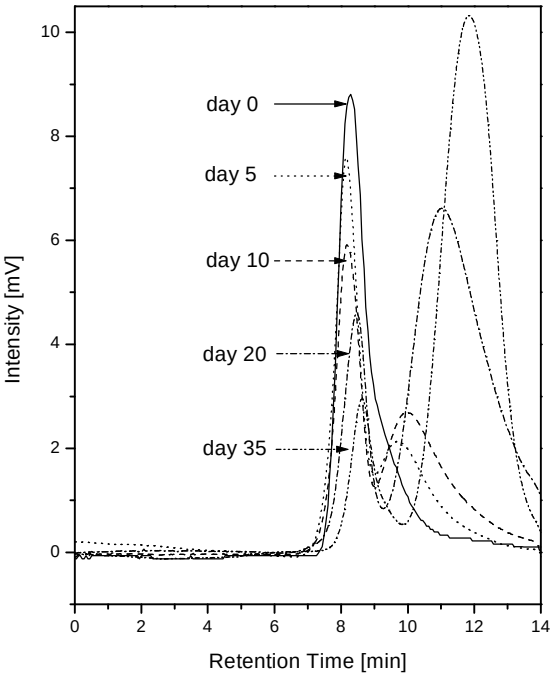
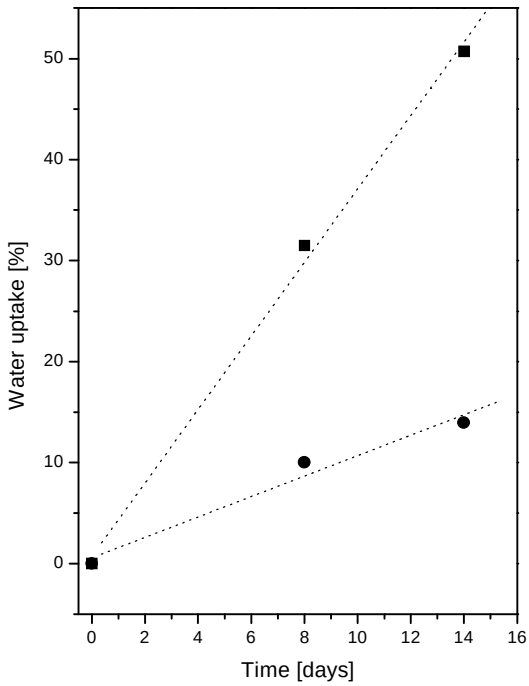


Figure 2c: Water uptake of HMW-PVA-g-PLGA, No 1(tab. 1) circle No 6 (table 1) square



Branched HMW-PVA-g-PLGA show reduced lag time in mass loss, because the rapid water penetration leads to an accelerated rate of ester bond hydrolysis. In case of linear PLGA high Mw materials cause prolonged lag phases without detectable changes in mass, delaying drug release [26].

Monomodal SEC traces of the HMW-PVA-g-PLGA became multimodal during in-vitro degradation experiments (fig. 2b). Since PVA is hydrolytically stable, chain cleavage only occurs in the branches, yielding in a mixture of more than one degrading species. Comb and linear breakdown products, as well as unchanged comb polyesters during the first days of incubation, rendered a quantitative interpretation of these mixtures very difficult. The faster hydration

of the hydrophilic backbone implies an initially preferred cleavage at the PLGA-backbone bonds. But the long PLGA chains may also undergo random and end chain scission of the ester bonds before they are cleaved from the backbone. The expected mixture of at least two different species was observed.

Compared to the HMW-PVA-g-PLGA the observed degradation and erosion pattern of polyesters with shorter PLGA chains (LMW-PVA-g-PLGA) changed significantly as shown in fig. 3a.

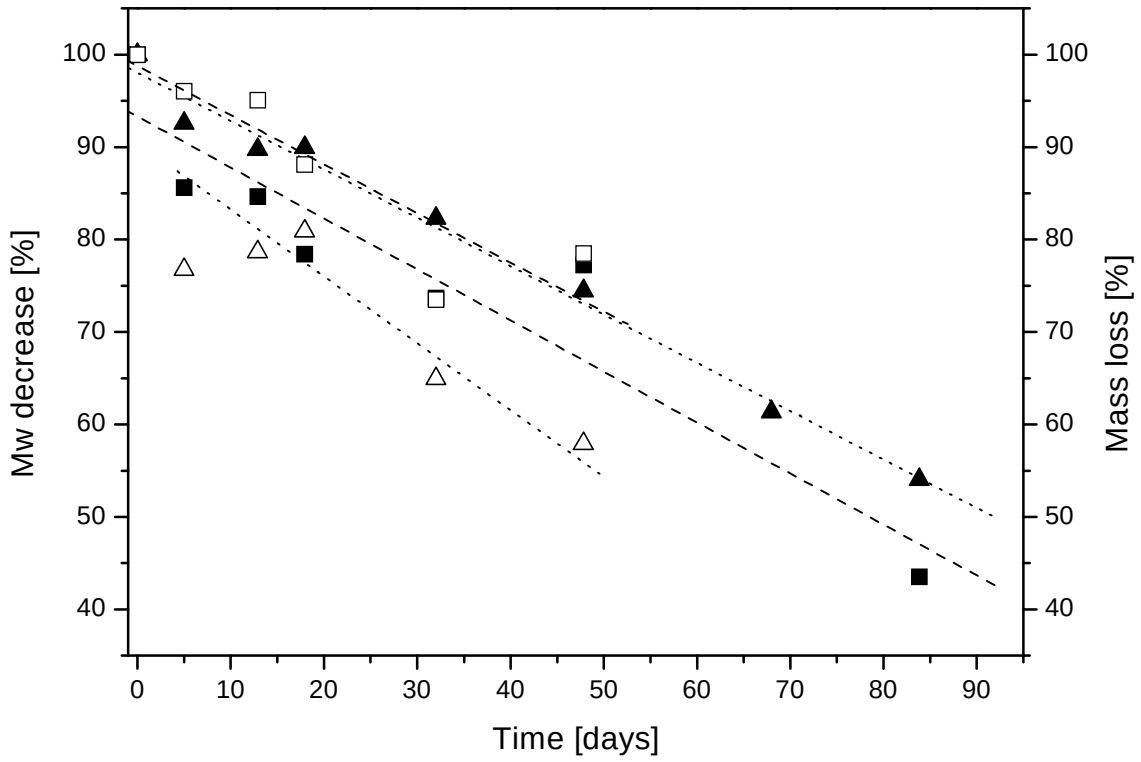


Figure 3a: Mass loss (erosion, full symbols) and loss of molecular weight (degradation, open symbols) of LMW-PVA-g-PLGAs, polymer No 3 (table 1) square, polymer No 4 (table 1) up triangle

No initial lag phase of mass loss was observed and the polymer chain cleavage occurred almost at constant rate. As described above, an average PLGA side chain consisted of ca. 6 dimers, equivalent to a chain Mn below 1'000 g/mol. Linear degradation products will become immediately water-soluble [27]. Consequently, no entrapment of breakdown products inside the polymeric matrix was to expect. The amphiphilic character of the LMW-PVA-g-PLGA and especially their degradation products resulted in a reduced solubility in DCM. Therefore, the eluent for SEC-SLS analysis was changed to acetone. Monomodal SEC traces of LMW-PVA-g-PLGA remained monomodal over time, suggesting a more homogenous mechanism of degradation, as shown in fig. 3b.

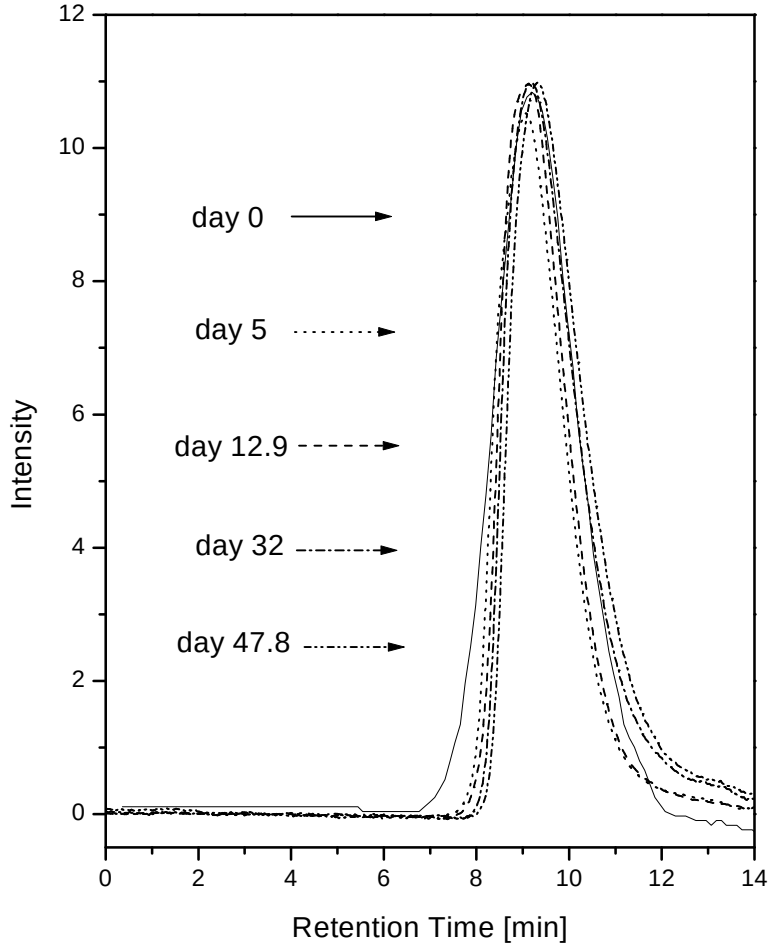


Figure 3b: SEC traces of LMW-PVA-g-PLGA (No 3, table 3) over the time

No entrapment of oligomers and cleavage products occurred, since they would have been dissolved and detected in acetone. Short PLGA chains and high amounts of the hydrophilic backbone PVA increased matrix hydrophilicity resulting in rapid water uptake of the polymer films (fig. 3c), which can be explained by the amount of unmodified PVA hydroxyl groups as determined by inverse gated decoupling ^{13}C-NMR (table 1). On average every second PVA-OH group was esterified. Compared to HMW-PVA-g-PLGA the rates of water uptake were found to be two to eight times higher.

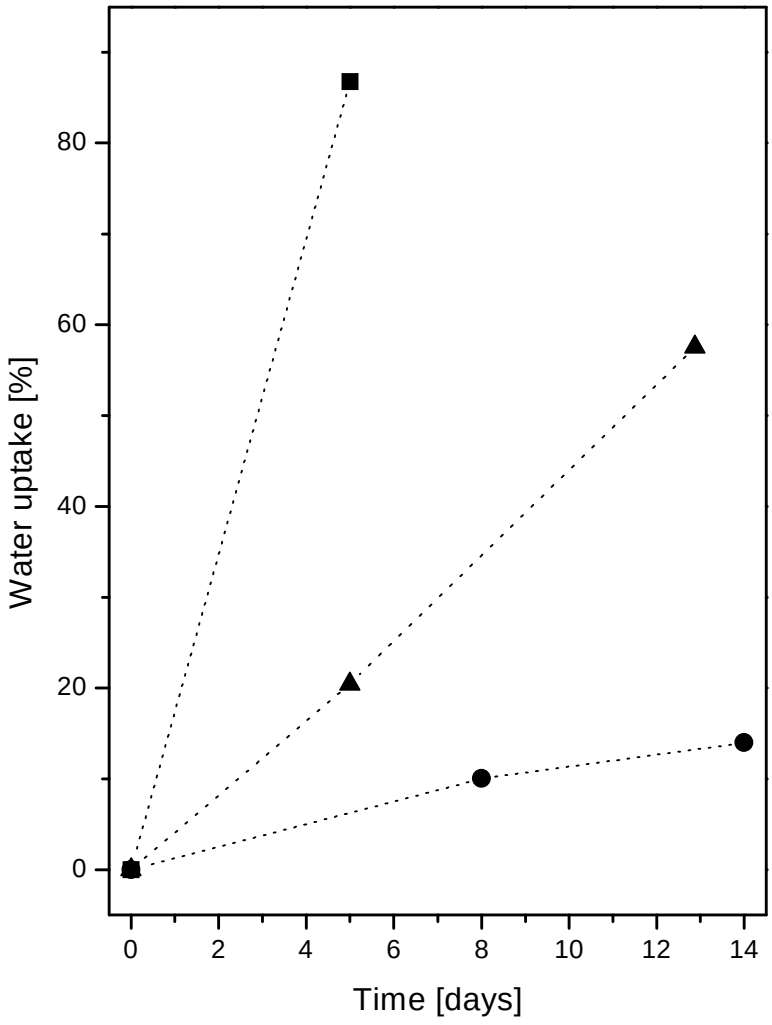


Figure 3c: Water uptake of LMW-PVA-g-PLGAs compared to HMW-PVA-g-PLGA, polymer No 4 (table 1) square, polymer No 3 (table 1) triangle, polymer No 1 (table 1) circle

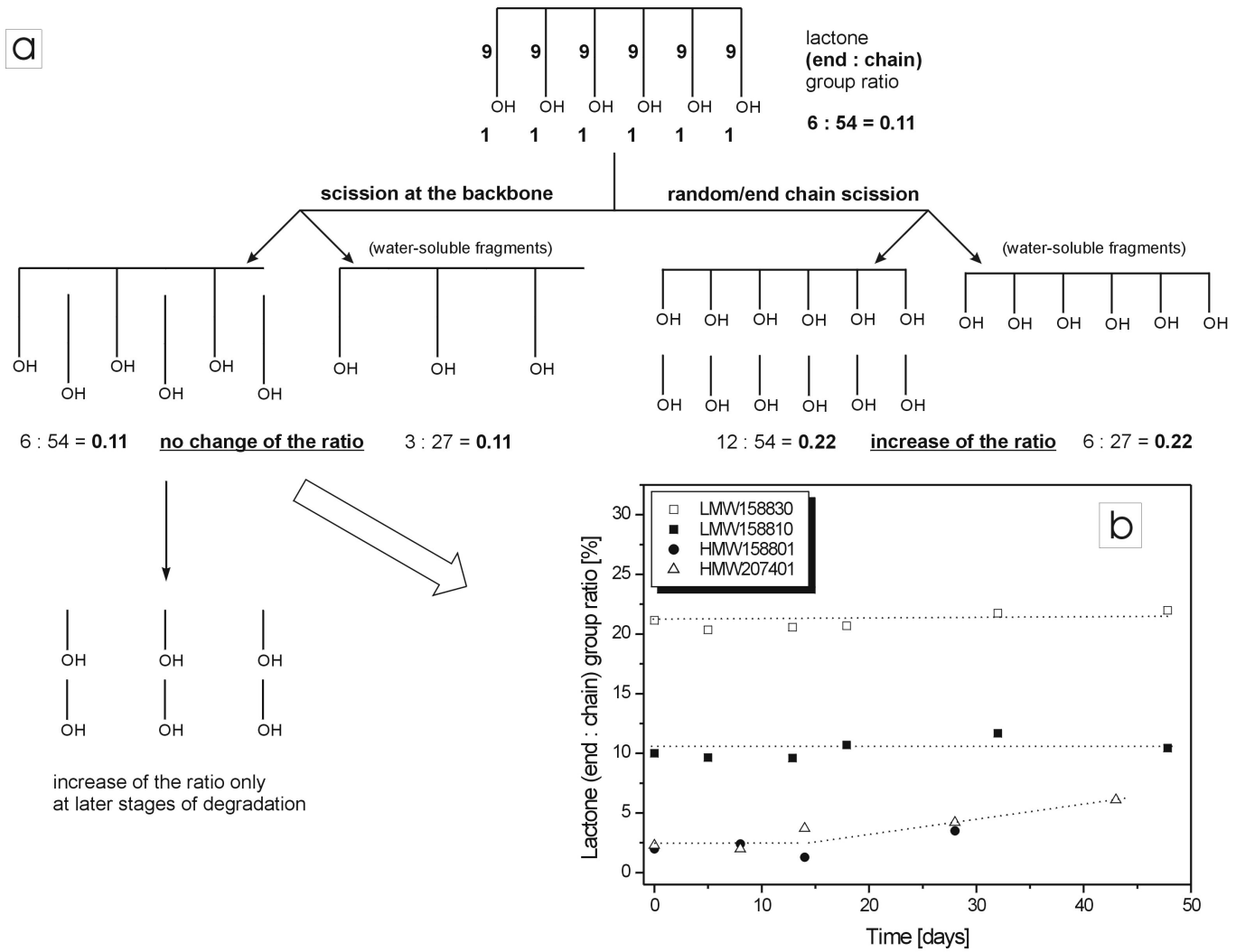


Figure 4: a) Possible chain scission mechanisms during degradation of PVA-g-PLGAs; b) Changes of the polymer structure with time as investigated by ^{1}H-NMR analysis indicating a non-random chain scission mechanism, **HMW-PVA-g-PLGAs:** polymer No 1 (table 1) full circle and polymer No 6 (table 1) open up triangle **vs. LMW-PVA-g-PLGAs:** polymer No 3 (table 1) full square and polymer No 4 (table 1) open square

Hypothetical Degradation Mechanism. Fig. 4a outlines the different possibilities of chain scission during degradation of the graft PLGA. A random and end scission of ester bonds leads to short linear hydroxyl terminated degradation fragments. As a consequence an increase of the total number of end groups, graft plus linear polymers, occurs. In contrast, a non-random scission in the vicinity of the connecting points of the side chains to the backbone results in a constant number of PLGA end groups with time. ^{1}H-NMR analysis was used to monitor the structural changes of the bulk degradation products by comparison of the signal intensities of the PLGA chain and end groups.

HMW-PVA-g-PLGAs showed a distinct lag time without visible changes, followed by an increase of the PLGA (end : chain) groups ratio at later degradation stages (fig. 4b). The absence of low molecular weight degradation products during the first weeks of polymer erosion in combination with an exponential Mw decrease seems to indicate a cleavage of long PLGA chains directly at the PVA backbone. This degradation behavior of HMW-PVA-g-PLGA deviates from the random chain scission, known for linear PLGA. When samples had lost about 70 % of their initial Mw, the increase of the ratio indicates that random chain scission begins to dominate the degradation pattern. By contrast, the PLGA (end : chain) group ratio of LMW-PVA-g-PLGA remained constant for more than 40 days under in-vitro conditions.

Consequently, the short PLGA chains are cleaved directly at the backbone and removed by dissolution in the buffer. This degradation mechanism differs from linear PLGA, avoiding accumulation of acidic breakdown products in the device, which could be an advantage for drug delivery systems containing sensitive proteins.

Thermal Properties and Sample Morphology. Thermo-chemical properties are an important factor for the degradation of polymers. In general, high glass transition temperatures (Tg) and/or high degrees of crystallinity reduce degradation rates of PLGA. Branched PVA-g-PLGA and PVA-g-PLA have lower Tg as well as a reduced degree of crystallinity compared to linear polyesters [22]. A linear relation of PLGA chain lengths and Tg was found, shorter chains led to decreasing values, which is in accordance with the free volume theory. With increasing thermal energy the chain ends will be able to rotate more readily than the rest of the chain. Consequently, the glass transition temperature is lowered. Using differential scanning calorimetry (DSC), the Tg of the degrading polymers in dry and hydrated state were investigated. The structure of the graft polyesters affected Tg of the dry samples during degradation and led to different decay rates of Tg for the HMW- and LMW-PVA-g-PLGA.

Fig. 5 outlines a fast and nearly constant Tg decrease of HMW-PVA-g-PLGA films over the time, which is in accordance with the fast decay of Mw of this type of polymer and the increase of the number of more flexible end groups. In contrast, the Tg of the LMW-PVA-g-PLGAs remained nearly unchanged over time. An explanation could be that in case of LMW-PVA-g-PLGAs water-soluble breakdown products are immediately dissolved in the buffer and cannot cause an internal plasticizer effect. We observed only one Tg in all samples both in dry and hydrated state, suggesting that in PVA-g-PLGA no phase separation occurred.

In the hydrated state Tg below 37°C were reached very rapidly. This means that under physiological conditions drug release and device degradation will occur in the rubbery state. While HMW-PVA-g-PLGA (polymer No 1, table 1) exhibited a Tg of ca. 30°C after one day of incubation, only 15°C were

determined for LMW-PVA-g-PLGA (polymer No 3, Table 1). In case of polymer 4 (table 1) Tg was too low to be determined even after 30 minutes of incubation.

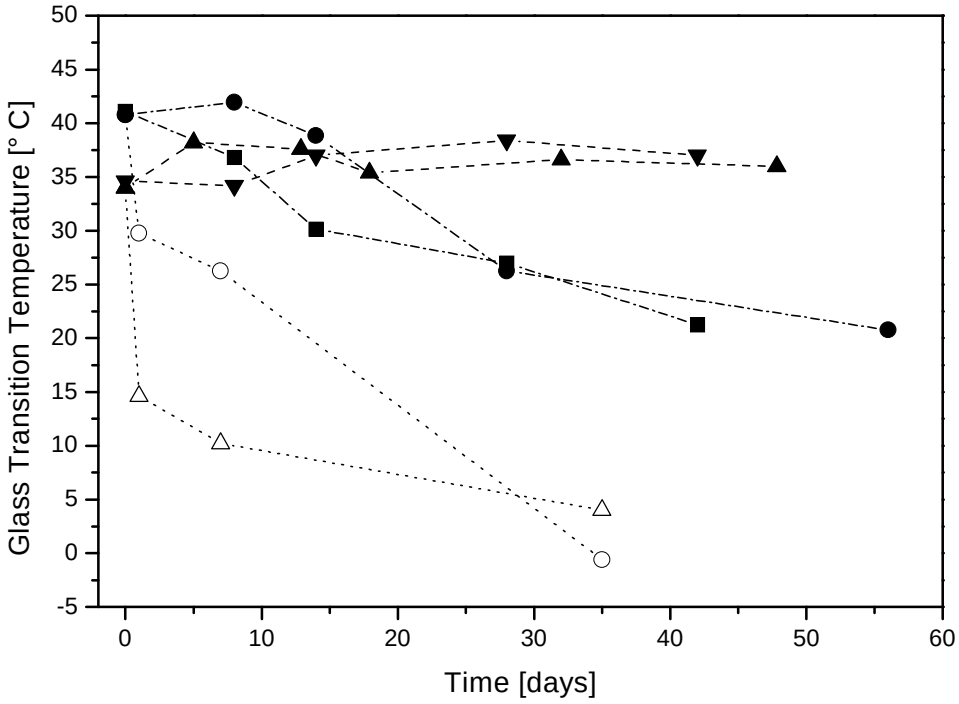


Figure 5: Glass transition temperatures (Tg) of the degraded films of the graft PLGAs with incubation time, dry samples = full symbols, wet samples = open symbols, **HMW-PVA-g-PLGAs:** polymer No 6 (table 1) square and polymer No 1 (table 1) circle **vs. LMW-PVA-g-PLGAs:** polymer No 3 (table 1) up triangle and polymer No 4 (table 1) down triangle

Morphology of PVA-g-PLGA films. Visual inspection of PVA-g-PLGA films incubated under in vitro conditions revealed changes of the morphology from a clear glassy to a white rubbery state as a function of polymer composition. While in the case of HMW-PVA-g-PLGA samples this change was observed in the course of several days, it occurred in a few hours with LMW-PVA-g-PLGA.

Morphological changes of the incubated films after freeze-drying were investigated by scanning electron microscopy (SEM).

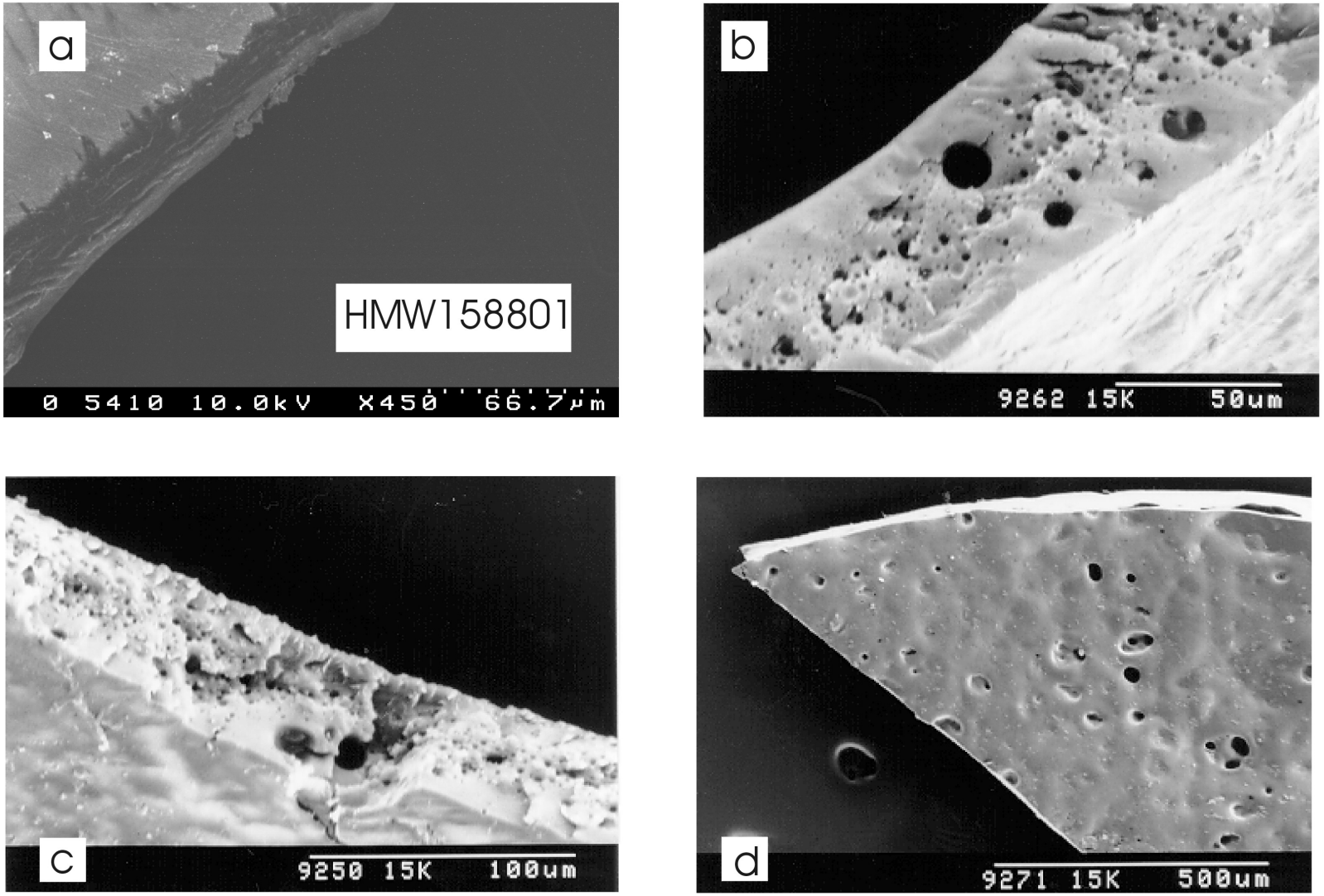


Figure 6: Change of film morphology of HMW-PVA-g-PLGA (polymer No 1, table 1) with time as investigated by SEM (a = day 0, b = day 5, c = day 12.8 and d = day 17.9)

Fig. 6 shows a time-series of SEM photographs for HMW-PVA-g-PLGA (polymer 1). No measurable changes in film thickness were found until the matrix was completely degraded. In the initially phase of degradation (fig. 6a) pore development of different sizes and shapes was observed. The density of pores seemed to increase both with time and distance from the film surface (fig. 6b after 5 d and fig. 6c after 12.8 d), while the surface structure remained smooth. After ca. 18 d the surface layer was deteriorated and became rough and porous, as shown in fig 6d. This degradation pattern is compatible with a bulk degradation mechanism for the HMW-PVA-g-PLGA, similar to PLGA. The morphological changes in degrading film samples are in reasonable agreement with the observed erosion rates, for instance after ca. 2.5 weeks mass loss was detected (fig. 2a).

By contrast, pore formation of LMW-PVA-g-PLGA samples started from the matrix surface, moving into the center of the film, as outlined in fig. 7. The initially smooth film surface (fig. 7a) was found to be porous after 5 d (fig. 7b). After 12 d (fig. 7c) no major changes in the inside of the matrix occurred. Moreover, an irregularly shaped layer of eroded material around a core of intact polymer after more than three weeks of incubation (fig. 7d) was observed. These data are compatible with a more surface front-like erosion mechanism, proposed for poly(anhydrides) [28,29].

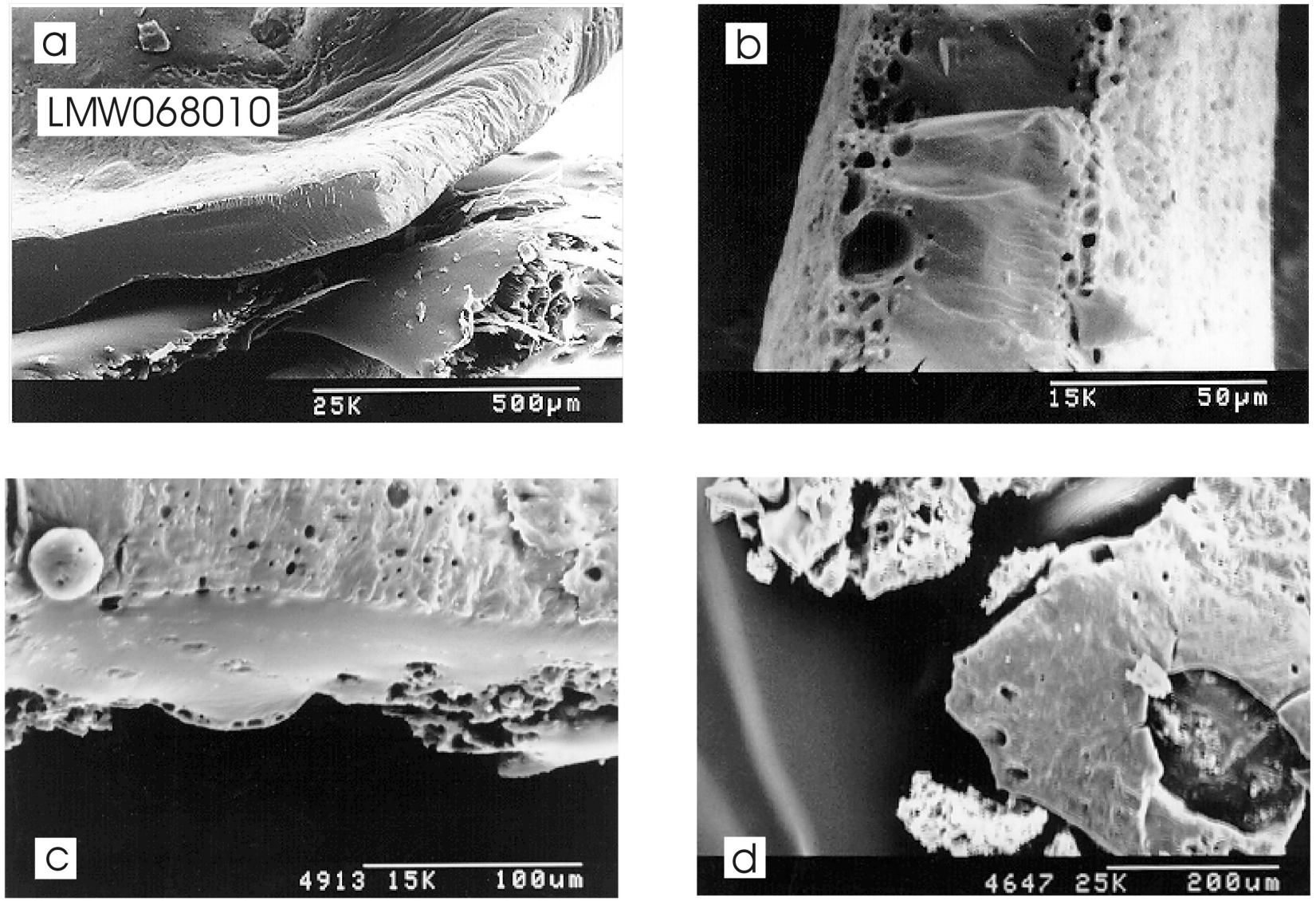


Figure 7: Change of film morphology of LMW-PVA-g-PLGA (polymer No 5, table 1) with time as investigated by SEM (a = day 0, b = day 5, c = day 12 and d = day 24)

Microsphere Drug Release. Graft PVA-g-PLGAs with different chain lengths and different PVA Mw were used to prepare biodegradable microspheres containing fluorescently labeled dextran (FITC-Dex) as marker substance, to investigate the influence of polymer properties on drug release behavior.

A modified W/O/W double emulsion method was employed to prepare microspheres at 4°C [12]. The microencapsulation with a theoretical drug loading of 1 % (w/w) did not pose any technical problems. The encapsulation efficiency was in the range of 70-90 % and yields were > 60 %. The average size was found to be ca. 10 µm for all polymers. FITC-dextran (Mw 40'000 g/mol), a fluorescent labeled hydrophilic polyglycan, is frequently used as a model compound for proteins. The typical biphasic release from linear PLGA microspheres was found, as outlined in fig. 8a. After an initial burst, the in-vitro release rate decreases to low values and recommences when the erosion of PLGA becomes noticeable, leading to the characteristic sigmoid release profile. The in-vitro profiles of FITC-dextran from the PVA-g-PLGAs were improved significantly (fig. 8a). After small initial bursts, almost continuous release rates could be obtained. The same structure property relationships found for the degradation and erosion behavior were also dominating the release of FITC-dextran from biodegradable PVA-g-PLGA microspheres. At constant Mw of the PVA backbone, shorter PLGA chain lengths resulted in a substantial increase of the release rates of the marker. While a change of the ratio hydrophobic PLGA to hydrophilic PVA backbone at comparable PLGA side-chain lengths resulted in a change from erosion controlled release to a pore diffusion type release with increasing Mw of the PVA backbone. In case of a 15'000 g/mol PVA backbone the maximum rate was observed after ca. 2 weeks, the polymer with a 20'000 g/mol PVA had already released nearly 80 % of FITC-dextran at this time.

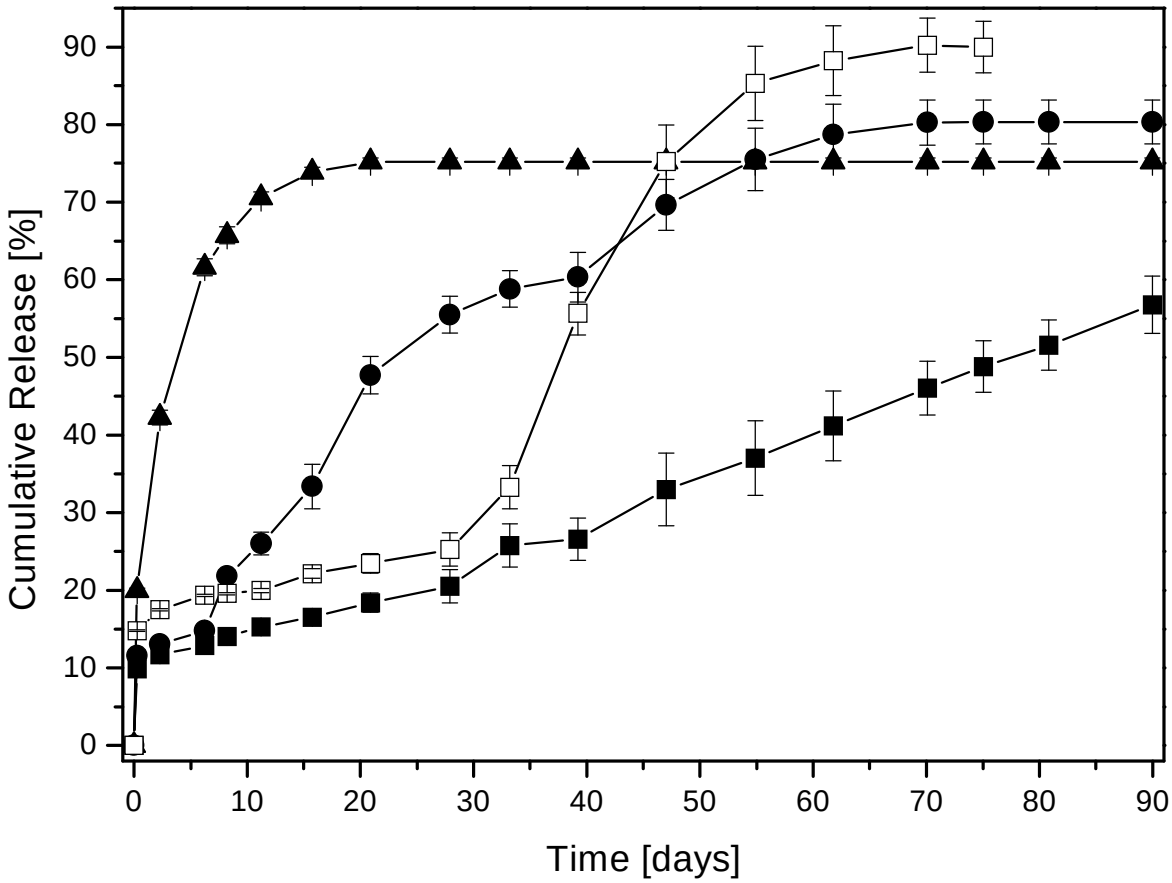


Figure 8a: Cumulative release of FITC-dextran from microspheres prepared from linear PLGA (polymer No 8, table 1) open square, HMW-PVA-g-PLGA (polymer No 1, table 1) full square, LMW-PVA-g-PLGA with 15 kg/mol backbone (polymer No 3, table 1) full circle, LMW-PVA-g-PLGA with 20 kg/mol backbone (polymer No 6, table 1) full up triangle

These profiles can be explained by different hydrophilicity of the PVA-g-PLGA. As investigated by inverse gated decoupling ^{13}C NMR ca. 50% of the hydroxyl groups of the 15’000 g/mol backbone were esterified, while in case of the 20`000 g/mol PVA nearly 75% free OH groups were found, allowing fast hydration and pore formation.

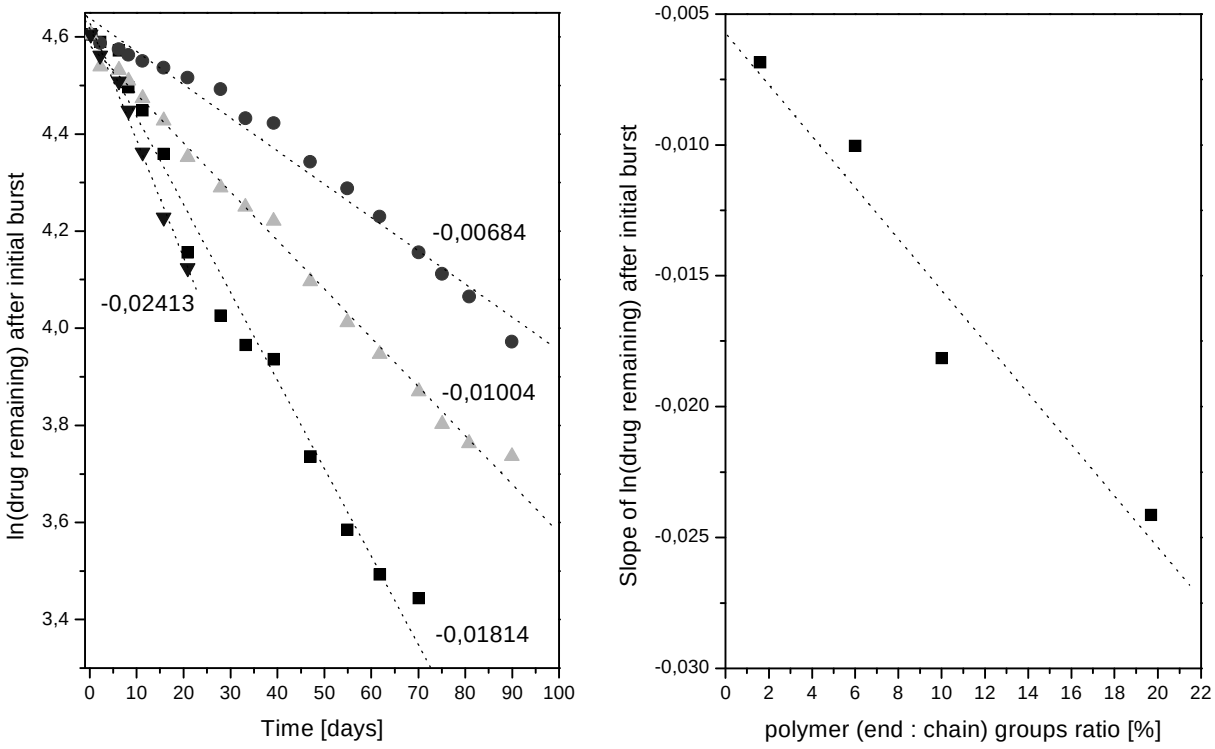


Figure 8b (left): FITC-dextran release rates from microspheres as a function of PLGA chain lengths, polymer No 1 (table 1) circle, polymer No 2 (table 1) up triangle, polymer No 3 (table 1) square, polymer No 4 (table 1) down triangle

Figure 8c (right): Slopes of the microsphere release rates (fig. 9b) as a function of PLGA chain lengths

A nearly linear relation of dextran release and polymer hydrophilicity was found, as outlined in fig. 8c, where the slope of the logarithmic rates of the drug remaining in the microspheres over the time after the initial bursts (fig. 8b) were plotted against the polymer composition as determined by ^{1}H-NMR. Shorter PLGA chains led to increasing release rates of FITC-Dex. The release was therefore directly related to the PLGA chain lengths and polymer hydrophilicity, respectively. Obviously the drug release rates from microspheres differed to some extent from the film erosion rates. The surface front erosion mechanism generates a layer of partially eroded polymer around a core of intact polymer,

influencing the diffusion of molecules. Moreover, this type of erosion mechanism is size dependent [28,29], that may lead to differences in film and microsphere erosion. The structure modifications of the novel polyester PVA-g-PLGA should allow the design of biodegradable parenteral drug delivery systems with release periods covering the range from ca. 30 to 90 days. The potential to switch the degradation mechanism from bulk to surface erosion is a feature of particular interest especially for the release of hydrophilic macromolecular drugs. Therefore, branched PVA-g-PLGA could be of useful for parenteral delivery systems of proteins.

4.5 CONCLUSIONS

Manipulation of both the three-dimensional structure and the hydrophilicity of polyesters by grafting PLGA chains onto PVA as backbone resulted in biomaterials with attractive properties, especially for the controlled delivery of hydrophilic proteins and peptides. It was possible to change the degradation and erosion profiles by parameters, such as PLGA chain lengths and composition as well as PVA molecular weight in a systematic manner. The degradation mechanism can be switched from bulk to a surface front erosion behavior.

The transition from bulk to surface erosion seems to be mainly influenced by the PLGA chain lengths. Grafting water-insoluble side chains onto PVA resulted in high molecular weight polymers exhibiting bulk erosion, while polymers bearing water-soluble PLGA chains seem to erode by a different mechanism. The faster rates, accompanied by high water contents and porous structures allowed the design of drug delivery systems with almost zero order kinetics covering the range from several weeks to several months. PVA-g-PLGA are therefore potentially useful for parenteral drug delivery systems.

4.6 ACKNOWLEDGEMENTS

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Chapter 5

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Chapter 6

Biodegradable semi-crystalline comb polyesters influence the microsphere production by means of a supercritical fluid extraction technique (ASES)

6.1 ABSTRACT

The Aerosol Solvent Extraction System (ASES) is a method based on solvent extraction using supercritical carbon dioxide for the preparation of microspheres. The ASES technology seems to be strongly affected by physico-chemical properties of biodegradable polymers, leading to incomplete or unsuccessful microsphere formation. The number of suitable polymers for ASES, such as poly(L-lactic acid) (L-PLA) and poly(β-hydroxy-butyric acid) (PHB) is rather limited for unknown reasons. Therefore linear and novel branched polyesters were synthesized and subjected to the ASES process to explore function property relationship.

The properties of these polymers as well as of the ASES products were characterized by NMR spectroscopy, differential scanning calorimetry, light scattering, wide-angle x-ray scattering and scanning electron microscopy. It appears that high degrees of polymer crystallinity are the key factor for successful microsphere formation using the ASES process. Under the conditions investigated two types of polymers were especially suitable: Semi-crystalline comb polyesters as well as comb polyesters in which crystallinity could be induced.

These novel polymers are of particular interest for the ASES encapsulation technology since they combine beneficial properties both controlling drug

release due to their three-dimensional architecture and faster biodegradability with sufficient mechanical stability to allow particle formation using supercritical carbon dioxide.

6.2 INTRODUCTION

Various techniques for microencapsulation have been described in literature. Most of them, such as organic phase separation [1,2], spray drying [3] or solvent evaporation [4], are based on the use of organic solvents for microsphere preparation. The investigation of alternatives to organic solvents, e.g. carbon dioxide, especially in its supercritical state, has attracted considerable interest recently. It is non-corrosive, nonflammable and nontoxic, properties, which seem to be ideally suited for pharmaceutical applications. The main advantage is its low critical temperature, which allows mild processing conditions for encapsulation of sensitive drugs like proteins and peptides. Furthermore, its physical properties, such as density, viscosity and dielectric constant, can be varied continuously [5,6].

Two main principles based on supercritical carbon dioxide for microsphere production can be distinguished, namely its use as solvent or as anti-solvent. In the RESS (rapid expansion of supercritical solutions) [5,7] and SFN (supercritical fluid nucleation) [8] process a drug or polymer/drug solution in a supercritical fluid (SF) is expanded across a nozzle at supersonic velocities, leading to instantaneous particle formation by precipitation. The gas anti-solvent precipitation (GAS) [5] bases on the rapid addition of a SF to a polymer/drug solution. In the precipitation with compressed anti-solvents (PCA) [9,10], the supercritical anti-solvent process (SAS) [11] and the aerosol solvent extraction system (ASES) [12,13] polymer solutions are sprayed into an excess supercritical carbon dioxide phase, leading to solvent extraction and polymer precipitation.

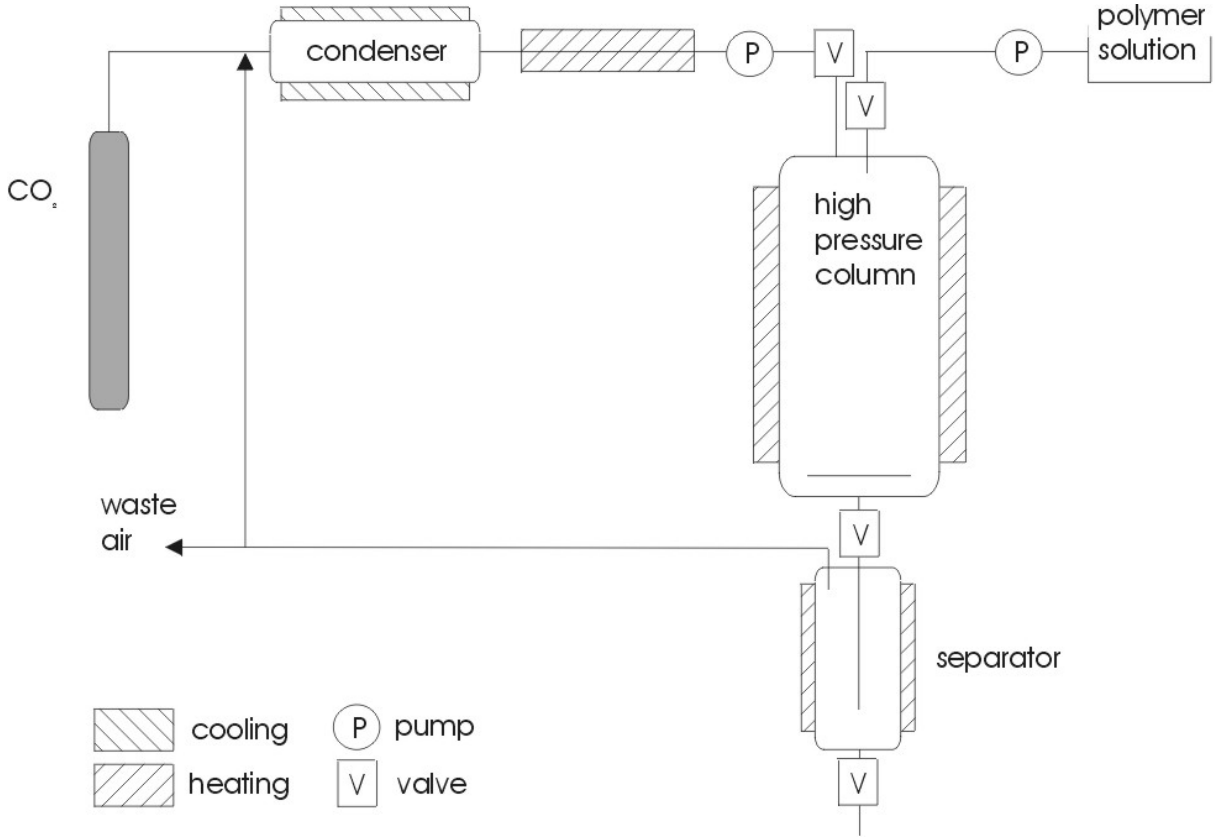
From a physical point of view ASES is located between the solvent displacement process (a liquid-liquid nanoprecipitation as described by Fessi [14]) and spray drying (a liquid-gas system) [3] and should be comparable to these methods to some extent. While particles can be produced with nearly every kind of polymer using these two methods, ASES seems to be limited to a few number of slow degrading polymers, such as L-PLA and β-PHB, only. Fast degrading PLGA does not lead to particle formation at all [12]. Since the reasons for this behavior are not clear, the aim of this study was to determine which polymer properties are needed in the ASES process.

Therefore, novel polymers, synthesized in our laboratory by melt grafting poly(lactone) chains, derived from L-lactide, D,L-lactide and glycolide, onto polyol backbones [15,16], were tested for microsphere production by means of the ASES process. The comb structure of the novel polyesters offered the unique possibility to tailor polymer properties by modification of polymer composition [15]. Polymer crystallinity, very high molecular weights, high thermo-chemical and -mechanical stability can be obtained. Furthermore, owing to the three-dimensional architecture and increased hydrophilicity these properties are not accompanied by a significant reduction of biodegradability and release rates of hydrophilic macromolecular drugs, such as proteins and peptides, known for linear polyesters with comparable properties [17].

6.3 MATERIALS AND METHODS

ASES

The principle of an ASES apparatus is shown in scheme 1. For a detailed description see ref. [12]. Liquid carbon dioxide is cooled below 10 °C in a condenser before it is pumped through a heater into a high-pressure column. By controlling the pressure through an inlet and an outlet valve as well as through the temperature, CO_2 is forced into the supercritical state. Suitable process parameters are e.g. 36°C and 150 bar. Polymer solutions in dichloromethane (usually 2.5 % w/v) were sprayed into the column by a separate valve.



Scheme 1: Experimental apparatus for the Aerosol Solvent Extraction System (ASES) process

After having passed the column the carbon dioxide is expanded to subcritical conditions into a separator vessel, where it is vaporized (leading to the

separation of gas and the extracted solvent). Afterwards the CO_2 can be recirculated to the condenser again or - as in this study - be discharged. The column was equipped with a metal sinter filter for particle collection. For product recovery the carbon dioxide is slowly discharged from the spraying column. Residual solvent content of the microspheres generally is below 500 ppm [18].

The SFC-Gilson SF3-TM system consisted of the following components: CO_2 pump: Gilson 308, modifier pump (for the injection of further liquid cosolvents, deactivated during the experiments): Gilson 306, Gilson 811C dynamic mixer, Gilson 821 pressure regulator (pulse dampening, heat exchanger, regulation valve), Gilson 831 temperature regulator, Rheodyne 7010 sample injection valve), stainless steel high pressure column with a volume of 115 ml, CO_2 flow rate 5 ml/min, injection interval 10 µl / 30 min.

Polymer syntheses

The synthesis and characterization of the polymers used in this study was reported earlier [15,16]. In brief, ring-opening melt polymerization of the lactones, L-lactide, D,L-lactide and glycolide, with stannous octoate as catalyst was used for linear polyesters synthesis (L-PLA, D,L-PLA). Comb polyesters were prepared accordingly in the presence of the core polyols, dextran sulfate sodium (DSS) [16] and poly(vinyl alcohol) (PVA) [15]. Polymer properties are summarized in table 1.

Sample Characterization

Size Exclusion Chromatography (SEC) was carried out relative to poly(styrene) reference materials [Merck] with '3rd order universal calibration'. 0.5 % (w/v) polymer solutions in DCM or acetone were injected into a thermostatted Merck-Hitachi system (columns: Lichrogel PS mix and Lichrogel PS 40, 10 µm) with a

differential refractometer (RI 71) as detector. Degassed eluents (DCM or acetone) were pumped with a flow rate of 1 ml/min.

For combined *SEC and Static Light Scattering*, SLS, analysis a MiniDawn light scattering detector [Wyatt Technology Corporation] (100 µl K5 cell, laser wavelength 690 nm, laser power 30 mW, three detecting angles (45°, 90° and 135°)) was added to above system.

Differential Scanning Calorimetry (DSC) was conducted in nitrogen atmosphere with a DSC7 calorimeter [Perkin Elmer] in sealed aluminum pans, relative to indium and gallium standards. Thermograms covered a range of -20°C to 200°C with heating and cooling rates of 10°C/min. Glass transition temperatures (Tg) were determined from the second run.

Nuclear magnetic resonance spectroscopy (NMR) was performed at 35°C with 6 % (w/v) polymer solutions in different deuterated solvents, like d-chloroform, d_6-DMSO, d_6-acetone. 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectra were recorded with a Jeol GX400 Delta N FT spectrometer, 500 MHz ^{1}H- and 125 MHz ^{13}C-NMR spectra with a Jeol LA500 eclipse+ Delta FT spectrometer.

Surface morphology and particle sizes were investigated by *scanning electronic microscopy* (SEM), Hitachi S 510, Tokyo, Japan) and *laser diffractometry* (Mastersizer X, Malvern Instruments) in triplicate.

Dynamic mechanic thermal analysis (DMTA) was carried out on a DMTA MK II equipment from PL Thermal Sciences (Polymer Laboratories Inc., USA) covering a temperature range from -100 °C to 100 °C and frequencies of 0.3, 1, 3, 5 and 10 Hz on either polymer films cast from 2.5 % (w/v) DCM solution on Teflon coated plates or on compression molded samples (80 °C).

Wide-angle x-ray scattering (WAXS) was performed with a wide-angle goniometer D-5000 (Siemens) at a wavelength of 1.54 Å (nickel filtered CuK_α, step scan mode, 2Θ range: 1° to 40°, step width: 0.05°, scanning time: 60 s).

Table 1: Physicochemical properties of the used polymers

No	**Polymer**	**OH : dimer** [mol : mol]	**Mw** (GPC) [kg/mol]	**D**	**Mw** (SLS) [kg/mol]	**Intr. Visc.**[2)] [dl/g]	**Tg** [°C]	**Tm** [°C]	**ΔHm** [J/g]	**DC**[3)] [%]
1	D,L-PLA	0 : 100	134	1.5	140	-	53.1	-	-	-
2	PVA(15)-g-D,L-PLA	1.025 : 100	260	2.5	4,688	0.65	44.3	-	-	-
3	- " -	4.1 : 100	180	2.5	-	-	43.9	-	-	-
4	- " -	11.4 : 100	125	1.8	-	-	37.7	-	-	-
5	PVA(15)-g-D,L-PLGA(3:1)	1.025 : (71.9 + 28.1)[1)]	235	2.8	-	-	42.1	-	-	-
6	PVA(15)-g-D,L-PLGA(1:1)	1.025 : (55.6 + 44.4)[1)]	287	3.4	-	-	40.8	-	-	-
7	L-PLA	0 : 100	101	1.7	105	1.14	57	174.2	52	56
8	DSS(8)-L-PLA	0.28 : 100	54.9	2.1	-	-	55.2	171.7	51.7	55
9	PVA(15)-g-L-PLA	1.025 : 100	274	2.5	4,570	0.53	57.6	163.8	52.3	56
10	- " -	2.05 : 100	277	2.7	-	-	54.3	154.9	43.7	47
11	- " -	4.1 : 100	166	2.8	1,877	0.37	52.0	134.3	31.7	34
12	- " -	11.4 : 100	125	1.7	1,010	0.31	43.3	-	-	-
13	- " -	28.8 : 100	98	1.8	445	0.26	37.3	-	-	-
14	PVA(49)-g-L-PLA	14.4 : 100	320	1.9	-	-	41.5	-	-	-
15	- " -	28.8 : 100	214	1.8	-	-	32.4	-	-	-
16	PVA(15)-g-L-PLGA(9:1)	1.025 : (88.5 : 11.5)[1)]	242	2.9	-	-	54.3	154.9	33.7	36
17	PVA(15)-g-L-PLGA(7:3)	1.025 : (70.2 : 29.8)[1)]	236	2.8	-	-	45.6	-	-	-
18	PVA(15)-g-L-PLGA(5:5)	1.025 : (53.8 : 46.2)[1)]	229	3.4	-	-	40.7	-	-	-
19	PVA(20)-g-D,L-PLGA(1:1)	2.1 : (55.1 + 44.9)[1)]	229	2.5	-	-	41.1	-	-	-
20	PVA(20)-g-D,L-PLGA(1:1)	5.5 : (53.4 + 46.6) [1)]	104	2.0	-	-	37.4	-	-	-
21	PVA(20)-g-D,L-PLGA(1:1)	21.9 : (52.0 + 48.0)[1)]	70.4	2.2	-	-	35.0	-	-	-

1) determined by 1H NMR 2) determined in THF at 25 °C 3) DC = Degree of crystallinity, rating it to the reference 100 % crystalline L-PLA (93.6 J/g) [30]

6.4 RESULTS AND DISCUSSION

From literature ASES seems to be limited to a few number of relatively slow degrading polymers, such as L-PLA and β-PHB. Faster degrading D,L-PLA and PLGA do not lead to particle formation at all [11,12]. There is some controversy in the literature if production parameters, such as spraying rate, polymer solution concentration [19] and carbon dioxide density/pressure [19-21] can influence the process of microsphere formation and particle sizes in ASES.

In the system used no control of the state of dispersion by either special nozzles or the use of emulsifiers was obtained. Thus, it is to be expected, that the negative results regarding microsphere formation will mainly be caused by properties of the polymers themselves. Therefore, in most experiments standard process conditions were chosen (36°C, 15 MPa). Moreover, only placebo microspheres were prepared, to exclude any stabilizing effects by encapsulated drug molecules [22,23].

The comb structure of the novel polyesters can be modified by reaction conditions and feed composition [15]. For one, the molecular weights can be manipulated by the ratio of polyol backbone to lactone in the feed, (e.g. polymers No 2-4 and 7-11, table 1). The resulting molecular weights are directly related to the amount of backbone incorporated. The less PVA-OH groups present during the polymerization, the longer the poly(lactone) chains grafted onto a single core molecule. The increasing molecular weights of the products are accompanied by a similar increase of thermo-chemical and -mechanical properties, as well. Secondly, decreasing molecular weights of the PVAs used as backbone cause a proportional decrease in the molecular weight of the resulting graft polymers owing to a reduction of the poly(lactone) chain number per molecule (e.g. polymers No 13 and 11, table 1).

Moreover, the chain composition can be manipulated by the selection and ratio of the used lactones, L-lactide, D,L-lactide and glycolide (e.g. polymers 2, 5-6 and 9, 16-18, table 1).

In case of linear polyesters, such as PLA and PLGA, increasing molecular weights and thermo-mechanical stability are always accompanied by a reduction of biodegradation rates [17]. Therefore, the undesired lag phases in microsphere drug release of hydrophilic drugs, such as proteins and peptides, are prolonged until erosion controlled drug release sets in. Owing to the three-dimensional architecture and increased hydrophilicity the novel comb polyesters showed improved degradation and drug release properties [16,24-26]. Moreover, these polymers offer the unique possibility to study the effects of very high molecular weights or high degrees of crystallinity.

To characterize the influence of physico-chemical properties, such as thermo-mechanical strength and polymer molecular weight on the formation of microspheres, a linear D,L-PLA and comb PVA-g-D,L-PLAs with different lactone chain lengths were prepared (polymers 1-4, table 1). 2.5 % [w/v] solutions of these polymers in dichloromethane (DCM) were spayed into supercritical carbon dioxide. In all experiments with these amorphous polymers no microspheres could be obtained. The best results were foam-like structures in case of the linear D,L-PLA. The graft polymer with the highest molecular weight (No 2, table 1) yielded in a similar result. Nevertheless, trends in the polymer precipitation behavior were observed. The lower the thermo-chemical properties of the polymers, the higher the tendency to produce molten structures, leading to glassy broken egg shell structures in case of polymer No 4 (table 1).

Variation of the polyester chain composition by copolymerization of D,L-lactide with glycolide (polymers No 2, 5 and 6, table 1), similarly changed the results from foam like to molten glassy structures. A higher amount of glycolide decreased the Tg of the polymers and probably increased their solubility and swelling behavior in supercritical carbon dioxide. Therefore, the higher the observed tendency for molten results. Obviously, neither polymer molecular weight, polyester type nor glass transition temperature, which for some polymers was more than 15°C above the process temperature, seem to be a key factor for microsphere formation using the ASES process.

To confirm literature experiments a linear 105 kg/mol poly(L-lactic acid) (L-PLA) was prepared by bulk polymerization and sprayed into supercritical carbon dioxide. The obtained particles with mean diameters in the range of ca. 2-5 µm were taken as reference. Comb PVA-g-L-PLA and DSS-L-PLA (polymers No 8 and 9 table 1), similarly prepared by bulk melt polymerization in the presence of the different polyols [15,16], were investigated next. With both types of polymers microspheres were obtained. Fig. 1 gives an example of particle sizes and morphologies. Since the properties of both linear (D,L-PLA and L-PLA) and comb polymers (PVA-g-D,L-PLA and PVA-g-L-PLA) were quite comparable except the different stereochemistry of the used lactones, polymer crystallinity seems to be of major influence in the ASES process.

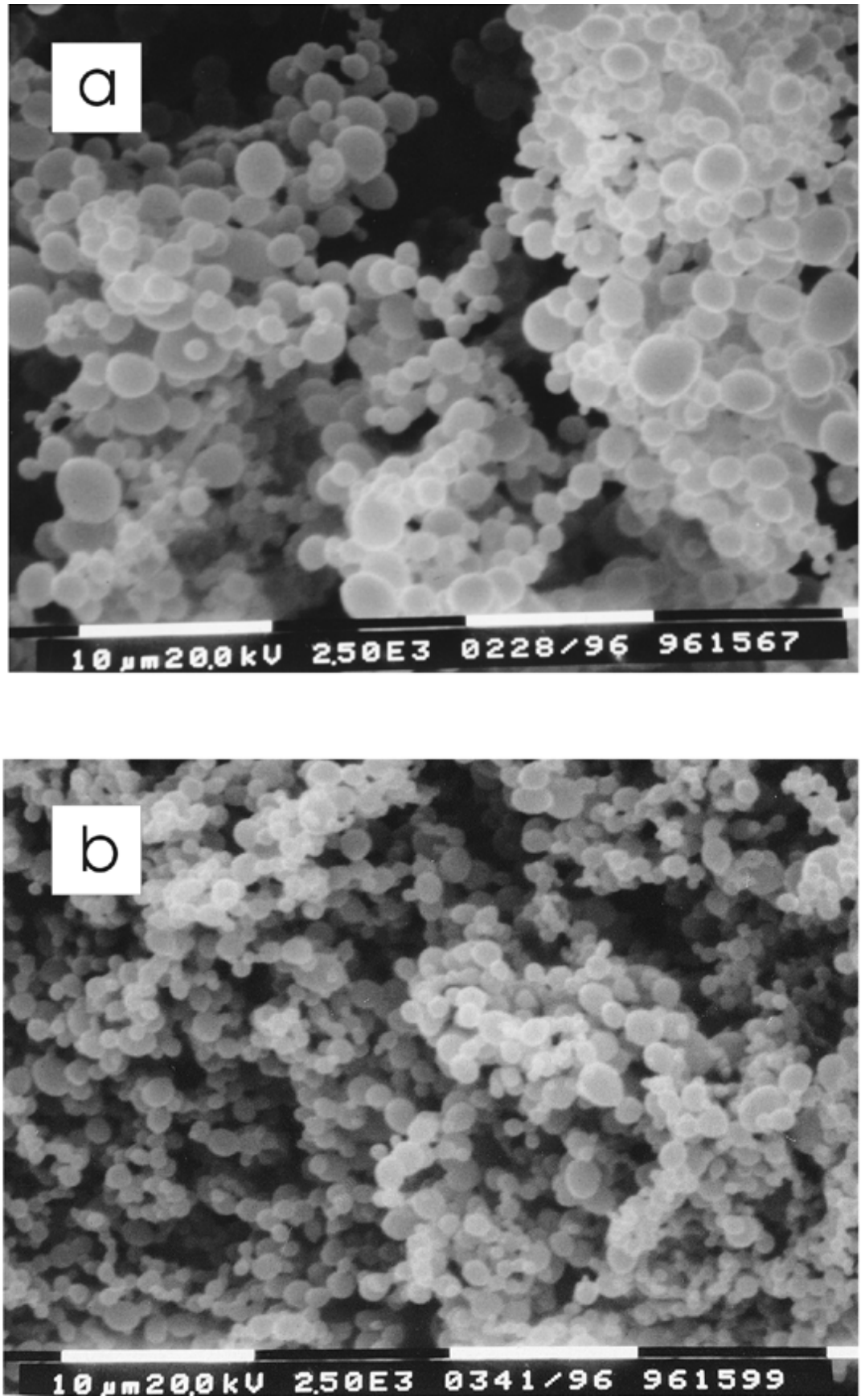


Figure 1: Microsphere morphology of linear L-PLA (a) and comb DSS-L-PLA (b)

To further investigate this influence on the general mechanism of particle formation, a series of PVA-g-L-PLAs was prepared (table 1, polymers No 9-13). This series of polymers was quite comparable to the first polymers investigated except for their semi-crystallinity.

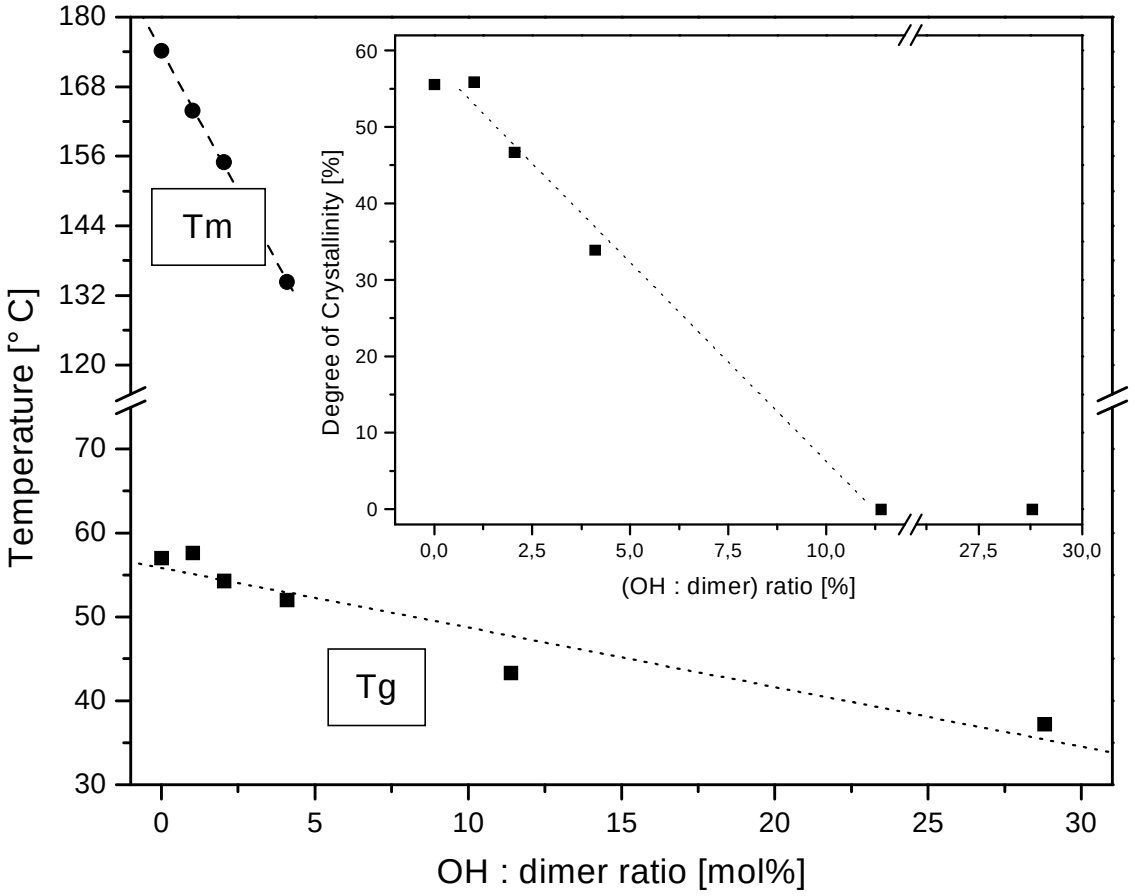


Figure 2: Thermo-chemical properties of comb PVA-g-L-PLAs as a function of poly(lactone) chain lengths (determined by DSC analysis)

Fig. 2 shows that the three-dimensional polymer architecture leads to reduced interactions between the molecules and lowers the thermo-chemical properties. The reduction of Tg and Tm was following the ratio PVA to lactone in the feed. Shorter poly(lactone) chains lead to a lower degree of crystallinity, as expected.

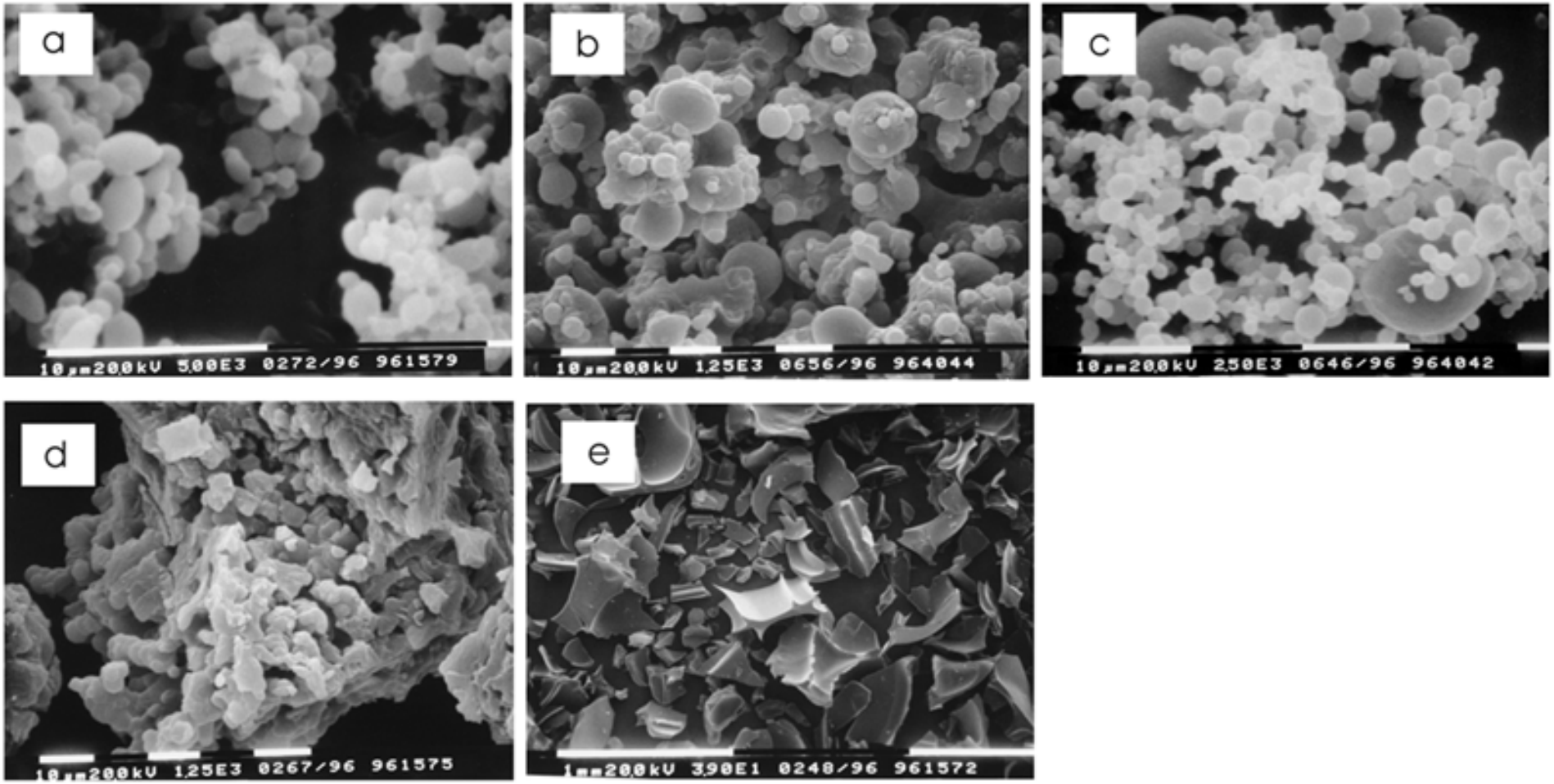


Figure 3: Microsphere morphology of comb PVA(15)-g-L-PLAs as a function of poly(lactone) chain lengths

The results obtained by ASES were also following the reduction of thermo-chemical properties with decreasing poly(lactone) chain lengths. The different morphologies of the obtained precipitates are outlined in fig. 3. With polymer No 9 (table 1) microspheres could be obtained (fig. 3a). But the tendency of particle formation dramatically decreased with a decreasing degree of crystallinity, leading to broken egg shell like structures (fig. 3e) in case of polymer No 13 (table 1). The reduction of L-PLA chain lengths led to a complete loss of crystallinity for this polymer, although no lactone racemization during synthesis was observed by NMR analysis.

Since the size and morphologies of the microspheres could not be determined by laser light diffraction in all cases, the following rating scale was employed for a quantification of the ASES results. A free flowing powder of microspheres was rated '2' (fig. 3a), microspheres which were agglomerated '1' (fig. 3b), foam like structures '0' (fig. 3d) and glassy and broken egg shell structures '-1' (fig. 3e). When this rating is applied to the series of PVA-g-L-PLAs described above, the trends in ASES precipitation behavior could be rationalized as shown in fig. 4.

Particle sizes and size distributions investigated by laser light diffraction confirmed these findings (table 2). It is worth noting, that by dividing the polymer concentration in half, which obviously leads to a reduction of solution viscosity, it was possible to improve the ASES result for polymer No 10 (table 1) from morphology '1' to '2' (fig. 3b to 3c). In case of the two following PVA-g-L-PLA polymers no improvement of the ASES result was possible either by manipulation of solution concentration or by carbon dioxide density (pressure variation: 9, 15, 25 MPa). These results indicate that these parameters do not have a significant influence in ASES as far as microsphere formation is concerned.

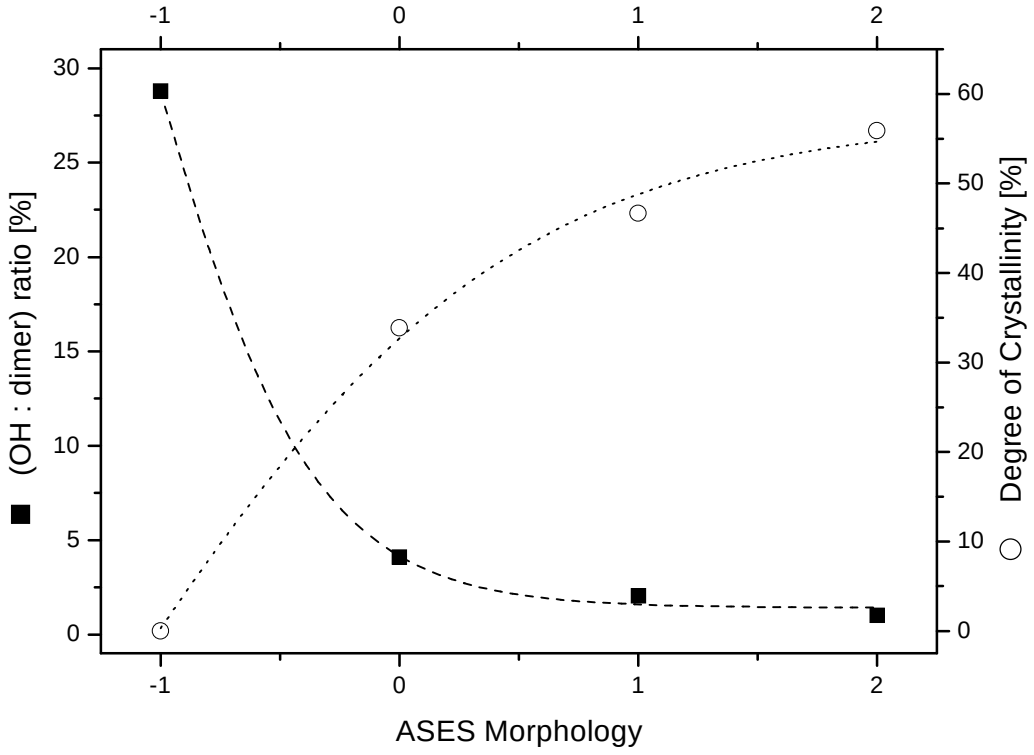


Figure 4: ASES results as a function of polymer degree of crystallinity, a free flowing powder of microspheres was rated '2' (fig. 3a), microspheres which were agglomerated '1' (fig. 3b), foam like structures '0' (fig. 3d), glassy and broken egg shell structures '-1' (fig. 3e)

Table 2: Sizes and Size distributions of the ASES particles prepared from semi-crystalline polymers

No	**Polymer**	**OH : dimer** [mol : mol]	**DC** [%]	**d(v, 0.1)** [µm]	**d(v, 0.5)** [µm]	**d(v, 0.9)** [µm]
7	L-PLA	0 : 100	56	1.76	3.90	6.78
8	DSS(8)-L-PLA	0.28 : 100	55	1.51	9.53	14.89
9	PVA(15)-g-L-PLA	1.025 : 100	56	1.21	3.57	7.29
10	- " -	2.05 : 100	47	n.d.	n.d	n.d.
11	- " -	4.1 : 100	34	3.76	>162	>176
13	- " -	28.8 : 100	0	1)	1)	1)

1) = out of range n.d. = not determined

DC = degree of crystallinity, rating it to the reference 100 % crystalline L-PLA (93.6 J/g) [30]

By increasing the molecular weight of the used core PVA by a factor of ca. 3 and therefore increasing the molecular weight of the final polyesters, too, similar trends in ASES precipitation were observed as outlined in fig. 5.

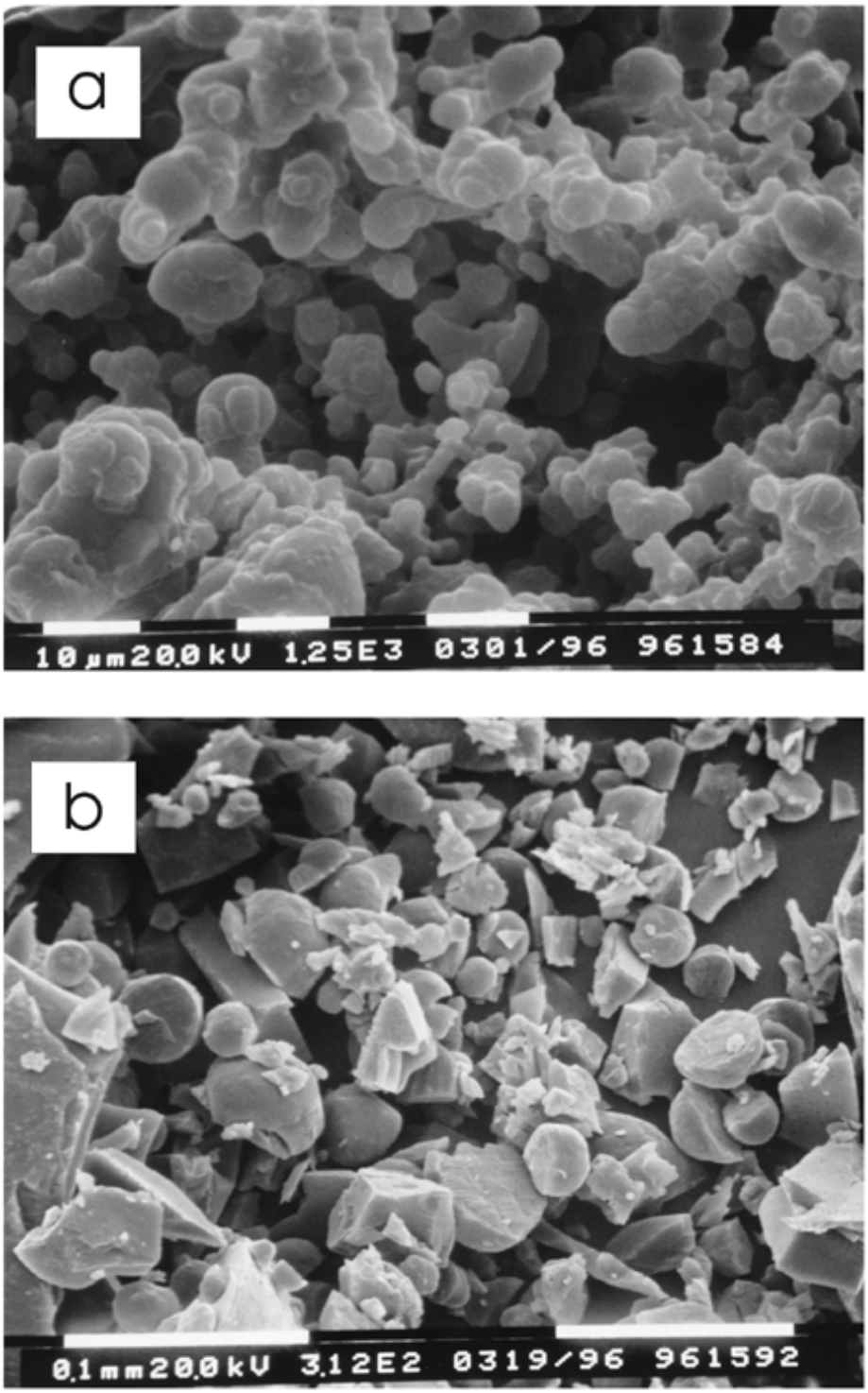


Figure 5: Influence of polymer molecular weight on the ASES microsphere morphology

A lower polymer molecular weight led to molten structures (fig 5b, polymer No 15, tab. 1), while a higher molecular weight resulted in foam like structures, where an initial particle formation tendency can be assumed (fig 5a, polymer No 14, tab. 1). These results confirmed, that a higher molecular weight of the polymers alone is not sufficient to significantly change the precipitation behavior of the polymers in principal, since no microspheres were obtained.

A possible explanation might be that in contrast to amorphous substances the solubility of crystalline substances is for one only limited. The contact with an anti-solvent leads to an immediate decrease in solubility and polymer precipitation. Secondly, segregation from a solution by addition of an anti-solvent is characterized by an immediate phase separation into a solid crystalline and a liquid phase. Therefore crystalline polymers can easily be precipitated in one step, in case of ASES promoting the formation of microspheres. The addition of a non-solvent to a solution of amorphous polymers leads to the formation of two liquid phases first, before a solid phase starts to precipitate. In corresponding phase diagrams of amorphous substances, e.g. described by Flory [27], there is only one polymer concentration (at the point of contact between spinodale and binodale) where the system is going directly from a stable to an unstable region, leading to immediate polymer precipitation. In all other cases a metastable region with supersaturation is reached, hindering the formation of small separate microspheres. Moreover, supercritical carbon dioxide was reported to have some solution properties towards amorphous polymers [28,29] leading to polymer swelling and thus a reduction of Tg. In combination with the used process temperature (36°C), results from molten particulate structures to broken glassy fragments become reasonable.

To further investigate the hypothesis, that the degree of crystallinity is the key parameter, the crystallinity of the comb polyesters was manipulated in a different way. By maintaining similar poly(lactone) chain numbers and lengths, but changing the chain composition by copolymerization of L-LA with GA, a series of PVA-g-L-PLGAs was obtained. Their properties are summarized in table 1 (polymers No 9,16-18). The expected reduction of their thermo-chemical properties (Tg, Tm, ΔHm) with increasing GA chain contents is outlined in fig. 6a. DMTA measurements conducted on polymer films cast from 2.5 % [w/v] DCM solution, showed the same trends for polymer thermo-mechanical strength. Owing to the lability of these films the onset temperatures of tan δ at the phase transition from the glassy to a rubbery state were plotted versus the polymer composition as outlined in fig. 6b.

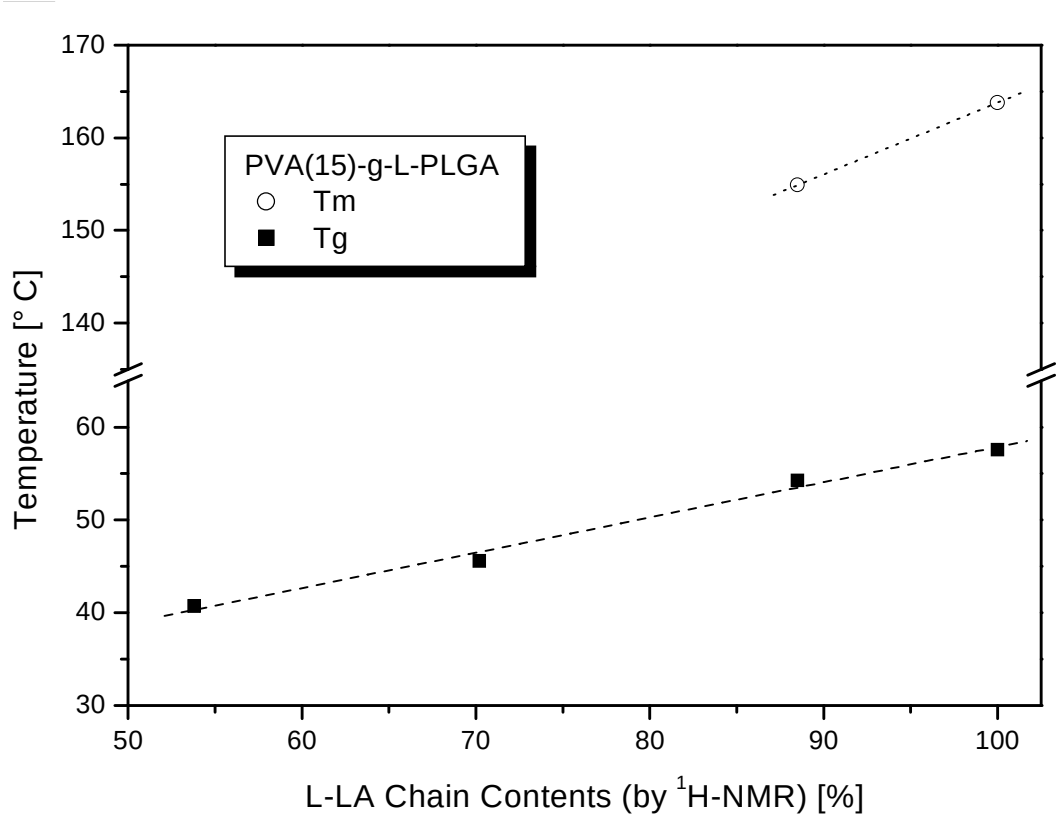


Figure 6a: Polymer thermo-chemical (DSC) properties as a function of poly(lactone) chain composition

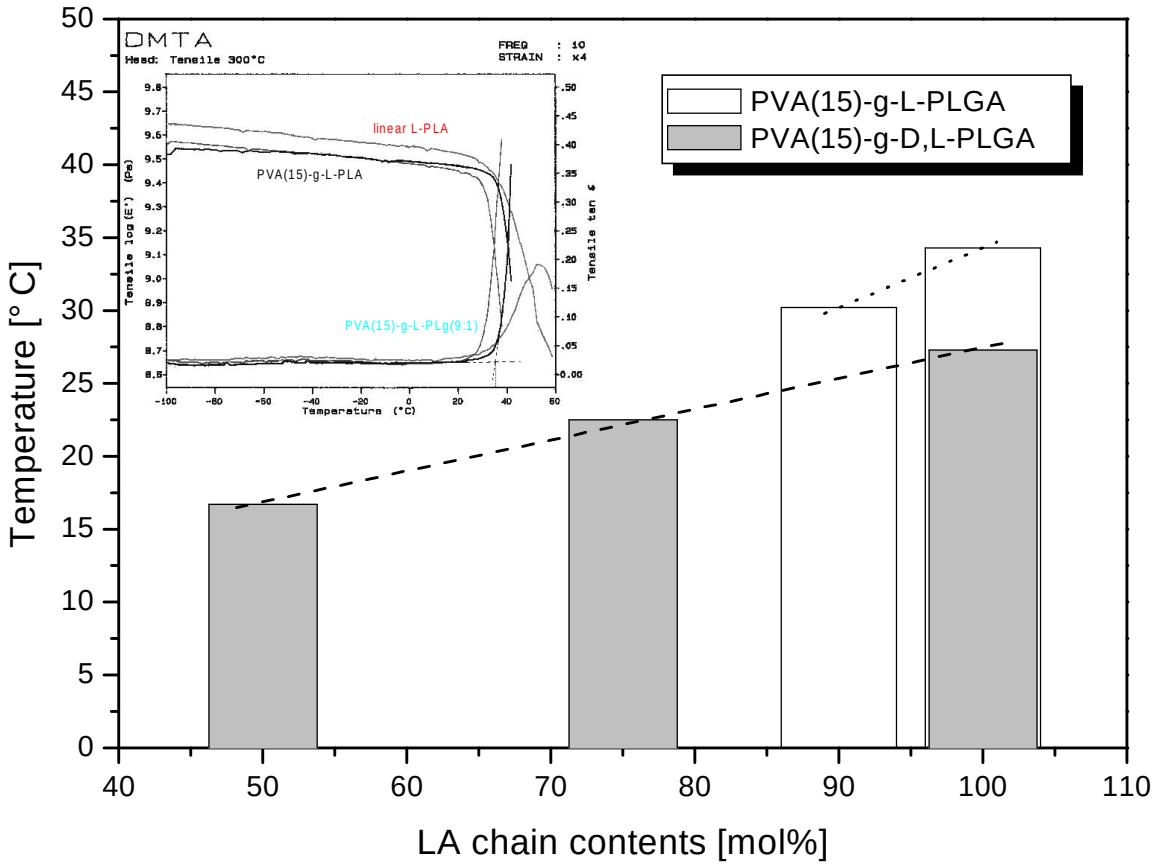
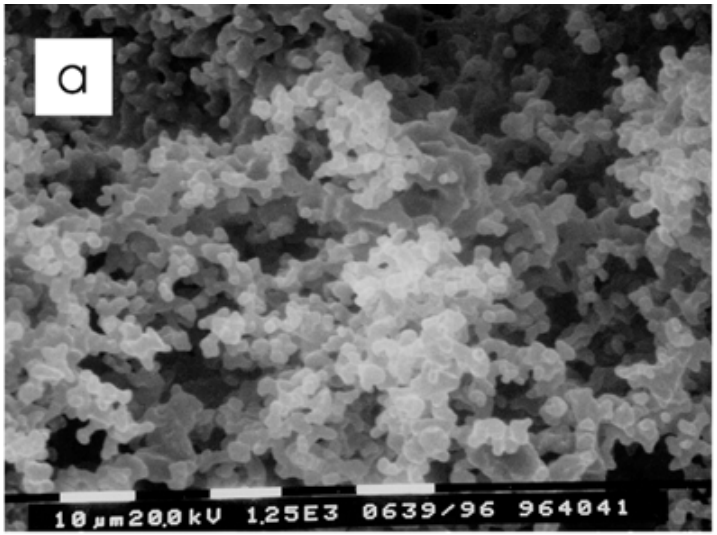


Figure 6b: Polymer thermo-mechanical (DMTA) properties as a function of poly(lactone) chain composition

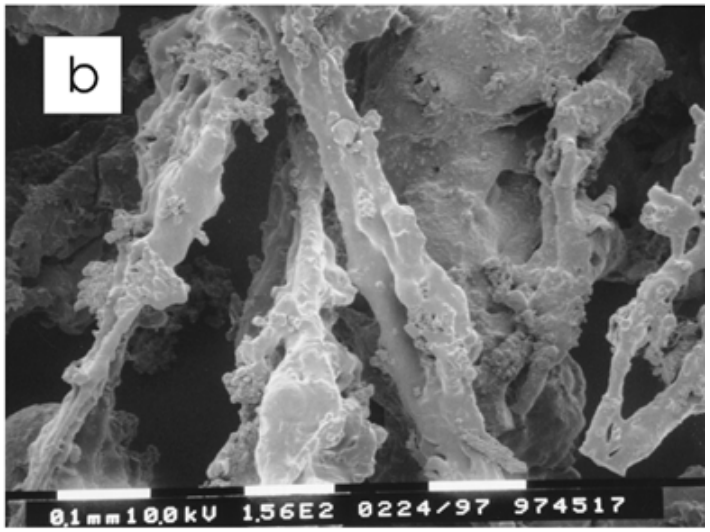
The linear L-PLA and polymer No 9 (table 1), whose properties were quite comparable, exhibited the highest strength, as to expect for semi-crystalline polymers. The reduction of thermo-mechanical properties was found to be a linear function of chain lengths and/or of chain composition, confirming the results obtained by DSC analysis. Shorter poly(lactone) chains reduced thermo-mechanical properties. In similar manner did the copolymerization of glycolide. The ASES results were equally following this trend. With decreasing values of Tg and Tm the obtained 'particle' morphologies were rated from 1 to -1 in decreasing order.

Taking all results in account, it makes sense to distinguish between factors, which enable a successful ASES microsphere preparation and parameters with a more limited influence, enabling only a small range of fine control. Neither polymer properties such as solution viscosity, generally reduced in case of the novel polyesters compared to linear PLA and PLGA (table 1), molecular weight and glass transition temperature, nor density of the supercritical carbon dioxide seem to be these key factors in ASES. Crystallinity, on the other hand, was found to influence the process of precipitation in a significant manner. Moreover, a ‘critical’ melt enthalpy of ca. 43.7 J/mol, equal to a degree of crystallinity of more than ca. 47 % (rating it to the reference 100 % crystalline L-PLA (93.6 J/g) [30]) could be derived for successful microsphere production.

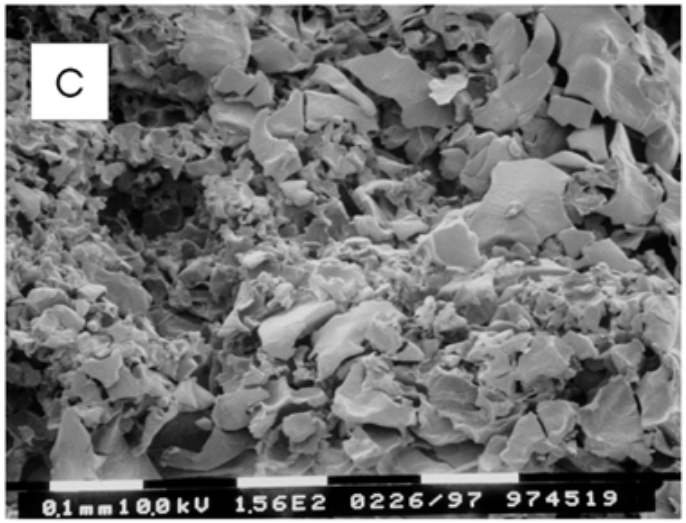
In most experiments described above a PVA core with a molecular weight of 15 kg/mol was incorporated into the polyesters. A slightly higher molecular weight of the backbone was found to have a significant influence on the process of ASES particle formation in case of faster degrading polyesters with an equimolar chain composition of D,L-lactide and glycolide. A higher molecular weight of the core PVA is equal to a larger number of lactone chains grafted onto a single backbone, leading to polymers with up to ca. 330 chains, in case of the used 20 kg/mol PVA (polymer No 19, table2). Surprisingly, the use of this polymer, with a lactone chain lengths similar to polymer No 6 (table 1), led to ASES particle formation, although the particles were partly agglomerated. The morphology (fig. 7a) was rated ' 1-2' compared to the results obtained with the comb L-PLAs and L-PLGAs. Although the molecular weight of this polymer was quite high, DSC as well as DMTA analysis confirmed much lower thermal properties, excluding them as key parameters for the particle formation tendency in ASES.



PVA-g-D,L-PLGA(1:1)_01



PVA-g-D,L-PLGA(1:1)_05



PVA-g-D,L-PLGA(1:1)_10

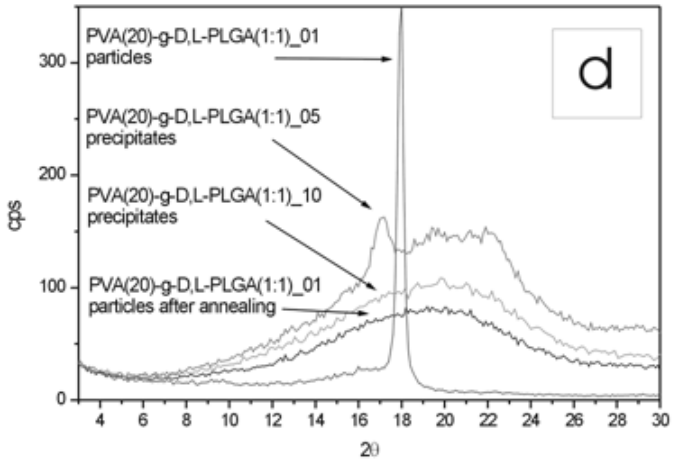


Figure 7: ASES microsphere morphology of PVA(20)-D,L-PLGAs as a function chain lengths and induced crystallinity (determined by WAXS analysis)

WAXS analysis of these particles revealed a high induced crystallinity (ca. 60 %). This crystallinity could not be detected either for a polymer film cast from DCM solution or for ASES particles after annealing above the polymer Tg. Therefore, it was a consequence of the ASES process itself. Most likely shear forces during the initial spraying process forced the polymer chains into chain crystallinity. A reduction of injected polymer concentration did not improve the results, neither did the variation of carbon dioxide pressure (density of the supercritical fluid).

Similar to the first experiments, a series of polymers (table 1, polymers No 19-21) with decreasing PLGA(1:1) chain lengths, but equal chain numbers was investigated. Similar to all other experiments a reduction of the chain lengths by a factor of about 5 led to foam-like fibrous structures, rated 0 (fig. 7b), and a further reduction to glassy fragments, rated -1 (fig. 7c). WAXS analysis of the ASES precipitates revealed that the induced crystallinity was equally a function of poly(lactone) chain length, as outlined in fig. 7d. The shorter the chains, the lower the degree of induced crystallinity. Phase separation, known for blends of PVA with PLGA [31], owing to the supercritical fluid, could be likewise explanation, but it should be an increasing function of the PVA contents to some extent, which was not observed.

Taking all results in account, crystallinity, either permanent or induced, can be reported to be the key factor in ASES processing.

It is likewise, that the situation will further improve, when a drug, suspended in the polymer solution, is coprecipitated and acts as nucleation agent. Therefore, the investigated novel polyester class may be of beneficial use in ASES. For the first time it was possible to obtain particles with a fast biodegrading polyester, which was found to continuously release protein drugs by pore diffusion from microspheres (prepared by a modified double emulsion WOW technique) [25,26].

6.5 CONCLUSIONS

The structure of the novel polyesters offered the unique possibility to investigate a pharmaceutical process by tailor-made polymer properties. Novel biodegradable comb polyesters were applied to monitor the influence of several factors, such as structure modification by variation of the poly(lactone) chain lengths, of the poly(lactone) chain number and of the use of different chain compositions. For the first time the key parameter of microsphere production by a supercritical fluid extraction process (ASES) could be related to polymer crystallinity. Moreover, the spectrum of processable biodegradable polymers could be expanded to comb-like polyesters, which have already shown their beneficial utility in controlled drug delivery, especially for protein and peptides.

6.6 ACKNOWLEDGEMENTS

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Chapter 7

Biodegradable comb polyesters containing polyelectrolyte backbones: A feasible carrier for the preparation of nanoparticles with defined surface structure and bioadhesive properties

7.1 ABSTRACT

A major challenge in oral drug delivery is to find suitable carrier systems for hydrophilic macromolecular drugs. Therefore, a new polymeric concept was investigated combining a modified three-dimensional architecture, increased hydrophilicity of poly(lactic-co-glycolic acid) (PLGA) and charged groups in a single polymer.

Biodegradable comb PLGA were synthesized by grafting short PLGA chains onto different poly(vinyl alcohol) (PVA) based core polyols, namely PVA, poly(2-sulfobutyl-vinyl alcohol) and poly(diethylaminoethyl-vinyl alcohol). The polyelectrolyte backbones were obtained by etherification of PVA with charge containing pendent groups.

The comb polymer structure could be confirmed by nuclear magnetic resonance, infrared spectroscopy, differential scanning calorimetry, elemental analysis and measurement of intrinsic viscosity. Nanoparticles (NP), as possible mucosal colloidal carriers, were prepared by controlled precipitation and investigated as a function of polymer composition.

The amphiphilic character and the three-dimensional architecture of the novel polyesters allowed the preparation of small nanoparticles even without the use of surfactants. Surface NMR, surface charge and hydrophobicity determination indicate a core-corona like NP structure, especially in case of negatively charged

polyesters. A structural model is proposed for the NP with an inner polyester core and an outer charged groups containing coating, depending on polymer composition and backbone charge density. The higher the polymer backbone charge density, the more pronounced its influence on the nanoparticle surface properties.

The possibility to prepare NP without the use of a surfactant as well as to design the NP surface characteristics by polymer backbone charge density and polymer hydrophilic-hydrophobic balance, will be a major advantage in protein adsorption, bioadhesion and organ distribution. This makes these biodegradable polymers promising candidates for colloidal protein and peptide delivery.

7.2 INTRODUCTION

Ongoing advances in the areas of biotechnology, biochemistry and gene technology generated a growing number of potent and highly specific hydrophilic drugs, such as peptides, proteins and oligonucleotides. Similar progress was not achieved, however, in peptide and protein delivery, especially by the oral route [1,2]. Nanoparticulate colloidal dosage forms have shown first promising results in case of small or lipophilic drugs [3]. But suitable polymeric carriers for transmucosal delivery of hydrophilic macromolecules remain a major challenge [4].

A number of techniques for the preparation of nanoparticles (NP) have been described in the literature [5-7]. Originally, NP are obtained by emulsion polymerization [8]. Inspite of the advantages of these polymerization techniques, such as control of sizes and size distribution, the drawbacks, namely use of toxic surfactants and catalysts, drastic reaction conditions, are prohibitive for sensitive drugs. Furthermore, the resulting polymers, such as poly(acrylates)

and poly(cyanoacrylates) are either non-degradable or of reduced bio-compatibility [9] compared to polyesters, such as poly(lactic-co-glycolic acid) (PLGA). Therefore, conversion of biocompatible polymers into NP, would be of particular interest.

Known preparation methods include emulsification solvent evaporation [10], solvent displacement [11,12] and interfacial phase deposition induced by salting out [13] or emulsification diffusion processes [13]. Solvent displacement is based on the precipitation of a polymer in solution by addition to a surfactant containing miscible non-solvent of the polymer. The method allows the preparation of NP without prior emulsification and is usually employed to incorporate lipophilic drugs into a carrier. The often observed precipitation of large amorphous polymer aggregates in the µm-range can only be overcome by surfactants influencing the surface characteristics of the resulting NP. Therefore, novel amphiphilic polymers avoiding the use of surfactants would be preferred. Synthetic or natural polymers, used for colloidal delivery systems, such as different types of poly(acrylate) derivatives, poly(urethanes), poly(styrene), aliphatic polyesters based on lactic and glycolic acid (PLA, PLGA), hyaluronic acid, chitosan [14], lack the possibility to adjust polymer properties to allow facile preparation of nanoparticules for drug delivery.

Therefore, we investigated a new synthetic polymer concept to overcome these drawbacks. Hydrophilic polymers, such as poly(vinyl alcohol) (PVA), are known to possess mucoadhesive properties, similar to poly(acrylates) [14]. By grafting short poly(lactide-co-glycolide) (PLGA) chains onto unmodified and charged groups containing PVAs, novel amphiphilic biodegradable polyesters were obtained [15-17]. These potentially mucoadhesive polymers were synthesized by ring-opening bulk polymerization of the cyclic lactones in the presence of the different polyol backbones (scheme 1). NP were obtained by

controlled precipitation. Their properties, such as size, surface charge and structure were investigated as a function of polymer composition, to demonstrate the utility of this novel carrier system especially for the preparation of colloidal drug delivery systems with designed surface characteristics.

7.3 MATERIALS AND METHODES

Materials

Poly(vinyl alcohol) (PVA) with a molecular weight of 15'000 g/mol and a degree of hydrolysis of 88% was obtained from Fluka. Before use it was rigorously dried at 80°C in vacuo to constant weight stored in a desiccator under vacuum at room temperature over P_2O_5. D,L-lactide and glycolide (Boehringer Ingelheim, S-grade) were recrystallized twice from dry ethyl acetate (refluxed over calcium hydride) and dried for 48 hours in vacuo directly before use. The melting points were 125-126°C and 82-83°C, respectively. Stannous octoate (Aldrich), 1,4 butanesultone (purum, Fluka), N-(2-chlorethyl)-N,N-diethyl-ammonium-chloride (Merck-Schuchard) and all other materials of analytical grade were used as received. Sodium hydride (Merck-Schuchard) was purified by three times extraction with pentane. DMSO (99.5 %, Riedel-de Haën) was dried over calcium hydride (Riedel-de Haën) and distilled under reduced pressure directly before use.

Polymer Syntheses

Polyelectrolyte Backbones. Sulfobutyl modified PVAs, P(SB-VA), and diethyl-aminoethyl modified PVAs, P(DEAE-VA), were prepared from poly(vinyl alcohol) under anhydrous conditions according Williamson in a dry nitrogen atmosphere, as described earlier [18,19] (scheme 1).

Briefly, 2.4 g (0.1 mol) purified sodium hydride were allowed to react with 50 ml dry DMSO at 20°C (cooling with an ice bath) under stirring until no

evolution of gas was observed. The resulting solution of the DMSO carbanion was added dropwise in the course of one hour to a solution of 5 g PVA (equal to 0.1 mol hydroxyl groups) in 100 ml dry DMSO at room temperature (cooling with an ice bath). After maintaining this temperature for one hour, either 13.6 g (0.1 mol) 1,4-butanesultone or 17.3 g (0.1 mol) N-(2-chlorethyl)-N,N-diethyl-ammonium-chloride in 20 ml dry DMSO were added dropwise in the course of half an hour. Finally, the temperature was raised to 60°C for additional 12 hours. Products were purified twice by precipitation from water in a 70 : 30 (v/v) acetone : hexane mixture and dried in vacuum at 35°C to constant weight. Degrees of substitution were determined by sulfur or nitrogen elemental analysis after ultrafiltration and lyophilization.

Ultrafiltration was performed four times on each sample (initial concentration: 200 mg polymer in 10 ml water) using an Amicon ultrafiltration stirring cell 8010 equipped with a YM1 filter membrane (Amicon, cut off = 1000 g/mol).

Sulfur Analysis of P(SB-VA) was performed by Schoeniger method (barium perchlorate titration using thorin as indicator), *nitrogen analysis* of P(DEAE-VA) with an autoanalyzer 185 (Hewlett Packard).

Polyesters. Biodegradable comb polyesters were prepared by grafting short poly(lactic-co-glycolic acid) chains onto the different polyols (PVA [15,16], P(SB-VA), P(DEAE-VA) [17]). In brief, ring-opening bulk melt polymerization of the lactones, L-lactide or D,L-lactide and glycolide, in the presence of the different core polyols with stannous octoate as catalyst was used (scheme 1). In a typical example 1.26 g PVA (equal to 0.025 mol hydroxyl groups), 7.00 g (0.048 mol) D,L-lactide and 5.64 g (0.048 mol) glycolide were charged under nitrogen into a rigorously dried 100 ml nitrogen flask which then was degassed at 50 - 55°C in a vacuum line for 1 hour, purging three times with dry nitrogen.

The flask was then immersed into a preheated oil bath (T = 170°C) for about 10 minutes to obtain a melt of monomers and backbone material. Then 3.9 mmol tin octoate as catalyst were injected under nitrogen and the reaction was allowed to proceed for three hours at 150°C. After cooling to room temperature, using a water bath, the products were dissolved in 50 ml of acetone and precipitated in 500 ml of cold water. The polymers were collected by filtration, washed with water, and dried at 35°C in vacuo for at least 48 hours until constant weight was obtained.

Polymer Characterization

Combined Size Exclusion Chromatography (SEC) and Static Light Scattering (SLS): 0.5 % (w/v) polymer solutions were injected into a thermostated (35 °C) Merck-Hitachi system (columns: Lichrogel PS mix and Lichrogel PS 40, 10 μm) with a differential refractometer (RI 71) and a MiniDawn light scattering detector [Wyatt Technology Corporation] (100 μl K5 cell, laser wavelength 690 nm, laser power 30 mW, three detecting angles (45°, 90° and 135°)). Chromatograms were obtained with degassed acetone at a flow rate of 1 ml/min.

Nuclear magnetic resonance spectroscopy (NMR) was performed at 35°C with 6 % (w/v) polymer solutions in different fully deuterated solvents (such as d_6-acetone, $CDCl_3$, d_6-DMSO). 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectra were recorded with a Jeol GX400 Delta N FT spectrometer, 500 MHz ^{1}H- and 125 MHz ^{13}C-NMR spectra with a Jeol LA500 eclipse+ Delta FT spectrometer.

Fourier Transform Infrared spectroscopy (FTIR) was recorded on a Nicolet 510 P FT-IR spectrometer (samples prepared as KBr disks).

Differential Scanning Calorimetry (DSC) was conducted with a differential scanning calorimeter (Perkin Elmer DSC 7) in sealed aluminum pans in a

nitrogen atmosphere, relative to indium and gallium standards. Thermograms covered a range of 0°C to 200°C with heating and cooling rates of 10°C/min. Glass transition temperatures (Tg) were determined from the second run.

Intrinsic viscosities were determined with an Ubbelohde viscosimeter (Schott Geräte, Germany) from dichloromethane or aqueous 0.5 N $NaNO_3$ solutions at 25°C with at least four different concentrations.

Nanoparticles (NP).

For *Nuclear magnetic resonance (NMR) surface analysis* NP were prepared in a dry nitrogen atmosphere by controlled polymer precipitation. 1 ml of a d_6-acetone solution (25 mg/ml) was injected dropwise into 2.5 ml of D_2O under stirring without the use of surfactants. Residual d_6-acetone was removed at 35°C by purging with nitrogen for 1 hour under stirring. Afterwards these dispersions were investigated by ^{1}H-NMR spectroscopy at 35°C with 1024 scans and compared with the spectra of redissolved particles (35°C, 256 scans).

For investigation of surface hydrophobicity and surface charge nanoparticles were prepared according to [11,12] by controlled polymer precipitation from acetone solution (250 mg / 10 ml) into 100 ml of water, containing Pluronic F68 (0.1 % w/v) [BASF] as surfactant, at 35°C with a flow rate of 2 ml/min under stirring (250 rpm). Residual acetone was removed in vacuum.

For visualization of the NP morphology freshly prepared NP were cast on silicon wavers and allowed to dry at room temperature for three days. Then they were investigated with a *field emission scanning electron microscope* S-4100 (Hitachi).

NP mean sizes and distributions were investigated by *photon correlation spectroscopy* (PCS) using a Zetasizer 4/AZ110 (Malvern Instruments, Malvern, UK) equipped with a 4 mW laser source, a 64 channel correlator and a multiangle photomultiplier device. Each sample was diluted with filtrated (Millipore 0,2 µm) distilled water until the appropriate concentration of particles was achieved to avoid multiscattering events and measured with a sample time of 30 ms for 30 min in serial mode. Each measurement was performed in triplicate. The mean values of the three measurement were used to carry out the statistical analysis. The PCS V. 1.26 -software was used to calculate particle mean diameter and width of fitted gaussian distribution.

The nanoparticle surface charge was determined by *electrophoretic light scattering* using a Zetasizer 4 (AZ 104 cell, Malvern Instruments, Malvern, UK). The NP dispersions were diluted (200 µg/ml) with filtrated distilled water (conductance 0,055 µS/cm) and the zeta-potential of the particles was measured.

Nanoparticle surface hydrophobicity was estimated by the binding constant of Rose Bengal [20]. Adsorption isotherms were measured in 0.1 M phosphate buffered solution at pH 7.4. After three hours of incubation at 25°C and centrifugation, the residual dye in the supernatant was quantitated photometrically (542 nm) against a calibration curve.

Table 1: Physicochemical properties of the polymers

No	Polymer	PVA DS [a] [%]/[mass%]	Backbone Mw [b] [kg/mol]	PLGA chain Mn [c] [g/mol]	PLGA Units per Chain [c]	Polymer Mn [d] [g/mol]	LA:GA [e] [mol%]	Tg [°C]	Solvent
1	P(DEAE-VA)-g-PLGA	20 / N=3.9	21.4	580	4.6	160'100	50 : 50	33.5	Acetone
2	PVA-g-PLGA	-	15.0	4000	31.7	1'254'000	51 : 49	39.7	DCM
3	PVA-g-PLGA	-	15.0	1100	8.8	360'300	51 : 49	37.0	1:1 (DCM : Acetone)
4	PVA-g-PLGA	-	15.0	590	4.7	170'200	50 : 50	34.0	Acetone
5	P(SB-VA)-g-PLGA	14 / S=6.8	19.9	590	4.7	171'700	53 : 47	28.7	Acetone
6	P(SB-VA)-g-PLGA	23 / S=9.7	24.1	580	4.6	160'000	53 : 47	34.9	Acetone
7	P(SB-VA)-g-PLGA	26 / S=9.8	25.6	870	6.9	219'800	52 : 48	31.7	Acetone
8	P(SB-VA)-g-PLGA	27 / S=10.0	26.0	840	6.7	210'700	52 : 48	29.8	Acetone
9	P(SB-VA)-g-PLGA	43 / S=12.3	33.6	1100	8.7	220'800	53 : 47	31.9	Acetone
10	P(SB-VA)-g-PLGA	27 / S=10.0	26.0	120	1.8	52'300	51 : 49	24.7	1:1 (Water : Acetone)
11	P(SB-VA)-g-PLGA	27 / S=10.0	26.0	65	0.5	40'200	52 : 48	25.6	Water

a) = from elemental analyis, first number = degree of substitution (DS), second number = elemental mass% (N = nitrogen, S = sulfur)

b) = from elemental analysis

c) = from 1H NMR analysis by intensity comparison of PLGA chain and end groups

d) = calculated from 1H NMR analysis assuming complete conversion of PVA hydroxyl groups

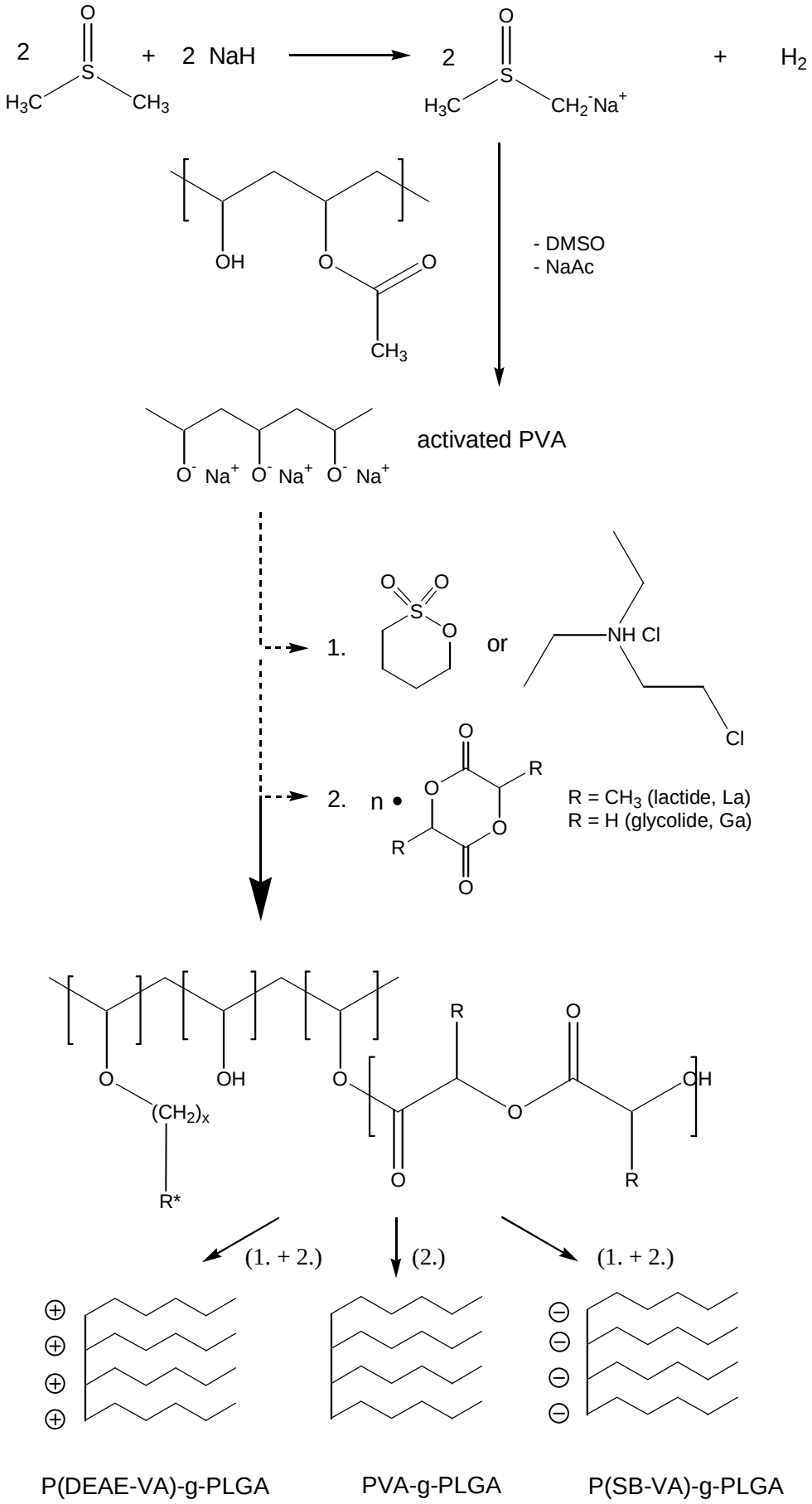
e) = from 1H NMR analysis

7.4 RESULTS AND DISCUSSION

In the area of colloidal drug delivery systems it is a major concern that when particles come in contact to physiological fluids or organs they may interact with other components. As such, the surface structure of the nanoparticles (NP) must be properly designed to maximize the potential for favorable and minimize disadvantageous interactions. To investigate the possibility of adjusting NP surface properties, such as hydrophilicity and surface charge, a novel class of amphiphilic polyesters containing hydrophilic uncharged and charged poly(vinyl alcohol) (PVA) backbones were synthesized.

Poly(vinyl alcohol) (PVA) is a non-toxic and inexpensive polymer widely used in drug delivery [21]. Applications range from its use as surfactant in the preparation of drug containing microspheres to hydrogel type drug delivery systems, due to its good protein compatibility and its mucoadhesive properties [22-24]. Recently we have studied PVA as backbone to modify structure and hydrophilicity of PLGA [15]. A novel polyester class with advanced properties needed for successful protein drug delivery was obtained [16]. These polymers showed improved bioerosion and protein drug release rates from microspheres [25]. In this study the polymeric concept was extended by the additional introduction of charged moieties into the PVA backbone.

PVA Modification. PVA was reacted according Williamson with either 1,4-butanesultone or N-(2-chlorethyl)-N,N-diethylamonium-chloride.



Scheme 1: Schematic representation of synthesis

The success of such modifications depends on a sufficient reactivity of PVA hydroxyl groups. Neither an aqueous system with sodium hydroxide base catalysis, nor potassium carbonate in DMSO allowed higher degrees of substitution, possibly due to insufficient reactivity and stability of PVA. Therefore, the activation of its hydroxyl groups was achieved with the carbanion of DMSO, produced by reaction with sodium hydride at 20°C, leading to the PVA alcoxide, as outlined in scheme 1. In all experiments equimolar amounts of the DMSO carbanion relative to PVA hydroxyl groups were required to achieve sufficient degrees of substitution. The modification with butanesultone was comparable to propanesultone [18,19].

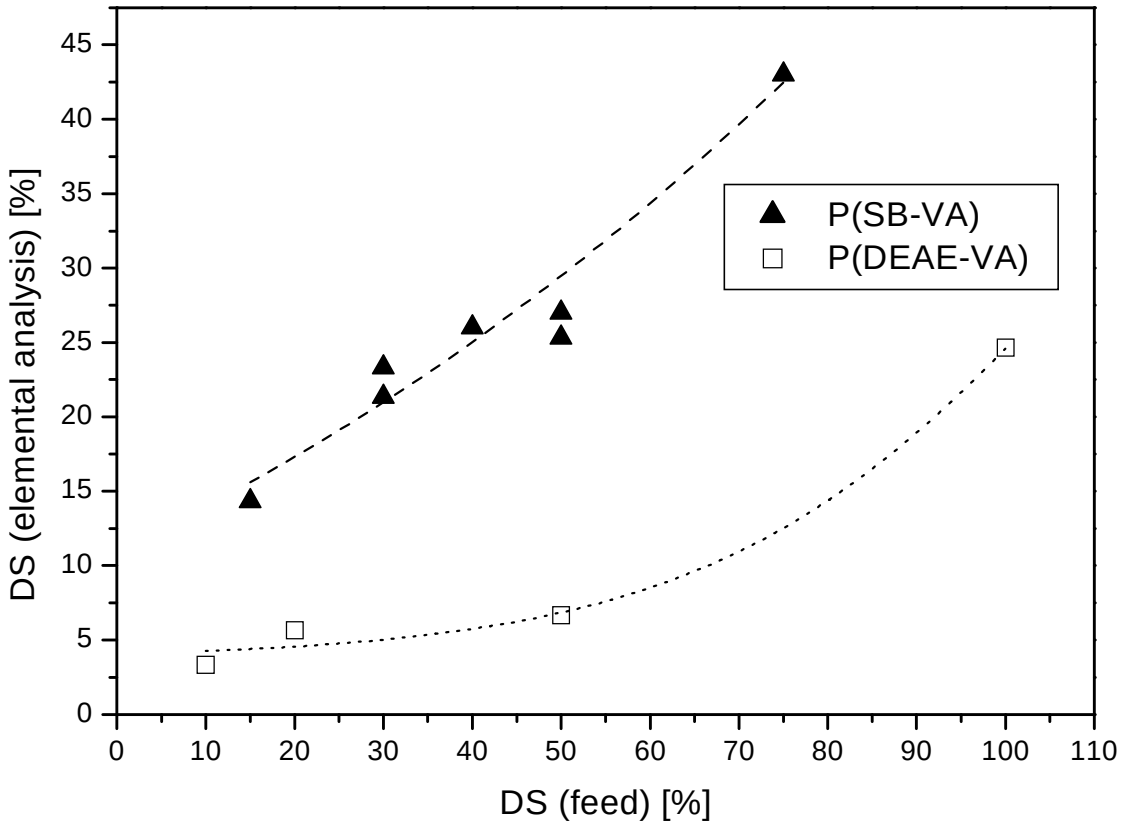


Figure 1: Theoretical and experimental degrees of substitution (DS) of the different core polyols

Fig. 1 shows an equimolar reaction for lower degrees of substitution of up to ca. 15 %. Possibly due to larger steric hindrance an excess of the sultone was

necessary, to generate polyelectrolyte backbones with higher degrees of substitution. A maximum degree of substitution of 43% could be achieved. A lower reactivity of N-(2-chlorethyl)-N,N-diethylamoniumchloride resulted in degrees of substitution of up to ca. 20%. Fig. 1 summarizes the complete results on theoretical and experimental degrees of substitution. The purified products were colorless solids, highly hygroscopic and showed good solubility in water as well as in DMSO. Yields were in the range of ca. 40 to 50% (ca. 80% if related to PVA actual degree of substitution). The increase in molecular weight by this reaction could be verified by increasing intrinsic viscosities. While 0.17 dl/g were found for the unmodified PVA in 0.5 N aqueous $NaNO_3$ solution, P(SB-VA) with highest DS exhibited 0.35 dl/g, which is in reasonable agreement with the molecular weight values calculated from elemental analysis, 15'000 g/mol for PVA and 33'600 g/mol for this P(SB-VA).

A typical ^{1}H-NMR spectrum of PVA in DMSO-d_6 is given in fig. 2b. Its methylene groups were found in the range of 1.5 ppm (hydroxyl terminated) and 1.7 ppm (acetylated). The acetyl CH_3 resonated at 1.9 ppm, **CH**-OH was found in the range of 4.1 ppm, the esterified **CH**-OCO in the range of 5 ppm and the hydroxyl groups themselves as broad signals at ca. 3.5 ppm. NMR analysis of the polyelectrolyte backbones confirmed the successful modification of PVA. In general, it revealed a reduced signal intensity of unmodified PVA hydroxyl terminated groups, due to etherification (**CH_2**-CH-OH 1.7 ppm and CH_2-**CH**-OH 4.1 ppm). Moreover, the etherified methylene protons (**CH_2**-CH-O-C and CH-O-**CH_2**) were found at ca. 1.9 ppm. In case of sulfobutyl (SB) modification the new signals of the butyl groups were visible at about 1.9, 3 and 3.7 ppm, as outlined in fig. 2a. The signals of the diethylaminoethyl (DEAE) substituent were found accordingly at 1.2 and 3.2 ppm (fig. 2b). It is worth noting that no signals of the PVA acetyl groups were visible after etherification, indicating that hydrolysis occurred during activation with the DMSO carbanion. Owing to the

broad and overlapping signals of PVA, NMR spectroscopy could not be used for a reliable quantification of the degrees of substitution.

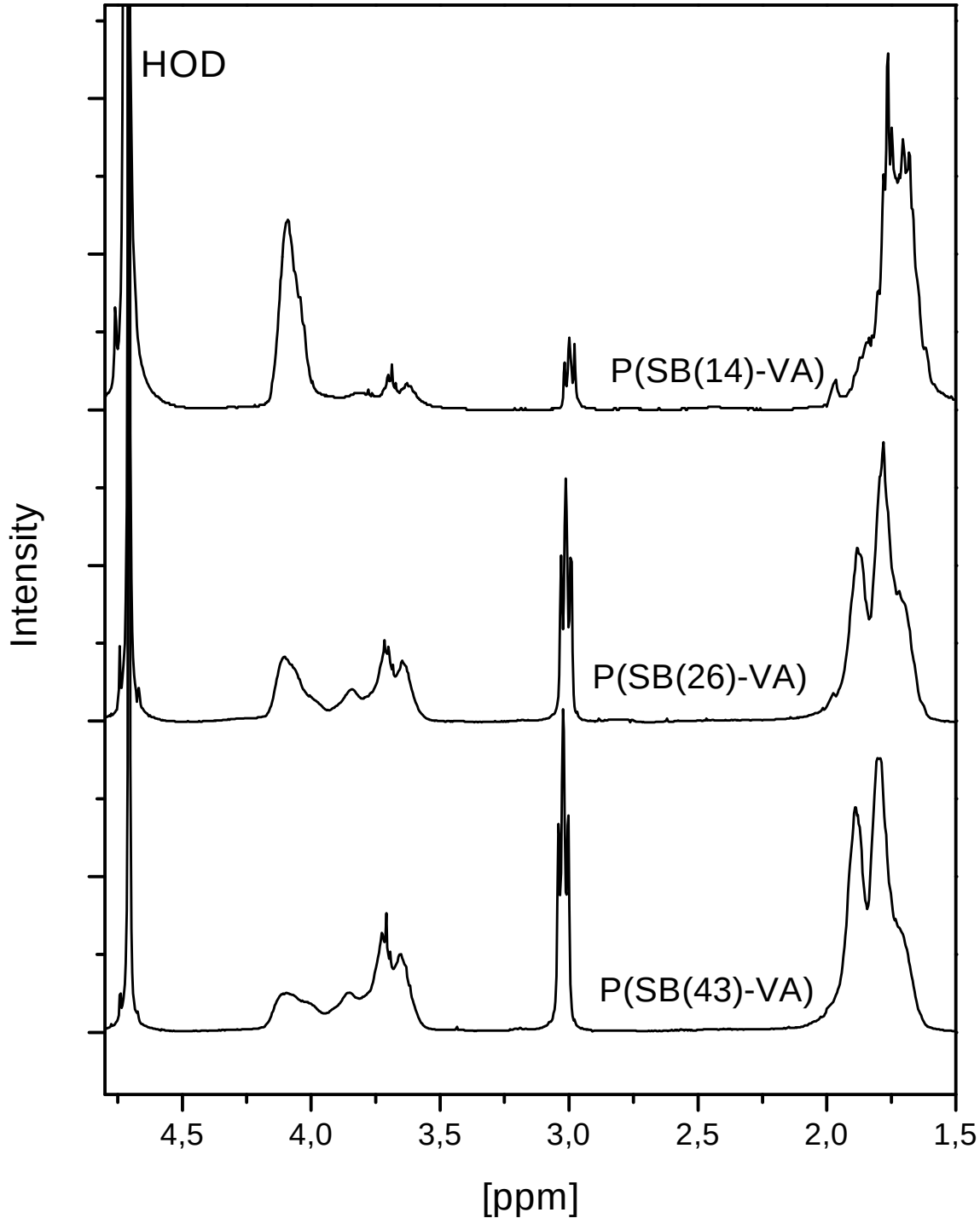


Figure 2a: 1H-NMR spectra of P(SB-VA) in D_2O as a function of DS

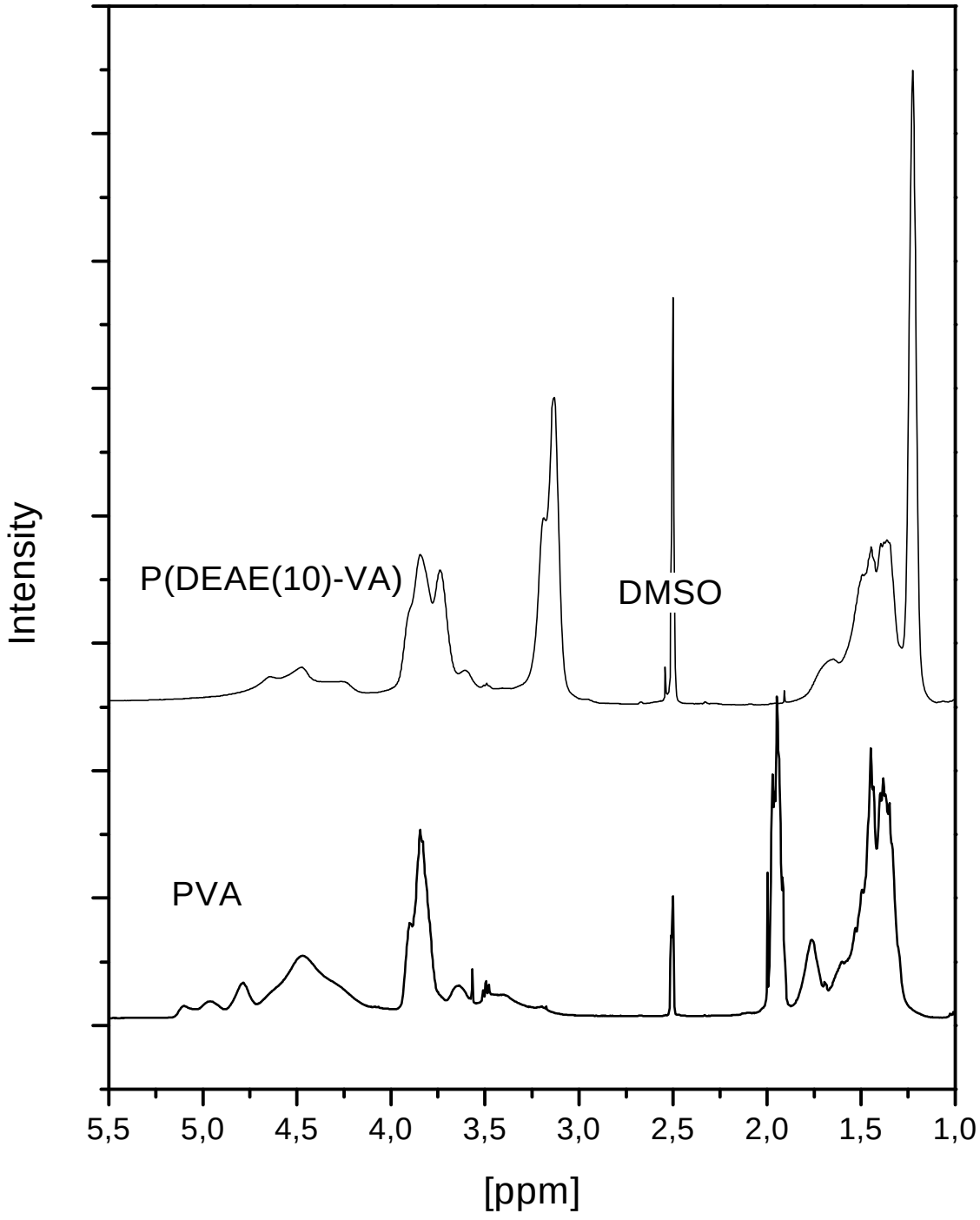


Figure 2b: 1H-NMR spectra of PVA and P(DEAE-VA) in DMSO-d_6

FTIR spectroscopy was a useful tool to visualize the backbone modifications, especially in case of sulfobutylation. Fig. 3 shows the fingerprint region of several modified P(SB-VA)s and the SO related vibrations were found at 1190 cm^{-1} (sym. str SO_2), 1040 cm^{-1} (str S-O) and 610 cm^{-1} (str S-C). Moreover, the

intensity of these peaks was a function of the degree of substitution and increased accordingly. The complete loss of CO vibrations confirmed that hydrolysis of PVA acetyl groups occurred during activation and modification, which is in accordance with the results obtained by Dolle et al. [18] as well as the results from NMR spectroscopy described above.

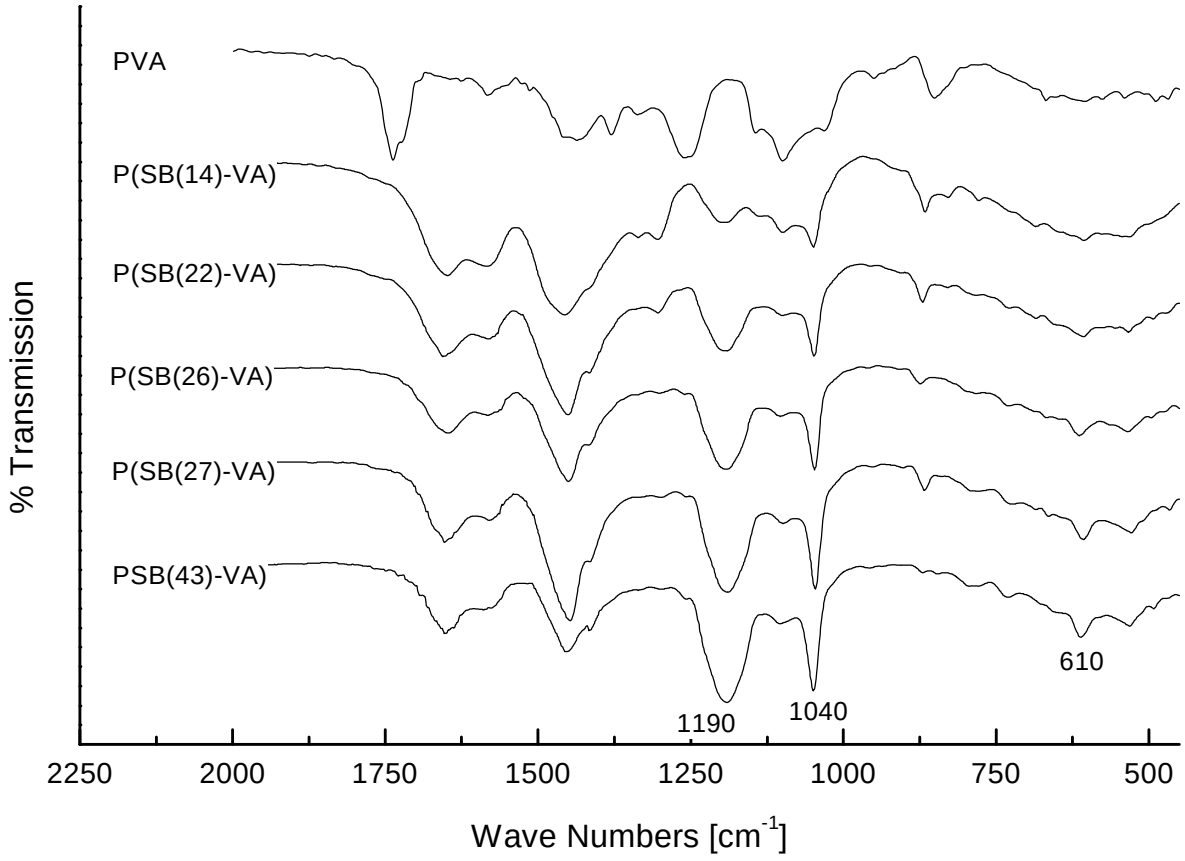


Figure 3: FT-IR spectra (fingerprint region) of the backbones as a function of SB degree of substitution

Polyester Synthesis. The incorporation of the charge modified polyols into brush-like grafted polyesters was performed by bulk melt polymerization of the lactones, lactide and glycolide, in the presence of the polyols using tin octoate as catalyst, as already reported for unmodified PVA as backbone [15,16]. The initial reaction temperature was set to 170°C for 10 minutes to achieve a

sufficient solubility of the backbones in the melt of the lactones, which could not be reached at lower temperatures. The reaction was then allowed to continue for additional three hours at 150°C. Bulk products were purified twice by dissolving in acetone and precipitation in water to remove unreacted backbone molecules, leading to final yields in the range of ca. 60 %, which was similarly observed in case of uncharged backbones [15].

In this type of polymerization the polyol hydroxyl groups are activated by tin octoate catalyst and start the first lactone ring opening reaction by insertion. Compared to unmodified PVA the number of hydroxyl groups of a single backbone molecule is reduced by etherification in case of the charge modified polyols, therefore the average number of PLGA units grafted onto a single OH-group in general is higher, leading to polyesters with less but longer PLGA chains, as confirmed by NMR analysis.

The feed ratio of backbone OH-groups to the lactones, lactide and glycolide, determines the molecular weight (Mw) of the final polyesters. The Mw could be systematically modified in the range from several million to less than 100'000 g/mol. These differences in Mw significantly influenced the properties of the polymers. Thermo-chemical properties, e.g. glass transition temperatures (Tg), were found to be a function of the PLGA chain lengths. Shorter chains resulted in decreasing Tg values [15,16]. In case of long PLGA chains, equal to high polymer Mw, polymer properties are dominated by these long PLGA chains and, therefore, are comparable to linear polyesters with high Mw. As a result these polymers are lipophilic and showed a good solubility in dichloromethane (DCM). By reducing the chain length to an average of ca. 5 - 10 PLGA units solubility shifted to more polar solvents, such as e.g. acetone. A further reduction resulted in even more amphiphilic polyesters, which were water-soluble (table 1). Since the intended NP preparation method based on good

acetone solubility, only polymers with an average PLGA side chain length of ca. 5 to 15 units were chosen for NP preparation.

In case of high SB degree of substitution of the backbones and short PLGA chains the SO related vibrations of the polyesters were again visible in the FT-IR spectra, as outlined in fig. 4. The spectra are plotted as a function of PLGA chain lengths, confirming the basic polymer structure. The shorter the PLGA chains the higher the peak intensities.

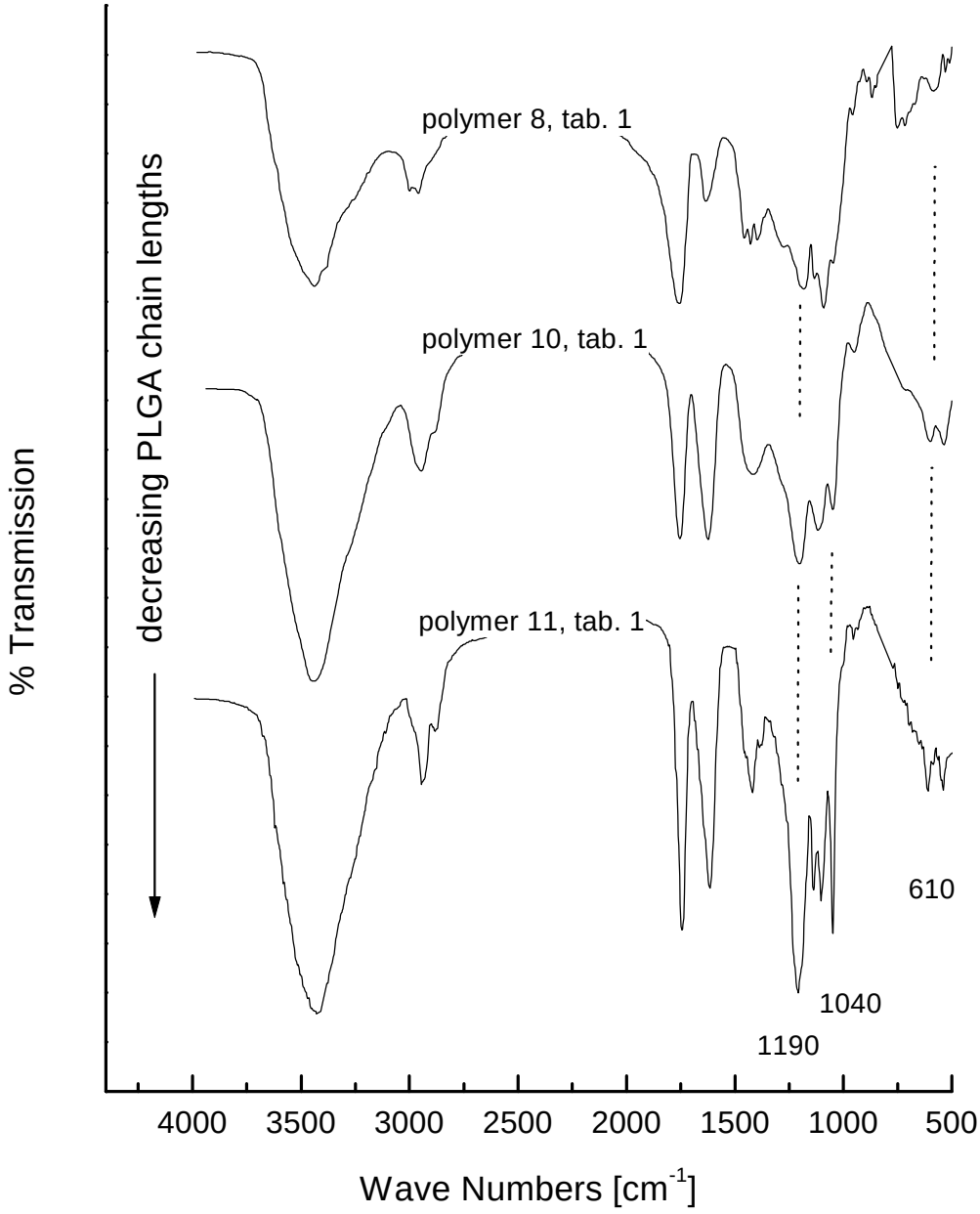


Figure 4: FT-IR spectra of P(SB(27)-VA)-g-PLGA as a function of PLGA chain lengths

In accordance with our previous reports on the synthesis of comb polyesters with unmodified PVA as backbone, NMR spectroscopy could be utilized for polymer structure determination. A typical example of a polyester with an unmodified PVA backbone is shown in fig. 5a. The spectra are dominated by the signals of the PLGA chains. Overlapping doublets are seen at δ = 1.45 ppm for the methyl groups of the L- and D-lactic acid (La) repeating units, the multiplets at δ = 4.8 ppm are assigned to the methylene groups of the glycolic acid (Ga) residues and at δ = 5.16 ppm the lactic acid CH appears confirming the basic chemical structure. In addition new signals for the corresponding PLGA end groups were found at δ = 4.2 ppm, 4.35 ppm (terminal **-CH_2**OH and **-CH**(CH_3)OH) and δ = 1.28 ppm (terminal -CH(**CH_3**)OH) indicating the three dimensional architecture of the polymers. The absence of COOH related signals shows the successful incorporation of the backbones and that no or less than 5% linear PLGA was produced during synthesis. Moreover, NMR analysis allowed the determination of the average PLGA chain lengths grafted onto the backbones by comparison of the intensities of the PLGA end and chain groups. Assuming complete conversion of the polyol backbones a maximum theoretical number average molecular weight (Mn) could be calculated, which in case of polyesters bearing uncharged PVA cores was in reasonable agreement with the values calculated from feed composition as well of those obtained by combined SEC and static light scattering analysis (SEC-SLS) [9,10,20].

These values are summarized in table 1. With increasing degree of substitution of the core PVA the number of unmodified hydroxyl groups decreases accordingly. Thus, a smaller amount of polymerization initiation and propagation centers is present during synthesis. Equal synthesis feeds, therefore, lead to a decreasing number of single PLGA chains per core molecule with increasing chain Mn, which could be confirmed by NMR analysis. While the

PLGA side chains of the polyester with an unmodified PVA backbone consisted of 5 units on average, 9 units were found in case of P(SB(43)-VA)-g-PLGA.

It was not possible to define a suitable solvent/SEC column combination for SEC-SLS analysis for the higher charged polymers. Interactions with the columns and a carry-over of the injected samples, prohibited this type of molecular weight determination. Therefore, intrinsic viscosities in DCM were used for estimation. Values ranging from 0.17 dl/g for the uncharged PVA-g-PLGA to 0.18 dl/g for the P(SB(43)-VA)-g-PLGA with the highest degree of SB substitution confirmed the results from NMR analysis. Less PLGA chains per single backbone molecule with increasing chain Mn led to comparable molecular weights depending on feed composition. The three-dimensional polymer architecture led to reduced interactions between the molecules, lowering their thermo-chemical properties, similarly observed for comb polyesters with either poly(saccharide) [26] or unmodified PVA [15,16,25] backbones. The glass transition temperatures (Tg) investigated by DSC were ca. 10 to 15°C lower compared to linear PLGAs and similar to those observed for uncharged PVA-PLGAs (table 1).

Nanoparticles (NP).

Nanoprecipitation of the comb polyesters from acetone solution into water was utilized to obtain narrowly distributed spherical NP. This preparation method is an effective and mild process to manufacture NPs avoiding emulsification and high shear forces [11,12].

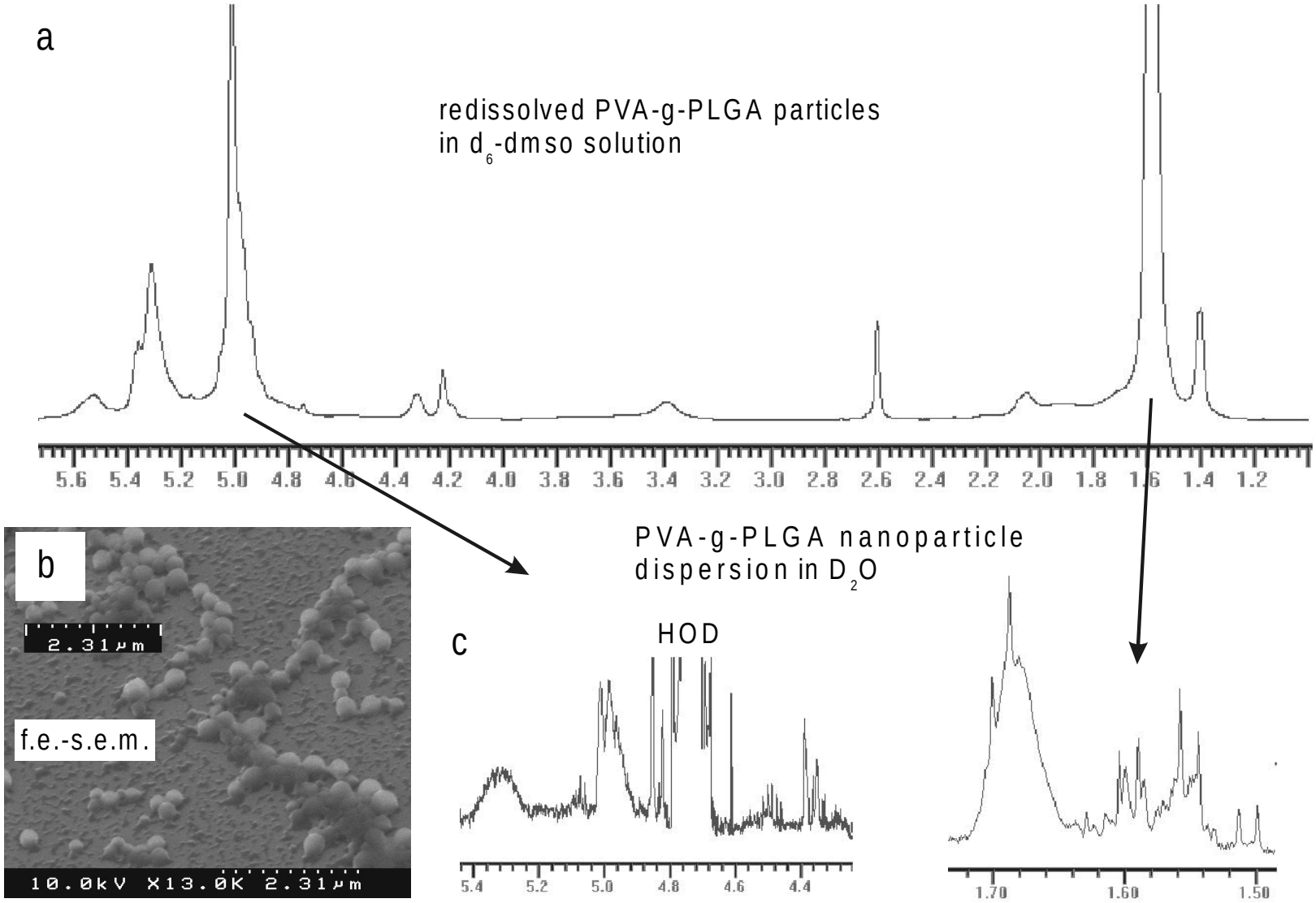


Figure 5: ^{1}H-NMR spectra of PVA-g-PLGA a) in solution and c) as colloidal dispersion, b) NP morphology

Fig. 5b gives an example of the NP morphology as observed by field emission scanning electron microscopy. The particles always showed a spherical shape with smooth surfaces and narrow size distributions, which could be confirmed by photon correlation spectroscopy (compare table 2). NP could be prepared even without the use of surfactants in the water phase, usually applied in the solvent displacement technique, indicating the amphiphilic surfactant-like character of these novel polyesters. Since the surface properties of the NP will not be masked by adsorbed surfactant molecules, its absence during NP preparation will be an advantage in protein loading by adsorption as well as in preservation of bioadhesive force.

Table 2: Surface characteristics of NP prepared from different polyesters

Polymer	**Intr. Visc.** [dl/g]	**Molecular weight** [a] **(Mn)** [g/mol]	**NP size** [b] **(z-Ave)** [nm]	**NP size** [c] **(z-Ave)** [nm]	**ξ-Pot.** [c] [mV]
PVA-g-PLGA_01	0.61	1'563'000	343.6	-	-
PVA-g-PLGA_05	0.31	n.d.	412.9	-	-
PVA-g-PLGA_10	0.17	140'400	513.4	119.3	-3.2
PVA-g-L-PLA_10	0.26	234'000	313.8	-	-
P(SB(14)-VA)-g-PLGA_10	0.20	n.d.	-	116.0	-14.4
P(SB(43)-VA)-g-PLGA_10	0.18	n.d.	202.9	104.2	-21.5

a) = From combined SEC and static light scattering analysis

b) = Prepared without the use of surfactant in the water phase

c) = Prepared with 0.1% [w/v] surfactant (Pluronic PF68) in the water phase

n.d. = not determined

Without the use of a surfactant in the water phase it was not possible to produce NP from commonly used linear PLGA. In contrast, comb polyesters with unmodified and therefore uncharged backbones enabled the surfactant-free preparation of NPs with sizes ranging from ca. 300 to 500 nm (table 2). Moreover, the situation further improved when comb polyesters with charge

containing backbones, especially negatively charged P(SB-VA)s, were used. A higher SB degree of substitution of the backbone leads to an increased amphiphilic character of the comb carrier polymers. Due to the higher amphiphilicity and low solution viscosities, NP with an average size of about 200 nm could be obtained. Their corresponding surface charge (zeta potential) was found to be ca. -57 mV. This result seems to be an indication for a core-corona NP structure leading to an orientation of the charged moieties of the backbone mainly onto the NP surface during precipitation.

A core-corona like structure with a polyester core and an outer hydrophilic backbone coating is already discussed for NP prepared from block-co-polymers consisting of poly(ethylene oxide) (PEO) and polyester blocks, PEO-PLGA and PEO_3-PLA [27]. To determine, if the comb polyesters exhibit a similar behavior, NP were investigated by ^{1}H-NMR spectroscopy in deuterated aqueous dispersion. These spectra were compared with those of the redissolved particles. Due to the nature of this experiment, only solubilized polymer parts, which, therefore, are able to free movements to some extent, will be visible in these spectra. In case of NPs prepared from uncharged PVA-g-PLGAs the NMR spectra revealed that the signal intensity of the more hydrophobic PLGA chain groups was significantly reduced compared to the intensity of their hydroxyl terminated end groups, as outlined in fig. 5c. Although the signals are only very weak and signal shifts cannot be excluded, a structural model is proposed, where the PLGA chain residues are part of the solid NP core, while their hydrophilic end groups are mainly oriented to the surface. During precipitation and NP hardening it is likewise that hydrophilic polymer parts are oriented to the outer water phase while the more lipophilic polyester residues form the inner NP core.

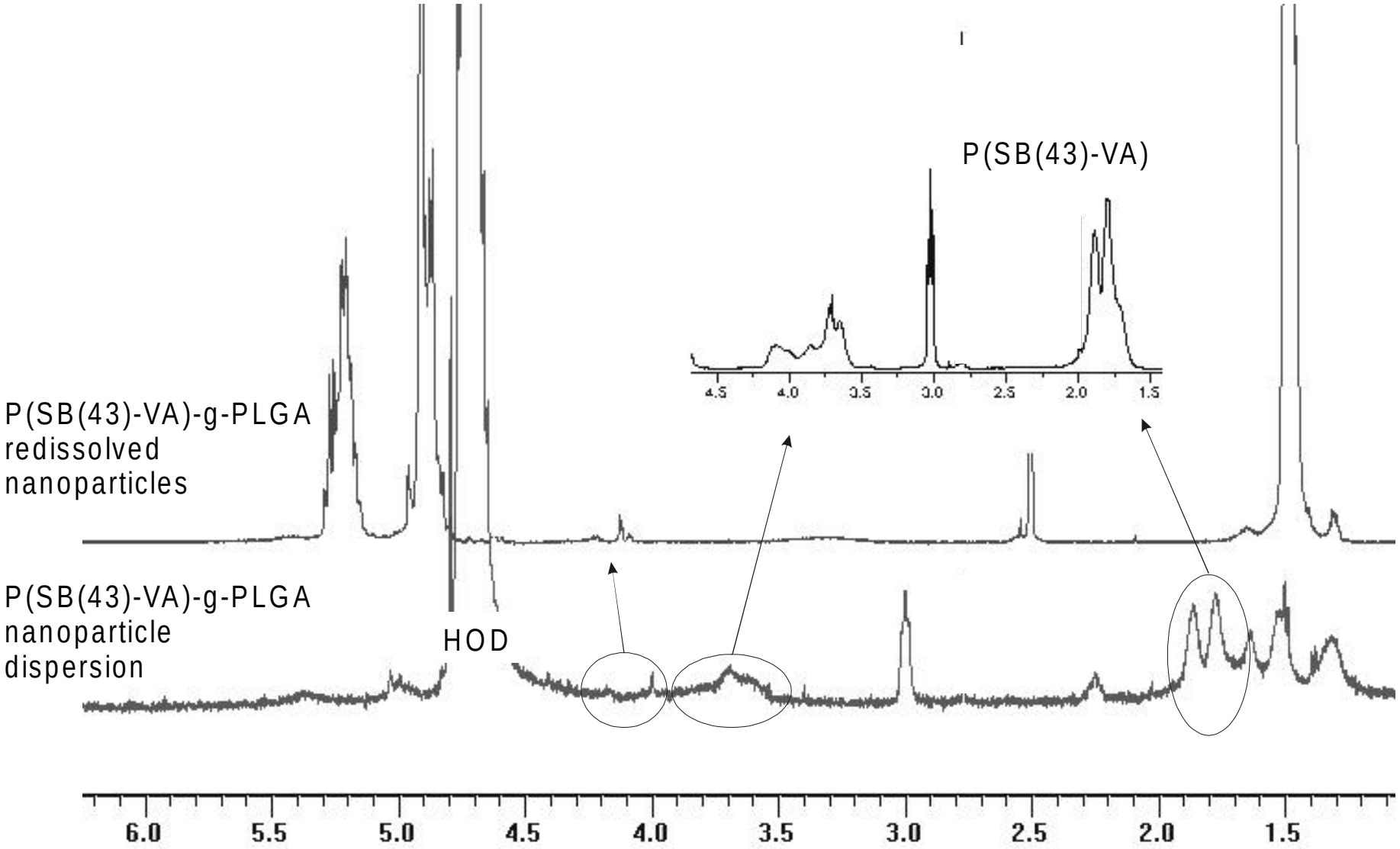


Figure 6: ^{1}H-NMR spectra of P(SB(43)-VA)-g-PLGA a) in solution b) as colloidal dispersion

In case of polymers bearing charged groups above described phenomena became even more pronounced. The NMR spectrum (compare fig. 6) not only revealed a similar behavior of the signal intensities of the PLGA chain groups and their hydroxyl terminated end groups, but also showed new signals, which could be assigned to the sulfobutylated PVA core. A core-corona structure of the NPs with a polyester core and outer backbone coating is the most likewise explanation. A physically stabilization during precipitation by negative attraction of the charged groups, reaching out in the outer water phase, seems to lead to this microdomain orientation.

Recent studies on mucosal particle resorption revealed a significant dependency on particle sizes. The smaller the tested nanoparticles, the higher their intestinal adhesion and absorption [5]. Especially sizes in the range of ca. 100 nm and smaller turned out to be most effective [2,6]. It was possible to produce nanoparticles consisting of the novel polyesters in the favorable size range by the addition of a low amount of Pluronic as surfactant [0.1 % w/v] to the water phase prior to precipitation (table 2) [11,12].

Even in the presence of the PEO-PPO surfactant, hydrated and therefore mobile charged groups were found on the NP surface by NMR analysis, as outlined in fig. 7. The NMR spectrum of the P(SB(43)-VA)-g-PLGA NP dispersion was dominated by the signals of the surfactant, dissolved in the water phase, but the charged backbone related signals are clearly visible, too, indicating the potential of this new polyester class. Even if low amounts of additional surfactant were employed, to produce very small NP, the charged groups at the NP surface were not completely masked by adsorbed surfactant, resulting in a preservation of the novel polymer properties.

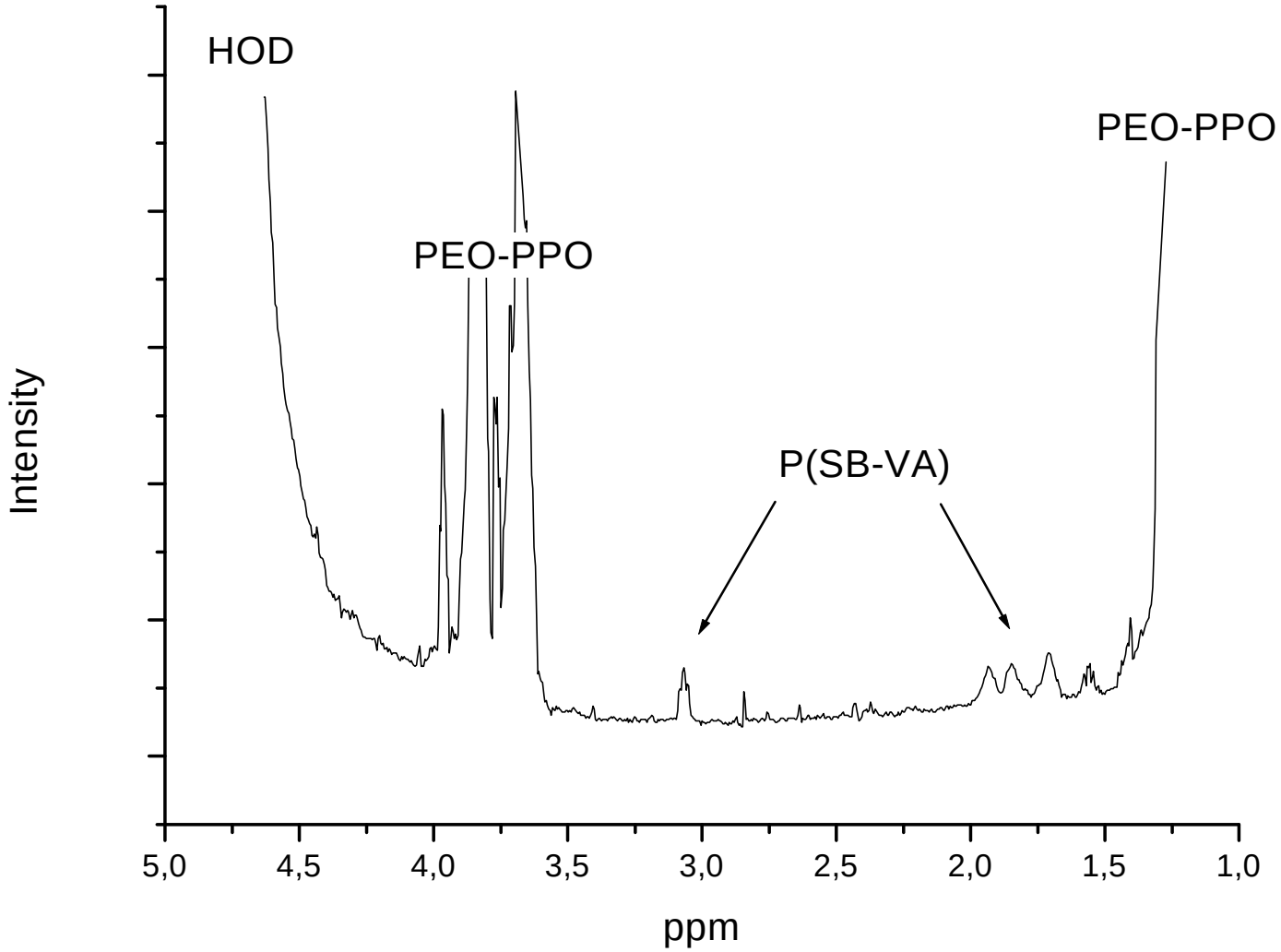


Figure 7: 1H-NMR spectrum of a NP dispersion prepared from P(SB(43)-VA)-g-PLGA using a 0.1 % [w/v] surfactant (Pluronic PF 68) containing water phase

To further investigate this result, the surface hydrophobicity of the NP as a function of polymer composition was estimated by adsorption of the lipophilic dye, Rose Bengal [20]. The resulting adsorption isotherms are given in fig. 8a. The amount of adsorbed dye per dry weight of polymer in mg is plotted against the equilibrium concentration. The obtained adsorption plateaus, as a measure for NP surface hydrophilicity, directly followed the polymer composition and the amount of charged groups in the backbone. The higher the backbone charge density, the obviously lower the NP surface hydrophobicity and, therefore, the lower the affinity to the lipophilic dye as well. These results are in reasonable agreement with the proposed rearrangement of the hydrophilic backbones to the surface of the nanoparticles during precipitation.

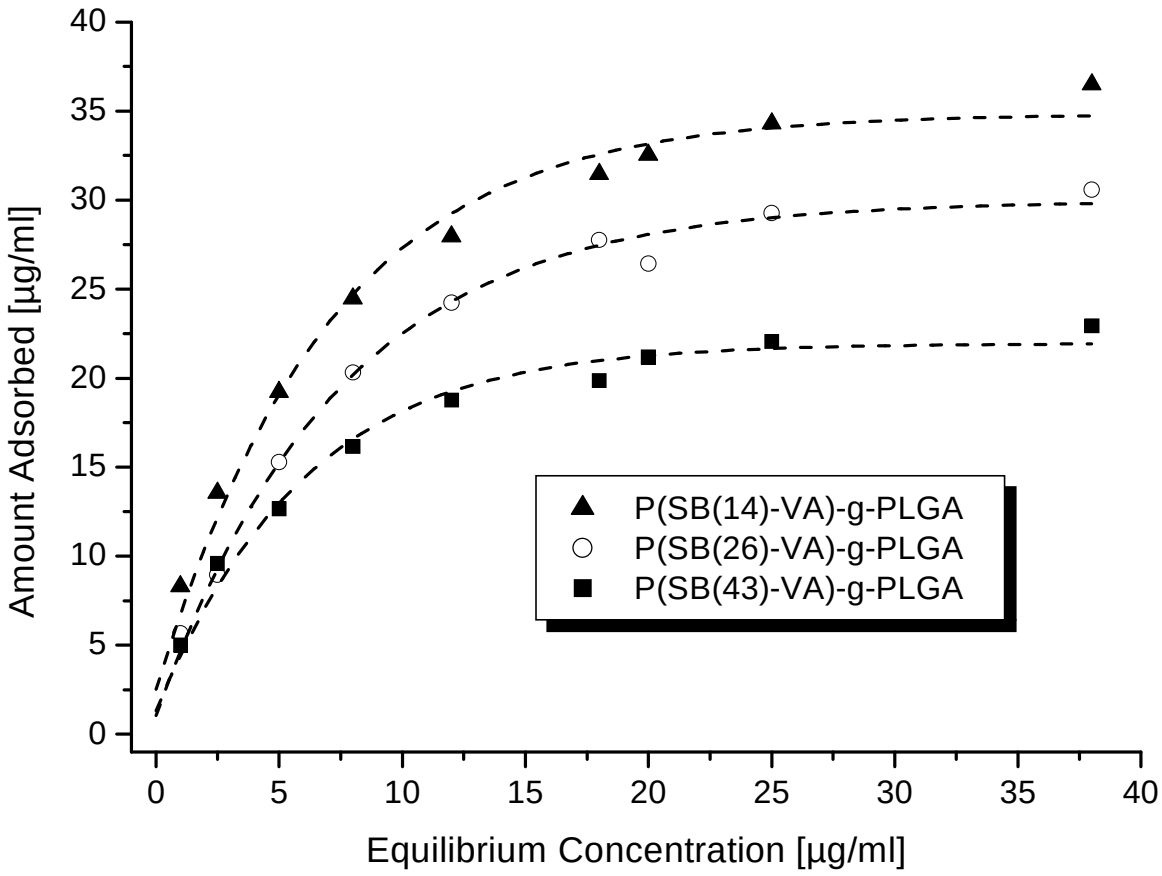


Figure 8a: NP hydrophilicity as a function of polymer backbone degree of charge modification (higher values equal to higher hydrophilicity)

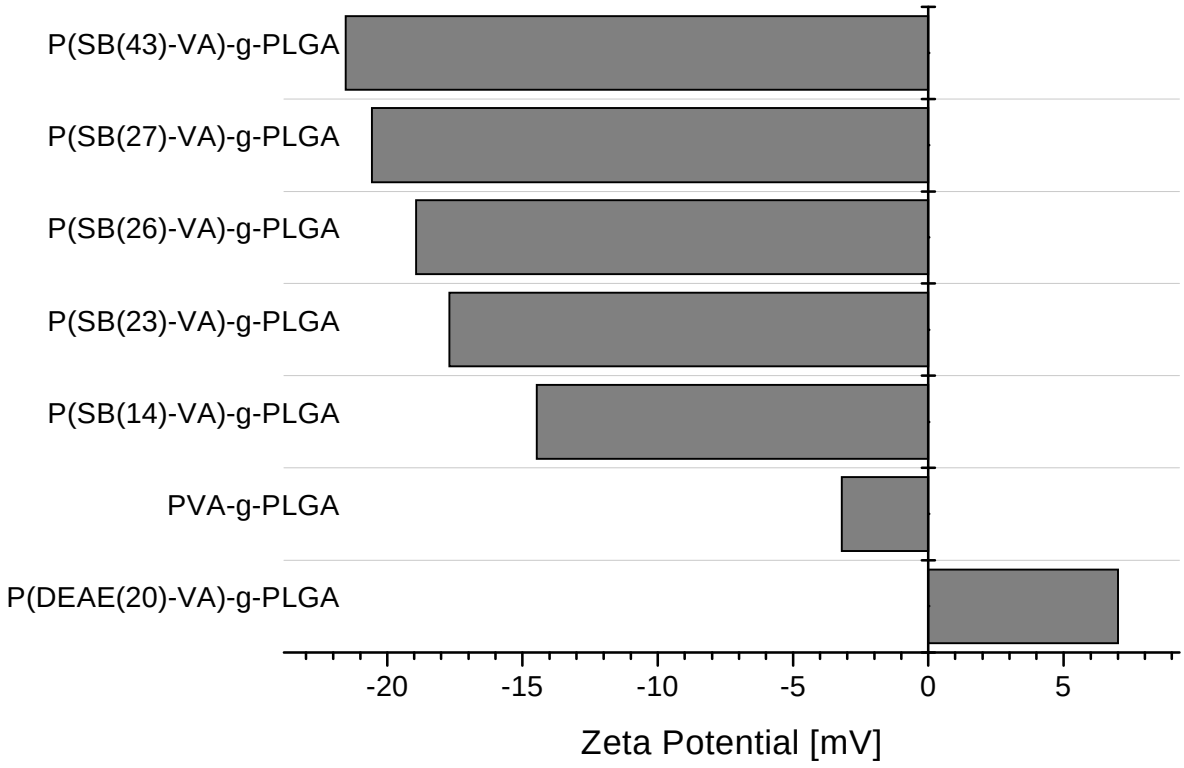


Figure 8b: NP surface charge (zeta potential) as a function of polymer backbone degree of charge modification

Not only the hydrophilicity increased with the use of more amphiphilic and higher charged polymers but also the total charge density of the particle surface. The observed zeta potentials (fig. 8b) were similarly a function of the initial degrees of substitution of the polymer backbones. The zeta potentials ranged from about -14 mV (SB(14)PVA-g-PLGA) to almost -22 mV (SB(43)PVA-g-PLGA), while only -12 mV were observed for carboxylic terminated PLGA NPs under the chosen conditions. In case of P(DEAE(20)-VA)-g-PLGA a positive zeta potential was observed. These results indicate for one, that the surfactant was adsorbed to the nanoparticle surface to some extent, compared to the zeta potential observed for the particles prepared without the use of Pluronic PF68. But, although all types of NPs had comparable sizes and size distributions, they exhibited different zeta potentials at identical concentrations, indicating that the amount of charged groups on the NP surface followed the degree of charge

modification of the polyester backbones. These results are in accordance with the proposed core-corona like NP structure and clearly demonstrate the beneficial utility of these novel carrier polymers. In contrast to linear PLGA these novel polymers allow an adjustment of NP surface properties.

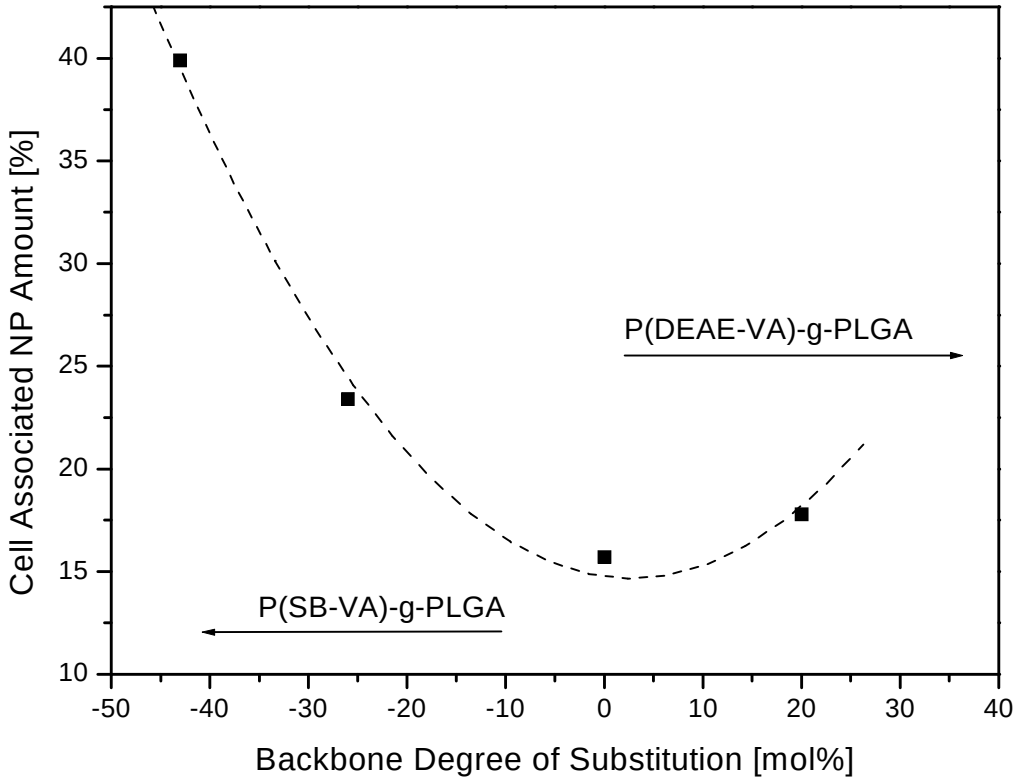


Figure 8c: NP Bioadhesion vs. NP properties

Recently, we reported the interaction of these nanoparticles with caco-2 cell monolayers [28,29], which are an often chosen in vitro tool for assessment of intestinal resorption [5]. In fig. 8c the NP cell association (cell mediated surface adhesion and intracellular uptake) after 120 minutes incubation of 0.76 µg NP per mm^2 cell growth area and intensive washing [29] are plotted against the polymer backbone degree of charge modification. The observed bioadhesion markedly depended on the type of polymer used in particle preparation. The higher the charge density of the polymer backbone the obviously higher the cell associated NP amount. A similar 2nd order relation was obtained if bioadhesion

is plotted against NP surface charge (zeta potential), confirming the influence of the charged groups in the NP surface structure as well as in bioadhesion.

The complete results on NP preparation, the influence of the charged backbones on adsorptive protein loading, bioadhesion in cell culture as well as initial in vivo experiments will be reported in the next parts of this investigation.

7.5 Conclusions

Combining a three-dimensional architecture, increased hydrophilicity and charged groups in a single PLGA polymer resulted in biomaterials with promising properties, especially for the preparation of colloidal drug carrier systems.

It was possible to manipulate the hydrophilic-hydrophobic balance of these polyesters by factors such as molecular weight, PLGA chain lengths and number as well as type and degree of charge modification. Acetone solubility and charge-modified backbones enabled the preparation of small NP with narrow size distributions without any mechanic emulsification procedures and even without the use of surfactants by nanoprecipitation.

Especially in case of the NP prepared from negatively charged polyesters a core-corona like NP structure with inner polyester core and an outer hydrophilic, charged groups containing coating is proposed. The possibility to tailor the NP surface characteristics by choice and degree of charge modification of the polyester backbone will be a major advantage in adsorptive drug loading and bioadhesive force. Therefore, this polyester class may be of particular interest for the preparation and formulation of colloidal mucosal carrier systems.

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7.7 Outlook: Biodegradation

Amongst the use of these polyesters with charged backbones for the preparation of nanoparticulate carriers, as described in the last chapter, biodegradability might also facilitate the preparation of parenteral drug delivery systems by means of microspheres or implants with interesting properties. Initial results (for preparation and analytical methods compare chapter 4) on in vitro polymer film degradation confirmed, that the charged backbone facilitates and accelerates hydrolytic cleavage of ester bonds compared to polymers with an uncharged PVA backbone, as outlined in figure o1.

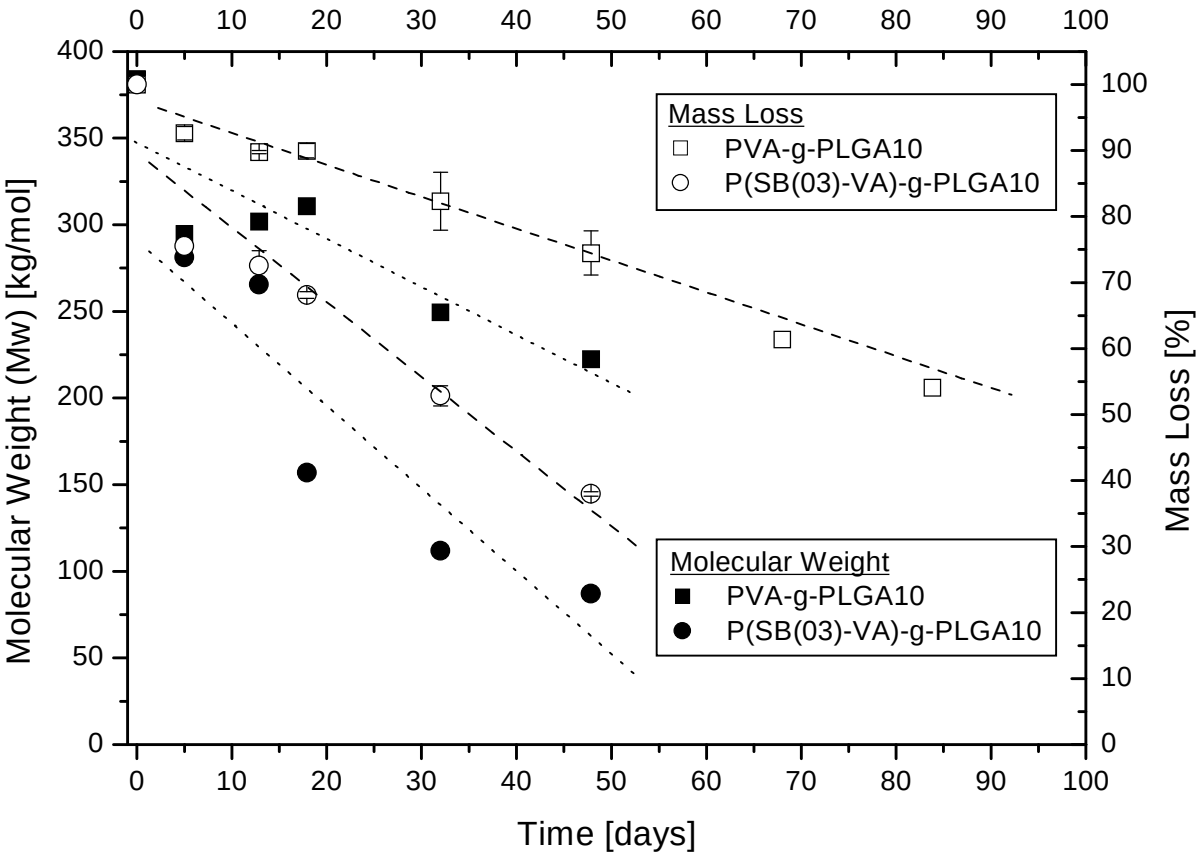

Figure o1: Degradation and mass loss of polymer films in PBS 7.4 prepared from negatively charged and uncharged comb PLGA

In contrast to linear PLGA (compare chapter 4) no initial lag phase of mass loss (erosion) was observed and the polymer chain cleavage (degradation) occurred almost at a constant rate. Therefore, it can be assumed that no inside-out bulk

erosion mechanism will occur, which in case of linear PLGA leads to the undesired lag phase in drug release. A significantly accelerated initial water penetration rate of the charge containing polyester is depicted in figure o2. The increased backbone hydrophilicity facilitates faster hydration and, therefore, promotes first polyester cleavage steps.

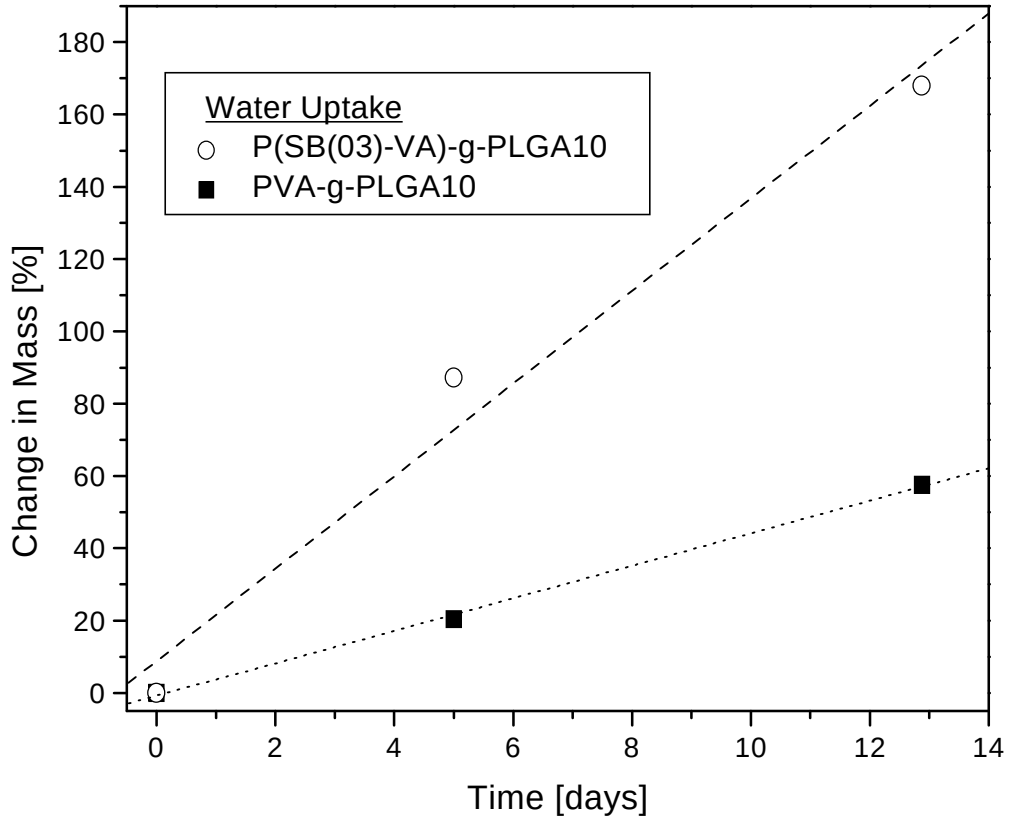

Figure o2: Water uptake of negatively charged and uncharged comb PLGA

As shown in figure o3 glass transition temperatures (Tg) remained nearly unchanged during degradation for at least 8 weeks. The shortness of the PLGA chains grafted onto the backbone leads to immediate chain water-solubility when cleaved. Therefore, they do not lead to internal plasticizer effects in the matrix, since they are washed out. In combination with the high number of connected chains to a single backbone, matrix stability is preserved for a longer time.

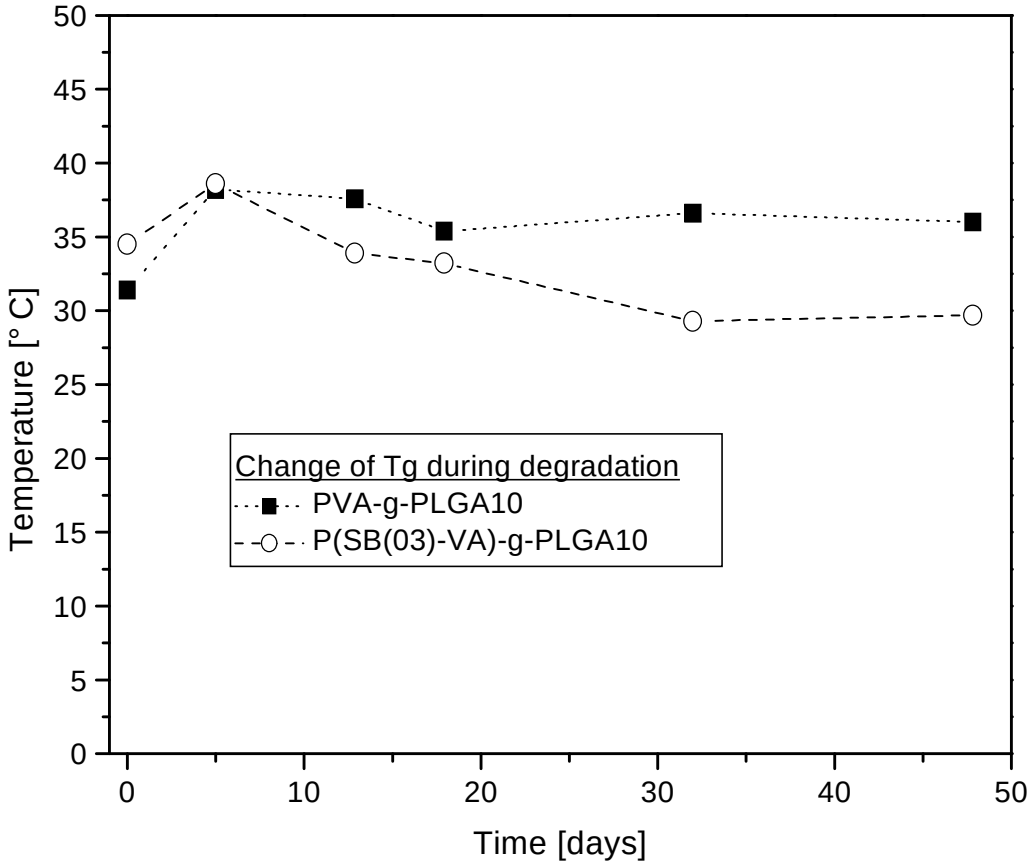

Figure o3: Tg changes (dry samples) during degradation

The already promising properties of PVA-g-PLGA, described in chapter 3-5, could be further improved by the introduction of charge into the polymer backbone.

Since comparable but faster degradation and erosion rates could be obtained with negatively charged PLGA compared to their uncharged counterparts, this class of polymers could be equally interesting for the preparation of PDS and their potential should be investigated in the future.

Chapter 8:

Self-assembling colloidal carriers for drug delivery: complexes of proteins with water-soluble polyesters obtained by brush-like grafting poly(lactic-co-glycolic acid) onto polyelectrolyte backbones

8.1 ABSTRACT

A self-assembling, polymer-based delivery system for proteins was obtained from water-soluble comb polyesters and a number of model proteins. The polyesters were prepared by bulk melt grafting of poly(lactic-co-glycolic acid), PLGA, chains onto poly(vinyl alcohol), PVA, or negatively charged poly(2-sulfobutyl-vinyl alcohol), P(SB-VA). Adjustment of the PLGA chain lengths by feed composition allowed to modify polymer properties, such as molecular weight and solubility. While polyesters with on average 5 PLGA units per chain showed good acetone solubility a further chain lengths reduction yielded water-soluble polymers. In aqueous solution a lower critical solution temperature was observed for these polyesters which equally could be manipulated by PLGA chain lengths.

Spontaneous formation of colloidal polymer-protein conjugates with a variety of proteins was observed. Sizes, ranging from ca. 100 nm to several µm, and 'drug loadings' of up to 200% (w/w) could be systematically manipulated by factors, such as pH, temperature and type of polymer. Complex formation was fully reversible enabling the preparation of drug release systems. Results on increased bioadhesion in Caco-2 cell culture as well as on successful oral in vivo vaccination in mice using tetanus toxoid-polymer conjugates suggest, that these polymers might be potent candidates for colloidal protein delivery.

8.2 INTRODUCTION

Recombinant DNA technology provided a wide variety of proteins, peptides and oligonucleotides, which may be of considerable therapeutic relevance [1,2]. Parenteral delivery of such hydrophilic macromolecules as drugs necessitates polymeric delivery systems, such as microspheres, implants and nanospheres [3]. Drug release rates and biodegradation need to be carefully controlled to provide save and efficacious devices for chronic therapy.

The protection of biologically active proteins against denaturation and enzymatic degradation is an important issue for all drug delivery systems. Possible strategies based on polymeric carriers for oral and parenteral delivery of proteins include: a) modification of biologically active compounds with polymers, b) encapsulation of the hydrophilic macromolecules into micro- or nanospheres and c) adsorptive drug loading onto the surface of nanospheres [4].

Covalent modification of proteins with polymers, e.g. by 'pegylation', can be used for altering and controlling their pharmacokinetics, biodistribution and often toxicity [5]. Especially anionic polymer-drug conjugates, parenterally applied, have shown persistently higher plasma levels, even gradually accumulating in peripheral tumors, whereas cationic ones are trapped by the liver and blood vessels and rapidly cleared from circulation [6]. There are two limitations of the polymer conjugation strategy: For one, the protein must contain suitable functional groups for derivatization. Secondly, modification of those groups may lead to a decrease or even loss of biological activity.

Problems associated with micro- and nanoencapsulation of proteins are for one general preparation parameters, e.g. the use of toxic emulgators in emulsion or dispersion polymerization [7] or the application of high shear forces during

emulsification processes [8], secondly polymeric issues, such as insufficient biocompatibility and biodegradability, balance of hydrophilic and hydrophobic moieties, etc. leading to insufficient drug release.

Especially in oral drug delivery very small lipophilic poly(styrene) nanospheres (NP) seem to allow mucosal particle absorption [9]. It was reported that negatively charged NP prepared from sebacid and fumaric acid copolymers [10] as well as anionic liposomes [11] showed substantial intestinal uptake. A promising strategy might be the use of polymeric carriers combining all properties described above, namely biodegradability and the possibility to engineer the NP surface by manipulation of the balance of hydrophilic and hydrophobic domains as well as surface charges. The ideal technological method would manage without emulsion or shear forces and, therefore, would be rather based on self-assembly. Such macromolecular self-assembling systems have attracted increasing attention as carriers for drug delivery [12-15].

Recently, we have utilized nanospheres with designed surface structure prepared from biodegradable comb polyesters, consisting of poly(lactide-co-glycolide) brush-like grafted onto charged or uncharged poly(vinyl alcohol) backbones for oral and nasal vaccination [16,17]. Here we report the preparation of water-soluble comb polyesters [18] and their potential use for protein delivery systems.

8.3 MATERIALS AND METHODS

Materials

Poly(vinyl alcohol) (PVA, Mw: 15'000 g/mol, degree of hydrolysis: 88 %) was obtained from Fluka, rigorously dried at 80°C in vacuo until constant weight was obtained and stored in a desiccator under vacuum at room temperature over P_2O_5. D,L-lactide and glycolide (Boehringer Ingelheim, S-grade) were

recrystallized twice from dry ethyl acetate (refluxed over calcium hydride) and dried for 48 hours in vacuo directly before use. The melting points were 125-126°C and 82-83°C, respectively. Stannous octoate (Aldrich), 1,4-butanesultone (purum, Fluka), and all other materials were of analytical grade and used as received. Sodium hydride (Merck-Schuchard) was purified by three times extraction with pentane. DMSO (99.5 %, Riedel-de Haën) was dried over calcium hydride (Riedel-de Haën) and distilled under reduced pressure directly before use. Fluorescently labeled bovine serum albumin (FITC-BSA) and cytochrom C (CytC) were purchased from Sigma, tetanus toxoid (Ttx) and human serum albumin (HSA) were kindly provided from Chiron-Behring. Dextran sulfate sodium (DSS) and diethyaminoethyldextran (DEAED) were purchased from Sigma.

Polymer Syntheses [16,18]

Polyelectrolyte Backbones. Poly(2-sulfobutyl-vinyl alcohol), P(SB-VA), was prepared from PVA under anhydrous conditions according Williamson in a dry nitrogen atmosphere, as reported recently [16,18]. Briefly, PVA was activated with the carbanion of DMSO, obtained by its reaction with sodium hydride, and etherified with 1,4-butanesultone at room temperature.

Ultrafiltration was performed four times on each sample (initial concentration: 200 mg polymer in 10 ml water) with an Amicon ultrafiltration stirring cell 8010 equipped with a YM1 filter membrane (Amicon, cut off = 1000 g/mol).

Sulfur Analysis of ultrafiltrated P(SB-VA)s was performed by Schoeniger method.

Polyesters. Biodegradable comb polyesters were prepared by bulk melt grafting poly(lactic-co-glycolic acid) onto the different polyols (PVA, P(SB-VA) [16,18]). In brief, ring-opening polymerization of the lactones, L-lactide or D,L-lactide and glycolide, in the presence of the different core PVAs with stannous

octoate as catalyst was used. Reaction time was 10 minutes at 170°C to achieve sufficient solubility of the polyols in the melt of the lactones, then the reaction was allowed to continue for additional 3 hours at 150°C. Purification of the water-soluble polyesters was performed by ultrafiltration.

Polymer Characterization

Combined Size Exclusion Chromatography (SEC) and Static Light Scattering (SLS): 0.5 % (w/v) polymer solutions were injected into a thermostatted (35 °C) Merck-Hitachi system with a differential refractometer (RI 71) and a MiniDawn light scattering detector [Wyatt Technology Corporation] (100 µl K5 cell, laser wavelength 690 nm, laser power 30 mW, three detecting angles (45°, 90° and 135°)). Chromatograms were obtained with degassed eluents at a flow rate of 1 ml/min. For DCM and acetone a Lichrogel PS mix and a PS 40 (10 µm) column [Merck] were used, for aqueous SEC analysis a column combination Suprema 10µ and Suprema linear 10 µ - 8x300 mm [PSS] was applied.

Nuclear magnetic resonance spectroscopy (NMR) was performed at 35°C with 6 % (w/v) polymer solutions in different fully deuterated solvents (such as d_6-acetone, $CDCl_3$, d_6-DMSO, D_2O). 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR spectra were recorded with a Jeol GX400 Delta N FT spectrometer, 500 MHz ^{1}H- and 125 MHz ^{13}C-NMR spectra with a Jeol LA500 eclipse+ Delta FT spectrometer.

Intrinsic viscosities were determined with an Ubbelohde viscosimeter (Schott Geräte, Germany) from aqueous 0.5 N $NaNO_3$ solutions at 25°C with at least four different concentrations.

Surface Tensions (σ) were analyzed in triplicate with a tensiometer from MGW Lauda according to the DuNoüy ring method with at least 8 different concentrations of aqueous polymer solutions at 25°C. Surface pressure (p) was calculated according to the following equation:

$p(sample) = \sigma(Water)-\sigma(sample)$.

Turbidity measurements as a function of temperature for LCST determination were preformed at different wavelengths with a Shimadzu UV-VIS spectrophotometer UV-160 or with a Zetasizer 4 (AZ110 cell, Malvern Instruments, 630 nm, 90°).

Polymer-Protein Complexes

Preparation (compare table 3). 100 mg polymer were dissolved in 1 ml of an isotonic (0.15 M) phosphate buffer saline solution (PBS) of know pH. In a typical example 100 µl of this polymer stock solution were transferred into a 1500 ml Eppendorf vial and diluted with 800 µl of PBS. Finally 100 µl of a 10 mg/ml protein stock solution in PBS were added and vortexed for 10 seconds. Each experiment was performed in triplicate. In case of uncharged polymers temperature was then raised from room temperature to 37°C for 1 hour.

Drug loading and release (at 37°C) were determined after centrifugation of the complexes in the supernatant photometrically at 280 nm (Ttx) and 491 nm (FITC-BSA) with a Shimadzu UV-VIS spectrophotometer UV-160 in triplicate.

Complexes containing FITC-BSA were optically investigated with a Standard Fluorescent Microscope (Zeiss, Germany) equipped with a Zeiss 490 / 525 nm FITC-filter.

For *visualization of the colloid morphology* freshly prepared complexes were cast on silicon wavers and allowed to dry at room temperature for three days. Then they were investigated with a field emission scanning electron microscope S-4100 (Hitachi) or a SEM CamScan 4 [Elektron-Optik Service GmbH, Germany]]equipped with a Voyager EDX analyzer [Noran Instruments, USA] with an ultra thin window enabling the detection of light elements. Freeze-fracturing samples were applied in the hole of a pair of gold specimen holders, snap-frozen in nitrogen slush (-210°C) and transferred to a freeze-fracturing apparatus (BAF 400 D, Balzer) equipped with an oscillating quartz monitor (QSG 301). The samples were fractured at ca. $5 \cdot 10^{-6}$ mbar and shadowed

immediately with platinum (2 nm, 45°) and carbon (20 nm). The replicas were cleaned in 6 % sodium hypochlorite for 1 hour, washed with water several times and mounted on copper grids. Transmission electron microscopy was performed with a Zeiss EM 10.

Colloid sizes and distributions were measured at 25°C in aqueous dispersion (200 µg/ml) by photon correlation spectroscopy (PCS) with a Zetasizer 4 (AZ110 cell, Malvern Instruments) in triplicate (90°, sample time 120 µs, serial mode, 4 mW laser, 64 channel correlator, multimodal analysis).

Isothermal titration calorimetry (microcalorimetry) was performed with an MCS-ITC equipment from Microcal Inc. (25°C, cell volume 1351.3 µl, stirring syringe 250 µl, 400 rpm, 10 µl injections every 250 sec.) and data processing with the software Origin 3.5 [Microcal]. Polymer solutions of known concentration were titrated at different solution pH with protein solutions of known concentration (compare table 3). All experiments were corrected by measured values of dilution enthalpy.

Non-reducing SDS PAGE and Native PAGE experiments were carried out with a PhastSystem (separation and development unit, LKB Pharmacia). Protein separation and gel development was performed with the Pharmacia methods ‘separation’ and ‘development technique files’. For protein separation 1 µl of the sample was applied onto foil supported poly(acryl amid) gels (PhastGel gradient 8-25, SDS-PAGE: PhastGel SDS buffer strips, Native PAGE: PhastGel Native buffer strips, Pharmacia). For calibration a high and low molecular weight kit for electrophoresis (Pharmacia) were used. After electrophoresis staining was performed with Coomassie Blue (PhastBlue R, Pharmacia), residual dye was removed by washing with a mixture of methanol : acetic acid : water (3 : 1 : 6), then gels were fixated.

For s*tatistical design and analysis* (factorial screening design with 3 center-points) the software STATGRAPHICS Plus for Windows 2.1 (Statistical Graphics Corp., Rockville, U.S.A.) was used.

Cell Culture [19]

Caco-2 cells were cultivated and seeded onto poly(carbonate) membranes (Costar, Transwell Cat. 3412) according to [19]. Cells were used for experiments between day 20 and 22 postseeding. Monolayers grown on permeable filter inserts were preincubated in HBSS for 30 min and 1.5 ml of the appropriate complex dispersion containing 2 mmol TRH as a permeability marker were applied to the luminal and 2.6 ml HBSS to the basolateral side. After predetermined time intervals up to 120 min 1 ml samples were withdrawn from the serosal compartment and the volume was replaced with fresh buffer.

a) After incubation with BSA-complexes monolayers were rigorously washed three times with HBSS, fixed with Formalin-HBSS (4 %) for 30 min, embedded in glycerol gelatin and imaged by confocal laser scanning microscopy (CLSM) for bioadhesion. The CLSM equipment consisted of a Zeiss Axiovert 100 microscope with Zeiss Neofluar 40x, 63x NA 1.3 oil objectives, a confocal laser scanning imaging system, and a helium Neon laser. The laser / filter settings (Exc/Emm) were BP 488/ LP 515- 525.

b) Cells after HSA-complex exposition were washed 3 times with buffer, fixed in 3% paraformaldehyde / 0.1% glutaraldehyde in PBS (pH 7.4) for 2 hours at room temperature and embedded in Lowicryl K4M (Plano, Wetzlar, Germany). Ultrathin sections were immunolabeled using polyclonal rabbit anti-human albumin antibodies, rinsed rigorously and incubated with an anti-rabbit- IgG, conjugated with 15 nm gold particles. After washing steps again samples were contrasted with uranyl acetate as well as lead citrate and examined with a Zeiss EM 10 electron microscope.

The integrity of the cell monolayer was determined at the beginning and at the end of all experiments by measuring the transepithelial electrical resistance (TEER) using an Endohm (WPI, Germany).

Animal studies

Animal studies were preformed according to [17]. Female Balb/c mice, 7-9 weeks of age, weighting 16-22 g, were obtained from Harlan-Winkelmann (Germany). 3 groups of mice were used in this experiment. Complex dispersions were compared to conventional alum-adsorbed as well as to free tetanus toxoid (Ttx). Mice were randomized, pooled into groups of 10 animals and immunized on three consecutive weeks (day 1, 8, 15) by peroral (p.o.) application of 200 µl 5LF Ttx containing colloidal polyelectrolyte complexes. The i.p. inoculations with 200 µl of Tetanol® were performed as a positive control. Animals were bled at week 0 and 4. All sera were assayed in duplicate for Ttx specific IgG and IgA antibody responses using an ELISA technique. Serial dilutions of sera were incubated on TTF 6 coated microtiter plate wells. Ttx specific antibodies were quantitated by incubating wells with heavy chain-specific peroxidase conjugated goat anti mouse IgG or IgA. Responses were quantitated by measurement of optical densities (OD) at 450 nm following incubation of wells with TMB. The results were expressed as reciprocal end-point sera titers representing the highest sera dilutions giving OD values of 0.2.

Table 1: Physico-chemical properties of the comb polyesters

No	Polymer	PVA DS [a] [%]/[mass%]	Backbone Mw [a] [g/mol]	PLGA chain Mn [b] [g/mol]	PLGA Units per Chain [b]	Polymer Mn [b] [g/mol]	LA:GA [b] [mol%]	Best Solvent
1	PVA-g-PLGA	-	15'000	4000	32	1'250'000	51 : 49	DCM
2	PVA-g-PLGA	-	15'000	1100	9	360'000	51 : 49	1:1 (DCM : acetone)
3	PVA-g-PLGA	-	15'000	590	5	238'000	50 : 50	acetone
4	PVA-g-PLGA	-	15'000	390	3	134'000	50 : 50	acetone
5	PVA-g-PLGA	-	15'000	(50)*	(0.8)*	(30'000)*	(50 : 50)*	water
6	PVA-g-PLGA	-	15'000	(37)*	(0.4)*	(26'000)*	(50 : 50)*	water
7	PVA-g-PLGA	-	15'000	(30)*	(0.3)*	(24'000)*	(50 : 50)*	water
8	PVA-g-PLGA	-	15'000	(21)*	(0.2)*	(21'000)*	(50 : 50)*	water
9	P(SB-VA)-g-PLGA	14 / S=6.8	19'900	590	5	172'000	53 : 47	acetone
10	P(SB-VA)-g-PLGA	14 / S=6.8	19'900	(50)*	(0,4)*	(33'000)*	(50 : 50)*	water
11	P(SB-VA)-g-PLGA	27 / S=10.0	26'000	840	7	210'000	52 : 48	acetone
12	P(SB-VA)-g-PLGA	27 / S=10.0	26'000	(120)*	(2)*	(52'000)*	(50 : 50)*	1:1 (water : acetone)
13	P(SB-VA)-g-PLGA	27 / S=10.0	26'000	(60)*	(0.5)*	(35'000)*	(50 : 50)*	water
14	P(SB-VA)-g-PLGA	43 / S=12.3	33'600	1100	9	221'000	53 : 47	acetone
15	P(SB-VA)-g-PLGA	43 / S=12.3	33'600	(80)*	(0.6)*	(35'000)*	(50 : 50)*	water

a) = from elemental analysis b) = from NMR analysis * = calculated from synthesis feeds since NMR signals were too broad for quantification

8.4 RESULTS AND DISCUSSION

The polymers used in this study (table 1) are characterized by a three-dimensional comb architecture and were obtained by brush-like grafting short poly(lactic-co-glycolic acid) (PLGA) chains onto hydrophilic macromolecular backbones [16,18]. They were prepared by bulk melt polymerization of the lactones, lactide and glycolide, in the presence of different polyols. Their hydroxyl groups effectively initiate this type of ring opening polymerization, as reported recently [20]. The backbone polyols used are for one an unmodified 15'000 g/mol poly(vinyl alcohol) (PVA) and secondly PVAs bearing negative charges. The charged groups were introduced by coupling 15'000 g/mol PVA with 1,4-butanesultone [16,18].

We have investigated structure modified PLGA for several years [16-23], since these polymers offer additional options to manipulate their balance of hydrophobic and hydrophilic domains. Especially variation of the PLGA side chain lengths grafted onto the backbone polyols allows to adjust properties, such as molecular weight (Mw), degree of crystallinity, glass transition temperature (Tg) and solubility [20]. The best solvent for polymers with a PLGA chain lengths in the range of ca. 10 to 30 repeating units was found to be dichloromethane. A reduction to on average 5 to 10 units increased polymer amphiphilicity and resulted in acetone solubility. Polymers with even shorter PLGA chains become water-soluble.

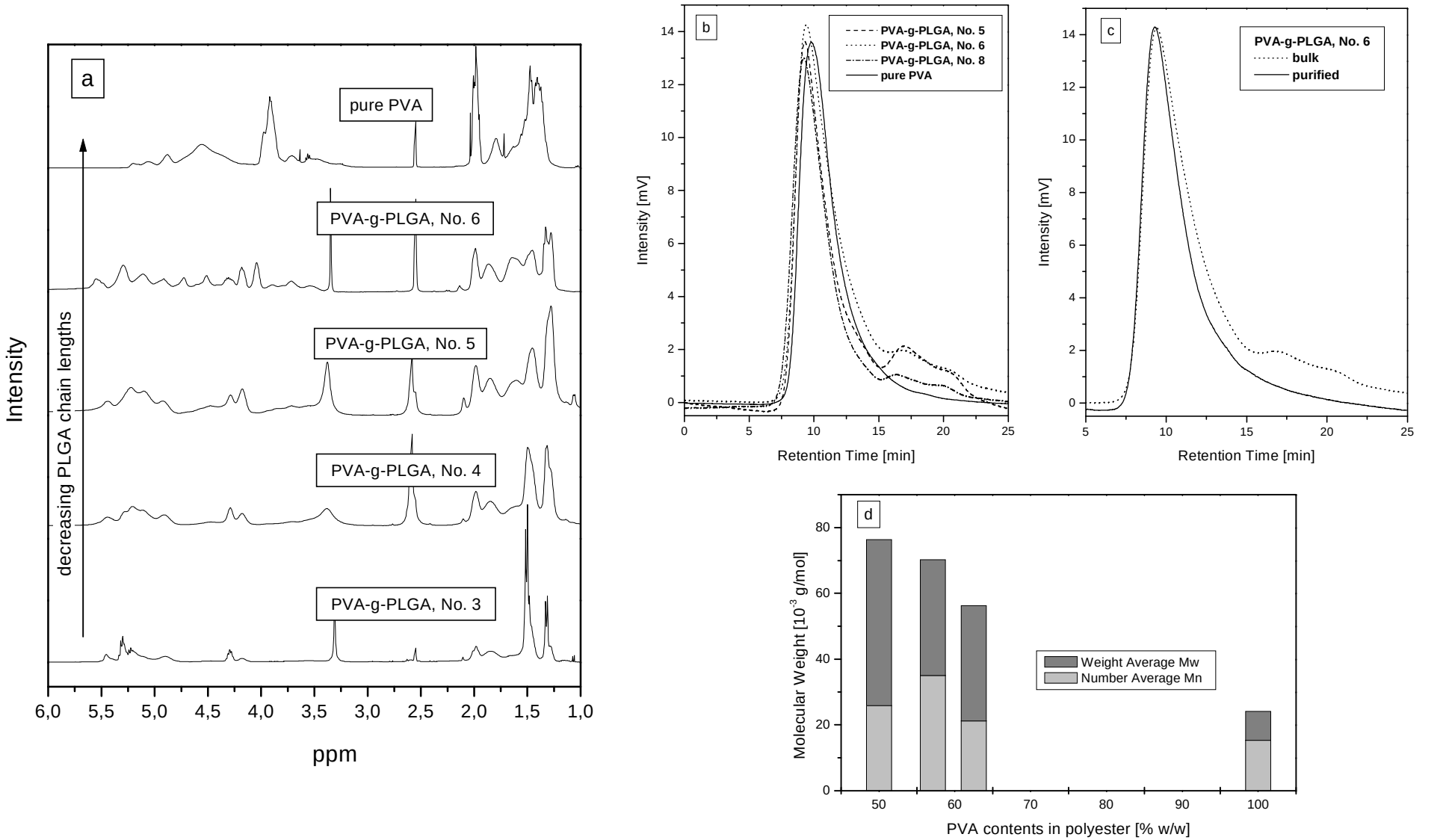

Figure 1: a) polymer ^{1}H NMR spectra as a function of PLGA chain lengths; SEC analysis of b) bulk polymers and c) after ultrafiltration, d) absolute Mw as a function of polymer composition

As outlined in fig. 1a, the reduction of the side chain lengths is manifested by an intensity decrease of the PLGA chain ^{1}H NMR signals (1.45 ppm = lactic acid methyl groups, 4.8 ppm = glycolic acid methylene groups and 5.16 ppm = lactic acid methine groups) and an accordingly intensity increase of the hydroxyl terminated PLGA end groups (4.2 ppm = terminal **-CH_2**OH, 4.35 ppm = terminal **-CH**(CH_3)OH and 1.28 ppm = terminal -CH(**CH_3**)OH). The PVA signals were found at 1.9 ppm = OCO-**CH_3**, 1.7 ppm = **CH**-OCO, and in the region of 1.3 to 1.5 ppm = **CH_2**. By comparison of the signal intensities of the PLGA chain and end groups the average PLGA chain lengths could be calculated.

The influence of the grafting reaction on polymer molecular weights can be seen in their SEC traces (fig. 1b) indicating that the water-soluble polymers all exhibited comparable Mw. Since reaction temperatures were relatively high (170°C/150°C) a small amount of by-products and/or monomers was visible at retention times of ca. 16 to 22 minutes. Their successful removement by ultrafiltration of the aqueous polymer solutions through a 1000 g/mol cut-off membrane is demonstrated in fig. 1c.

SEC analysis is not the method of choice to determine molecular weights of comb polymers, since it underestimates the Mw due to smaller polymer hydrodynamic volume in solution compared to linear reference material [20]. Neither application of 3rd order calibration with pullulan nor PVA standards yielded reasonable values. Therefore, some selected polymers were analyzed by a combination of SEC and Static Light Scattering (SLS), to characterize their effective molecular weights. The values ranging from several hundred thousand [20] g/mol to significantly below are listed in table 2, confirming, that the Mw directly followed synthesis feeds, as outlined in fig. 1d, the more polyol present, the lower the polymer Mw.

It was not possible to find a suitable column/eluent combination for SEC-SLS analysis of the charged polymers, which strongly interacted with the SEC columns. Therefore, intrinsic viscosities determined from aqueous 0.5M $NaNO_3$ solutions were used as qualitative measure, indicating that the negative polymers used in this study exhibited molecular weights quite comparable to the uncharged polymers in the range below 100'000 g/mol (table 2).

Table 2: Light scattering analysis and intrinsic viscosities

No*	**Polymer**	**Mol. weight Mn** [a)] [g/mol]	**Intr. Viscosity** [dl/g]
1	PVA-g-PLGA01	1'563'000	0.61 [b)]
3	PVA-g-PLGA10	182'800	0.17 [c)]
4	PVA-g-PLGA30	140'400	0.11 [c)]
5	PVA-g-PLGA50	25'900	-
6	PVA-g-PLGA57.1	35'040	0.14 [d)]
7	PVA-g-PLGA62.5	21'210	-
9	P(SB(14)-VA)-g-PLGA10	-	0.20 [c)]
10	P(SB(14)-VA)-g-PLGA50	-	0.14 [d)]

* numbers referring to table 1

a) from combined SEC and static light scattering analysis

b) DCM as solvent c) acetone as solvent d) aqueous 0.5 $NaNo_3$ solution

Not only good or bad water-solubility, but a much more distinct solution behavior was observed. A lower critical solution temperature (LCST), polymer precipitation at higher temperatures, was found, already known for the polymer backbone PVA. As outlined in fig. 2a, where turbidity of the polymer solutions is plotted against temperature, LCST (as turning point of the slopes) was also a linear function of the PLGA chain lengths, just as all other physicochemical properties.

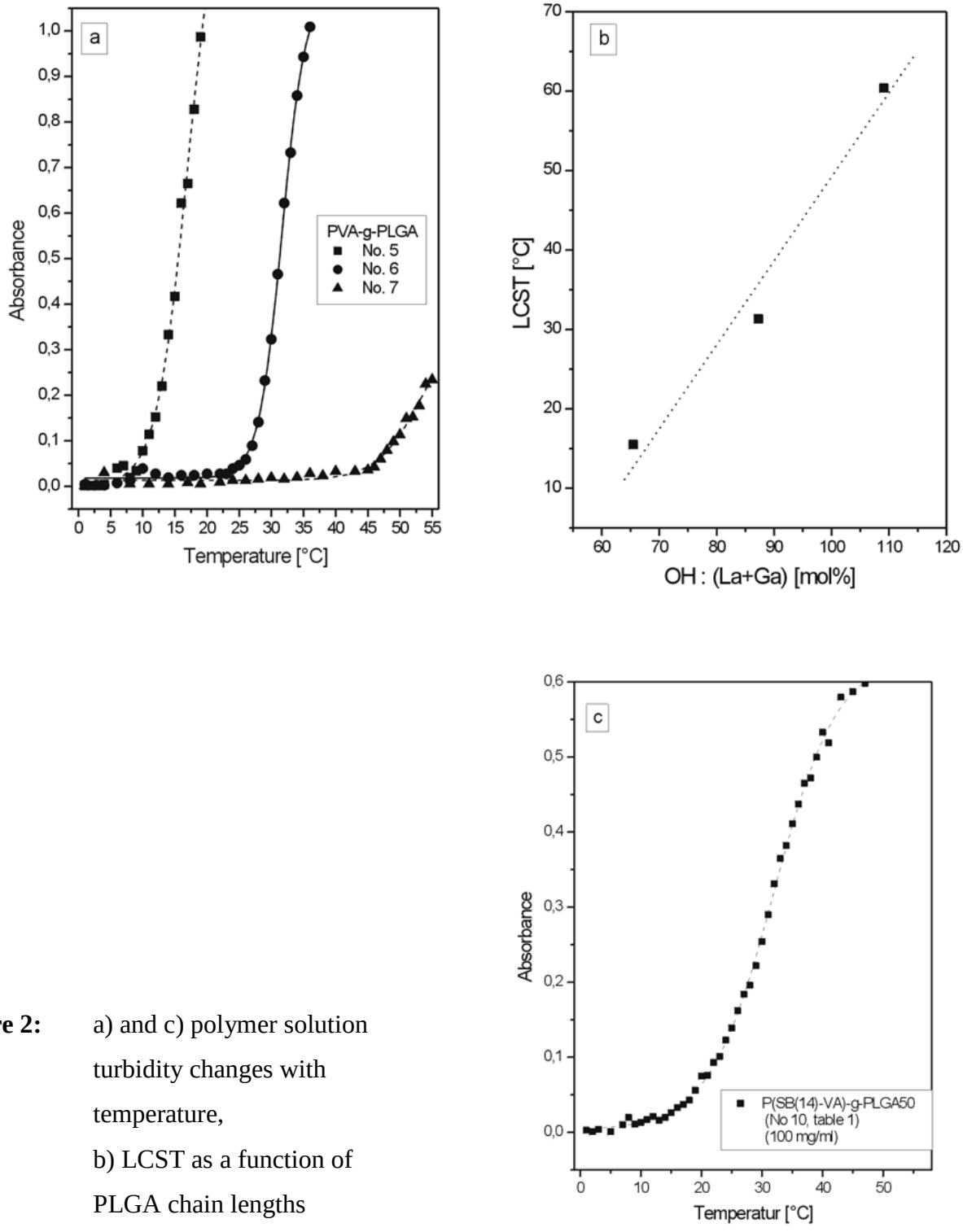

Figure 2: a) and c) polymer solution turbidity changes with temperature,
b) LCST as a function of PLGA chain lengths

The shorter the side chains, the more polyol like the polymers and, therefore, the better their solubility, causing higher precipitation temperatures (fig. 2b). Nevertheless, these temperatures were in a quite attractive range near physiological conditions. At high polymer concentrations temperature-induced precipitation was even visible for polymers with an anionic backbone (fig. 2c), although they generally exhibited better solubility owing to the charged groups, which raised LCST to high values. The existence of a LCST offered a first novel possibility to a prepare hydrogel type of protein delivery system with these polyesters: Combining a protein and a polymer solution, then raising the temperature above LCST, e.g. by parenteral application, resulted in polymer aggregation and precipitation partially including protein molecules.

While in case of the uncharged polymers larger inclusion-like aggregates with e.g. fluorescently labeled bovine serum albumin (FITC-BSA) using the temperature switch were observed (compare fig. 3a), the situation changed, when a negatively charged polymer and a protein solution were combined. In this case temperature independent spontaneous self-assembly of much smaller complexes occurred, an example is given in fig. 3b. In both experiments small dot-like fluorescence accumulation was visual, while in case of a pure protein solution as control only weak indifferent fluorescence occurred. These observations could be confirmed by various high-resolution microscopic techniques, such as field emission scanning electron (fig 3c,d) and freeze fracture transmission electron microscopy (fig. 3e). The anionic polymers yielded very small colloids with sizes of only a few hundred nm (fig. 3d,e), while larger precipitates in the µm-range were found in case of the uncharged polymers (fig. 3d).

Table 3

Experiment	Polymer concentration	Protein concentration	Preparation Buffer / Volume	Figure
Visualization	PVA-g-PLGA57.1 (No 6, tab. 1) P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	FITC-BSA - 1 mg/ml	PBS 3, 1000 µl	3a 3b
	PVA-g-PLGA57.1 (No 6, tab. 1) SB(14)PVA-g-PLG_50 (No 10, tab. 1) 10 mg/ml	CytC - 1 mg/ml	PBS 3, 1000 µl	3c 3d
	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	BSA - 1 mg/ml	PBS 3, 1000 µl	3e
Complexation	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	DSS - 1 mg/ml DEAED - 1 mg/ml	PBS 3, PBS 7.4, PBS 9 1000 µl	5a,b
	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	BSA - 1 mg/ml	PBS 3, PBS 7.4, PBS 9 + 0.25M NaNO3 + 0.50M NaNO3 + 1.00M NaNO3 1000 µl	5c-f
Size	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1)	CytC, compare figure	PBS 3, PBS 5, 500 µl	6a
Drug loading	compare figure 10 mg/ml	BSA, 0.66 mg/ml	PBS 4, 1000 µl	6b
	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	BSA, compare figure	PBS 3, 1000 µl	6c
Microcalorimetry	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml 0.5 mg/ml, 1.25 mg/ml 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml	CytC - 10 mg/ml BSA - 10 mg/ml tetanus toxoid - 16.6 mg/ml	PBS 3 (PBS 5) PBS 3 + 0.25M NaNO3 PBS 3 + 0.50M NaNO3	7
	PVA-g-PLGA57.1 (No 6, tab. 1) 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml	BSA - 10 mg/ml		
Drug loading and release	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) 10 mg/ml	FITC-BSA - 1 mg/ml	Preparation in PBS 3 / 1000 µl Release in PBS 3, PBS 6, PBS 7.4	8a,b
	P(SB(14)-VA)-g-PLGA50 (No 10, tab. 1) PVA-g-PLGA50 (No 5, tab. 1) PVA-g-PLGA62.5 (No 7, tab. 1) 10 mg/ml	tetanus toxoid - 2 mg/ml	Preparation in PBS 4 / 1000 µl Release in PBS 6	8c

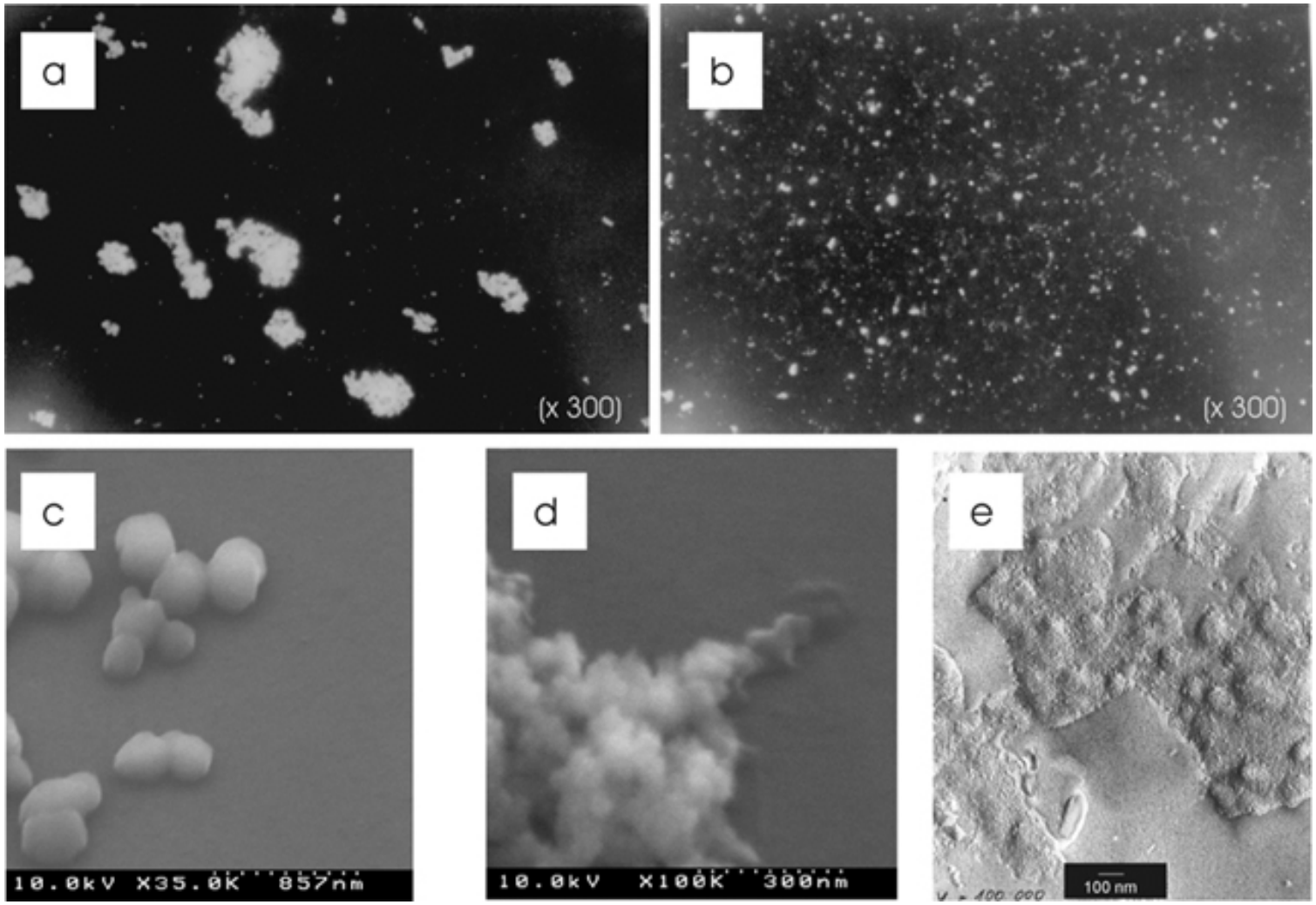

Figure 3: Complex morphology a) uncharged polymer + FITC-BSA, b) anionic polymer + FITC-BSA, c) uncharged polymer + CytC, d) anionic polymer + CytC, e) anionic polymer + BSA [a)+b) standard fluorescence micrograph, c)+d) field emission scanning electron micrograph, e) freeze fracture transmission electron micrograph]

The complex composition was investigated after purification and isolation by several centrifugation and washing steps. The presence of the polymer could be easily detected by ^{1}H NMR analysis after complex redissolving in deuterated DMSO (data not shown). The presence of the protein, in this case iron containing cytochrome C (CytC), was confirmed with an electron microscope equipped with energy dispersive x-ray microanalysis (EDX) successfully allowing the detection of the protein iron atom in the complexes at ca. 6.25 to 6.5 keV. An example is given in fig. 4.

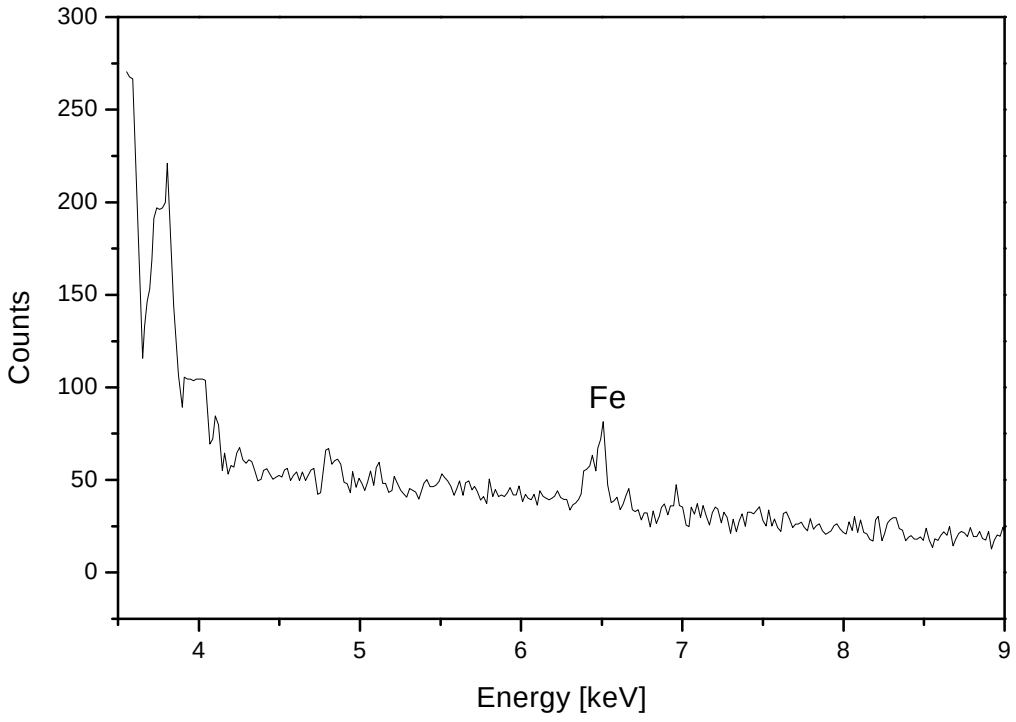

Figure 4: Energy Dispersive X-Ray analysis of CytC-Polymer complexes

To prove the complexation character of the spontaneous self-assembly seen with the anionic polymers, their reaction with a positively and a negatively charged dextran, diethylaminoethyl-dextran (DEAED) and dextran sulfate sodium (DSS), was investigated as a function of solution pH by dynamic laser light scattering (DLS). As outlined in fig. 5a the two negative partners did not interact in any way.

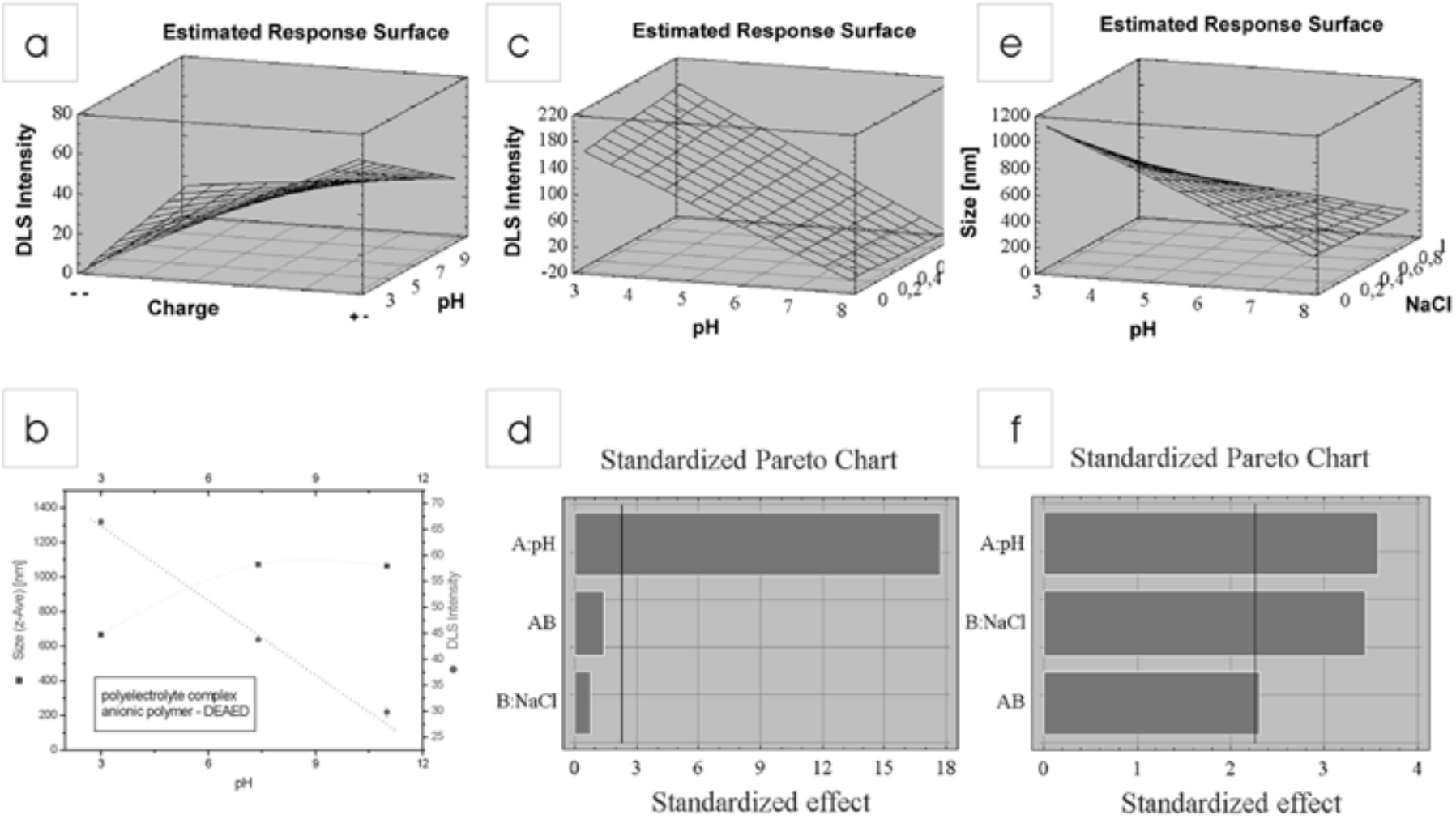

Figure 5: Complexation of charged dextrans with an anionic polymer a) DLS intensity and b) sizes as a function of solution pH, effect of solution pH and ionic strength on the complexation of BSA with an anionic polymer: c)+d) DLS intensity and e)+f) sizes

The solutions stayed clear and showed neither Tyndall effect nor turbidity at all. In contrast, a distinct increase of DLS intensity indicated the spontaneous complex formation between the anionic polymer and the cationic poly(saccharide). Moreover, this reaction was strongest at pH 3, linearly decreasing with increasing pH values, indicating the role of the charged groups in the polymer backbone, which become saturated and neutralized at higher pH.

Applied to a protein in solution a pH above its point of isocharge (PI) will cause a negative overall charge, and similar to the anionic dextran no complexation should occur. At a pH below its PI it should be able to interact with the negative polymer due to its positive charge. The sulfonic acid group of the polymer is a very strong acid and will be dissociated over a broader pH range, enabling the complexation of a wide variety of proteins with different PI values. In fig. 5c/d a factorial experimental design is showing this expected influence of solution pH and ionic strength (amount of additional NaCl) on the complexation reaction of BSA and an anionic polymer at a constant polymer to protein ratio. The DLS intensity, as an indirect measure of number (and sizes) of the formed polyelectrolyte complexes between polymer and protein, decreased with increasing pH value. The strongest reaction was seen at the lowest pH investigated (pH 3), where the polymer is negatively and the protein positively charged. The intensity was significantly decreasing at a pH near the PI of the protein, where its overall charge is zero, further decreasing with increasing solution pH. At ca. pH 6-7 no reaction was observable.

These results demonstrate one of the major advantages: if applied parenterally, the negative charge of the polymer and moderate pH in the range of physiological conditions will prevent complexation of serum albumin being anionic itself.

While the ionic strengths of the buffers used had no major influence on the DLS intensity - the pure complex formation - a distinct dependency on the amount of additional NaCl was found for the sizes of the self-assembled colloids (fig 5e/f). Especially at low pH up to values near the PI of BSA this effect was strongest. The sizes were decreasing with increasing ionic strengths, which can be explained by the high affinity of small ions to the charged groups of the polymer as well as the protein. At the examined ratio of polymer to protein (10 : 1 w/w), an excess of polymer, the overall charge of the complexes seemed to be not completely saturated. This could lead to secondary aggregation between the complexes themselves [11] and, thereby, cause larger aggregate sizes. This effect of course will occur to a less degree, if the charges are masked by small counterions to some extent. Drug loading was equally a linear function of solution pH, while no influence of the ionic strength of the solution was observed. The highest loadings were achieved under acidic conditions linearly decreasing with increasing pH, reflecting the results on complex formation and sizes. Investigation of the complex stability by supplementary addition of sodium chloride to increase the solution ionic strengths after complex formation revealed no major changes. Neither colloid sizes nor DLS intensity were influenced, except for the dilution effect, indicating the very strong bonding between the complex partners. Being able to control the number and sizes as well as, therefore, the drug loading of the complexes by factors, such as solution pH and ionic strengths, will be another advantage in controlled drug delivery. It is well known that especially small particles are most effective in e.g. mucosal (oral, nasal) application [10].

If an excess amount of polymer was used the polymer to protein ratio had no significant influence on the colloid sizes. As outlined in fig. 6a ratio-independent sizes in the range of 200 to 300 nm were observed for CytC complexes, if solution pH was kept clearly below the proteins PI. Moreover,

under these conditions it was always possible to achieve effective drug loadings in the range of 90 to 100% of the protein amount, as demonstrated in fig. 6b. These results gave first evidence for a stoichiometric reaction, which could be confirmed by the yields, determined after centrifugation, washing and drying (compare fig. 6c). Yields were linearly increasing the more protein present. Even with the inclusion-like complexes prepared from uncharged polymers above their LCST in the range of 1 to 20% (mass protein/polymer) 80% of the protein were coprecipitated in these hydrogels (fig. 6b).

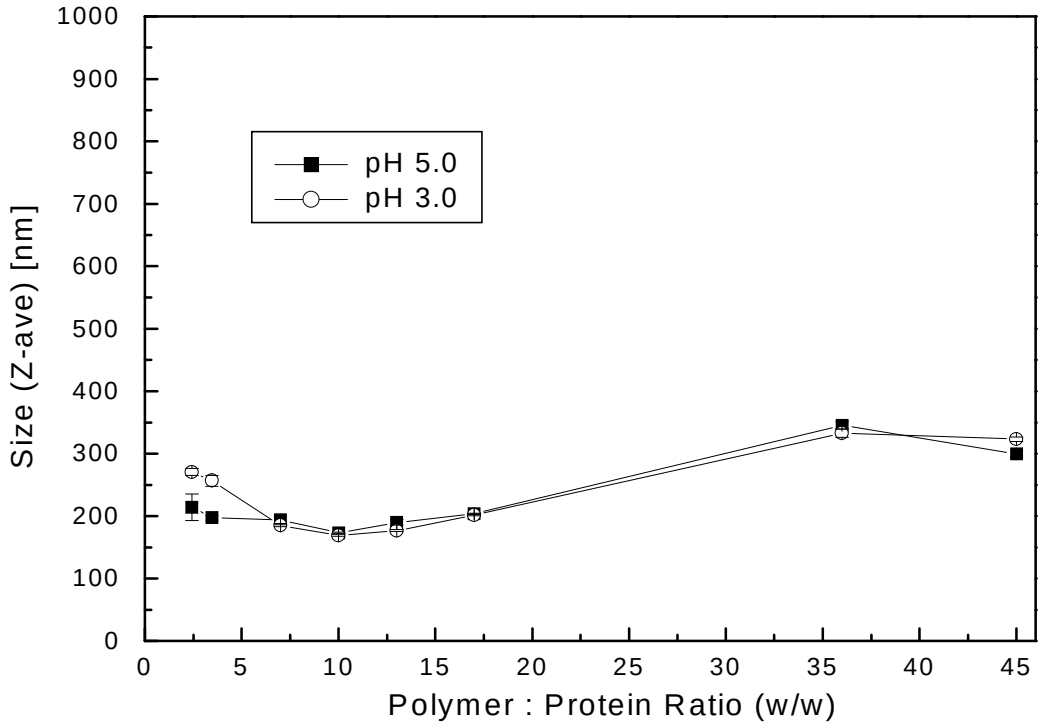

Figure 6a: Influence of polymer to protein ratio on the complexation of CytC

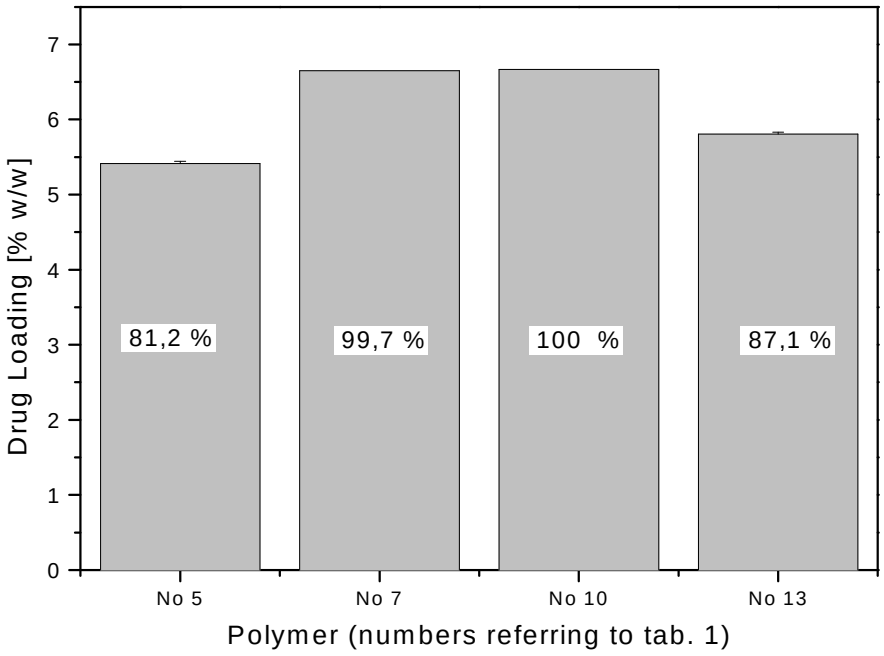

Figure 6b: BSA loading as a function of polymer type,

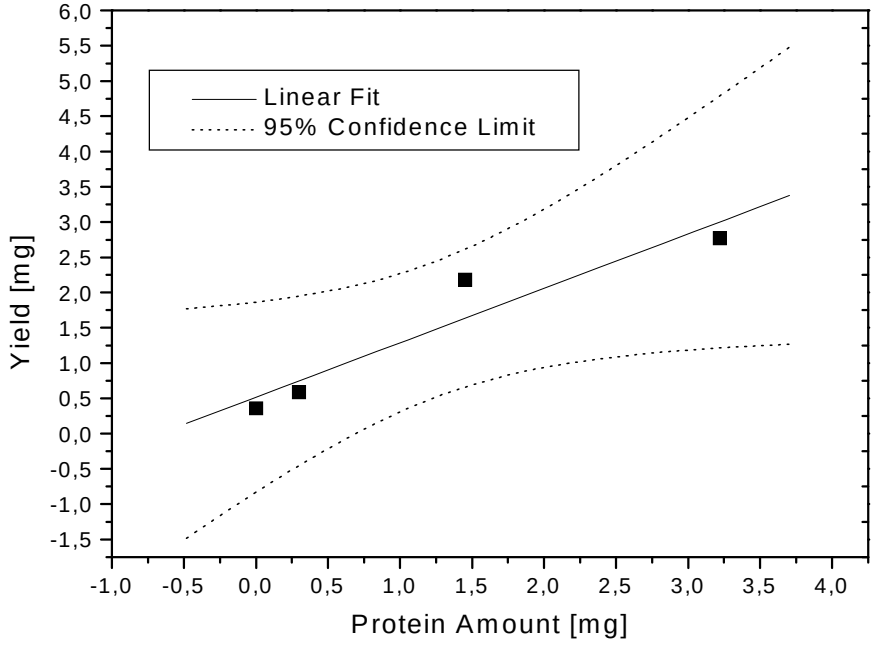

Figure 6c: Yields of BSA complexes as a function of initial amounts

To further investigate the nature of the reaction between the polymers and the proteins, isothermal titration calorimetry (ITC) was performed. Polymer solutions of known concentration and pH were degassed and titrated with protein solutions under stirring at constant temperature. The resulting heat changes after each injection were determined against a water filled reference cell. Titration of the uncharged polymers with proteins revealed no major heat changes except dilution effects, indicating that at constant temperature below polymer LCST nearly no reaction occurred. Similar results were reported for PVA and alkylated PVA hydrogels, investigated by monolayer method [24]. In case of the negative polymers strong heat changes during titrations were observed, due to the complexation reaction. The heat changes associated with the complexation were endothermic and decreased monotonically upon successive protein injections as outlined in fig. 7a. After about 10 injections the heats became the same as those observed for the dilution of the protein in pure buffer. Fig. 7b gives a typical example of the obtained plot of the energy changes derived from the titration experiments after subtracting the heat of dissolution of the protein in buffer. These plots allowed the determination of the heat of complexation (first plateau of the slopes) and the stoichiometry of the reaction (turning point of the slopes). These results are in reasonable agreement with literature data e.g. the adsorption of albumin onto negatively charged surfaces [25-28]. The complexation is driven by a large increase in entropy, which can be explained by the release of a high number of water molecules, dehydration of the macromolecules during contact formation. From a series of experiments with different proteins a dependency of the complex composition and the size/molecular weight of the protein was found (fig 7c), already postulated from the drug loading results described above. It is worth noting, that the mean values and standard deviations in this plot are not derived by repeating a single experiment for several times, but by series of experiments using different concentrations of the two complex partners.

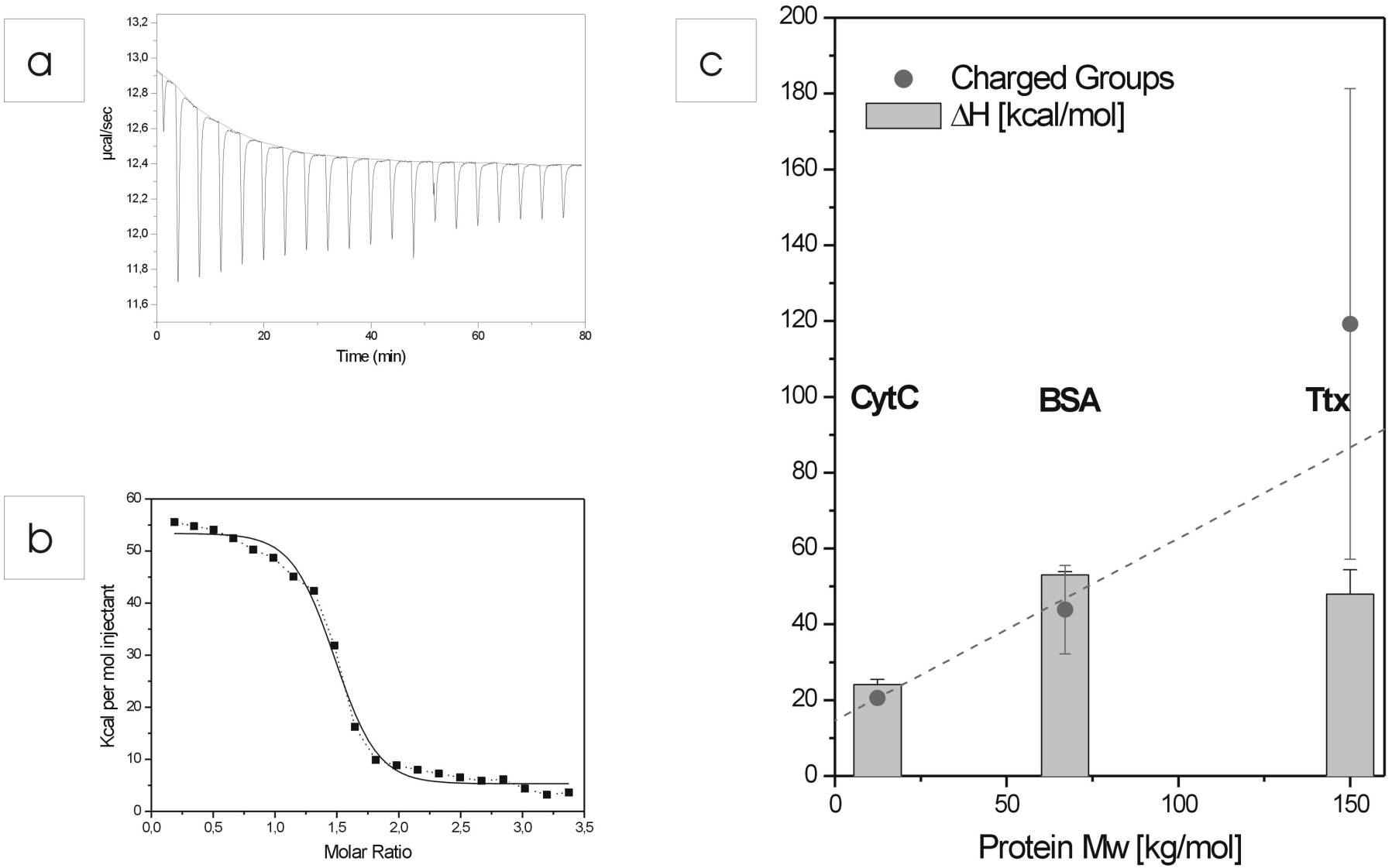

Figure 7: Isothermal titration calorimetry of P(SB-VA)-g-PLGA (No. 10, tab. 1) with different proteins

In case of 12'000 g/mol CytC two protein molecules seem to share one polymer molecule, which in the plot expressed by the amount of charged groups present in the backbone. One polymer contains ca. 42 sulfobutyl groups, two molecules ca. 84 etc. For the complexation of a single CytC molecule ca. 20 of the 42 charged groups of one polymer seemed to be necessary. A 1 : 1 reaction was found for BSA and a 2 : 1 reaction for tetanus toxoid (Ttx) with an approximate Mw of ca. 150'000 g/mol. In other words, a maximum drug loading of 50% (w/w) for Ttx could be achieved, for BSA 100% (w/w) and even 200% (w/w) in case of CytC, demonstrating the potential of this type of drug delivery system, especially compared to microspheres, which normally enable drug loadings in the range of about 1 to 10%.

Drug Release and Stability. To investigate the influence of pH on the complex stability and protein decomplexation, drug release, a series of FITC-BSA complexes was prepared at pH 3 and purified by three centrifugation/washing cycles. The purified samples were then immersed in buffer solutions of different pH (3, 6, 7.4) at 37°C. At pH 3 negligible protein release was observed. As outlined in fig. 8a the complexes once prepared remain stable at least for several days. Raising pH to 6 resulted in a slow but continuous release of the complexed protein. A further increase of pH to physiological conditions (PBS 7.4) caused a fast and nearly linear release of the complete protein amount in less than 24 hours.

The released proteins were investigated after centrifugation in the supernatant with regard to their stability by native and SDS poly(acryl amide) gel electrophoresis (PAGE). Compared to the original protein they were still in a non-aggregated, native form as demonstrated in fig. 8b. The complex formation obviously was fully reversible, the presence of the polymers successfully

inhibited protein self-aggregation and denaturation even under very acidic conditions and at elevated temperatures.

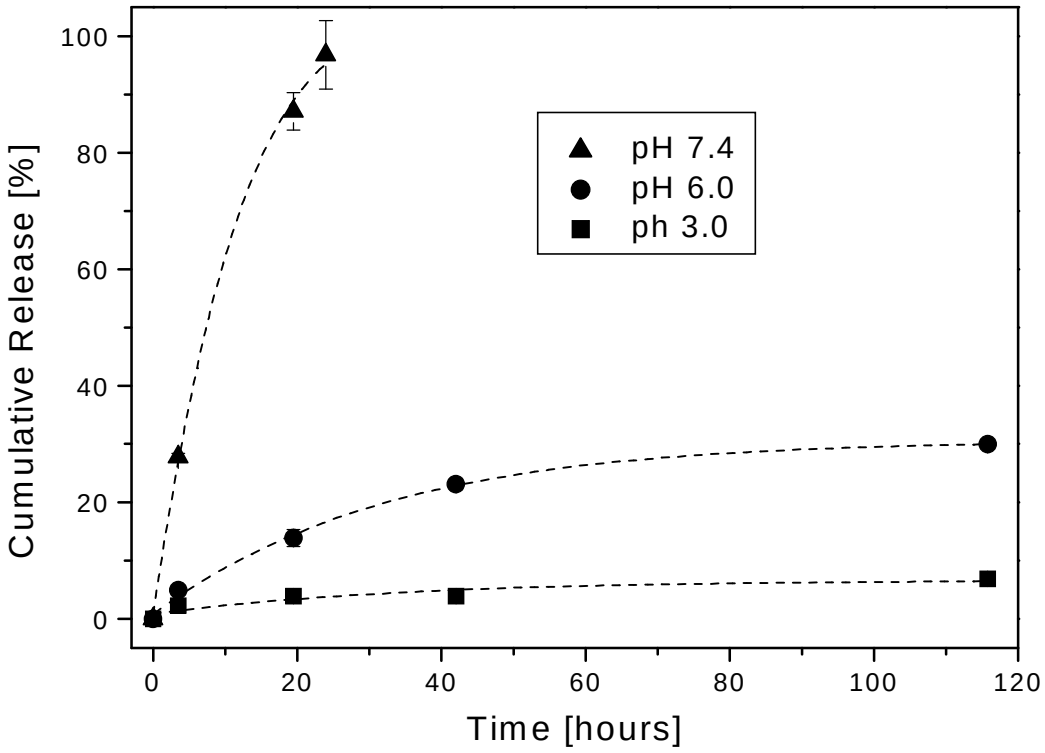

Figure 8a: Release of BSA at different pH values (polymer No. 10, tab. 1)

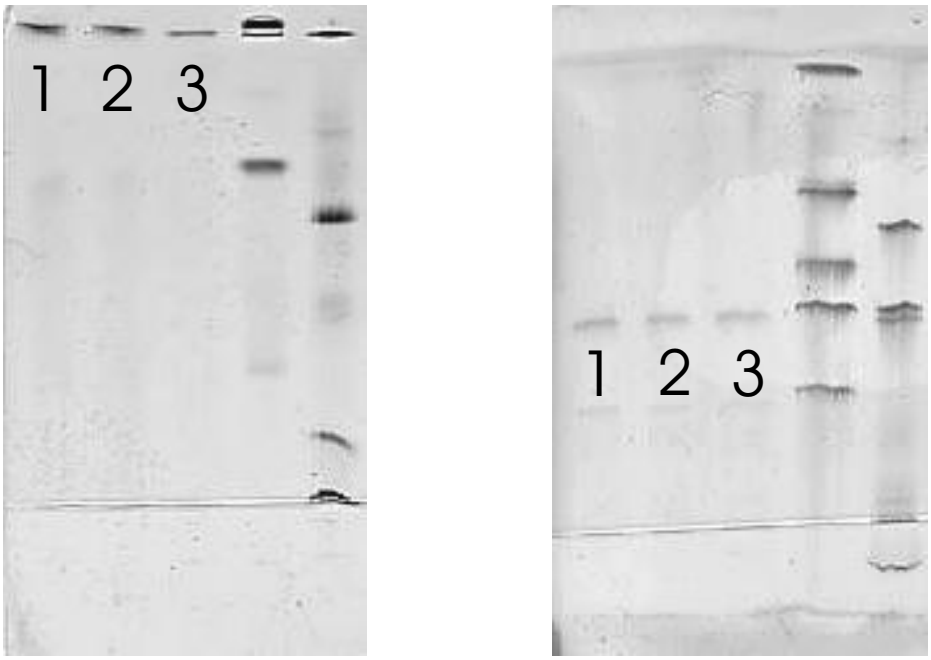

Figure 8b: Protein stability - (1) original BSA, (2) decomplexed BSA at pH 6, (3) decomplexed BSA at pH 7.4 (from release experiment in fig. 8a)

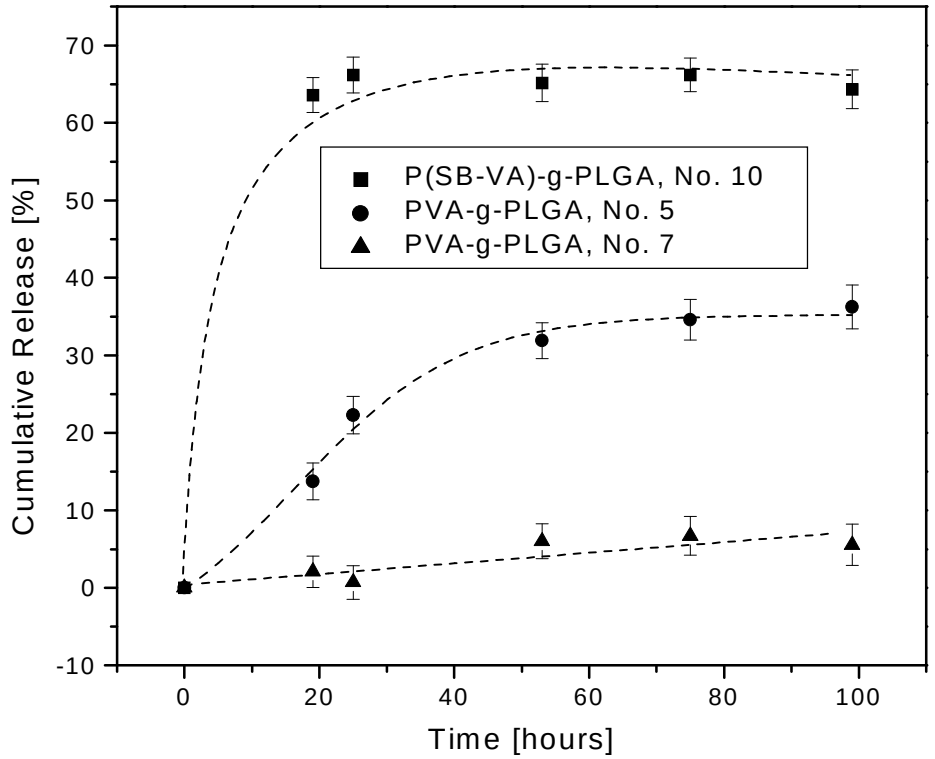

Figure 8c: Release of tetanus toxoid (Ttx) as a function of complex polymer type

Comparable results were obtained with tetanus toxoid containing colloids (fig 8c). No release at acidic pH, significantly accelerated at pH values above the PI of the protein. The release from the inclusion-like complexes with uncharged polymers was found to be pH independent and much slower. It seemed to be mainly an adsorption/desorption equilibrium, controlled by the polymer composition. Higher amounts of the hydrophilic backbone in the polymer (equal to shorter PLGA chains) enabled slightly faster release through the porous hydrated PVA domains, while in case of higher Mw polymers release rates were reduced.

Possible Applications. Bioadhesion might be one useful property of the complexes, since the polymers themselves were found to possess surface activity. Determination of the surface pressure and the CMC as outlined in table 4 showed that the comb polyesters still exhibited values quite comparable to the polyol backbones themselves, which are known for their surfactant-like properties. Therefore, the polyelectrolyte complexes might be a potential tool for mucosal vaccination. Moreover, protein complexation seemed to increase protein stability and, therefore, could possibly alter its bioavailability and biodistribution in parenteral application.

Table 4: Surface activity of the polymers

Backbone	**Surf. Pressure [mN/m]**	**CMC [mmol]**
PVA	27.5	0.89
SB(14)PVA	18.8	0.57
PVA-g-PLGA	27.8	0.42
SB(14)PVA-g-PLGA	28.1	0.58

As for the first concept, bioadhesion was investigated by cell culture incubation experiments [19]. Caco-2 cell monolayers were used as an in vitro tool to assess the binding of these complexes to intestinal epithelium. This well characterized model of the human intestinal mucosa has been widely used to study NP and antigen interactions due to its similarity to absorptive intestinal cells. The cell monolayer was incubated for two hours with an initial concentration of (2000 : 50) µg (polymer : protein)/ml, then washed three times with PBS 7. After fixation it was investigated with CLSM or TEM imaging (fig. 9).

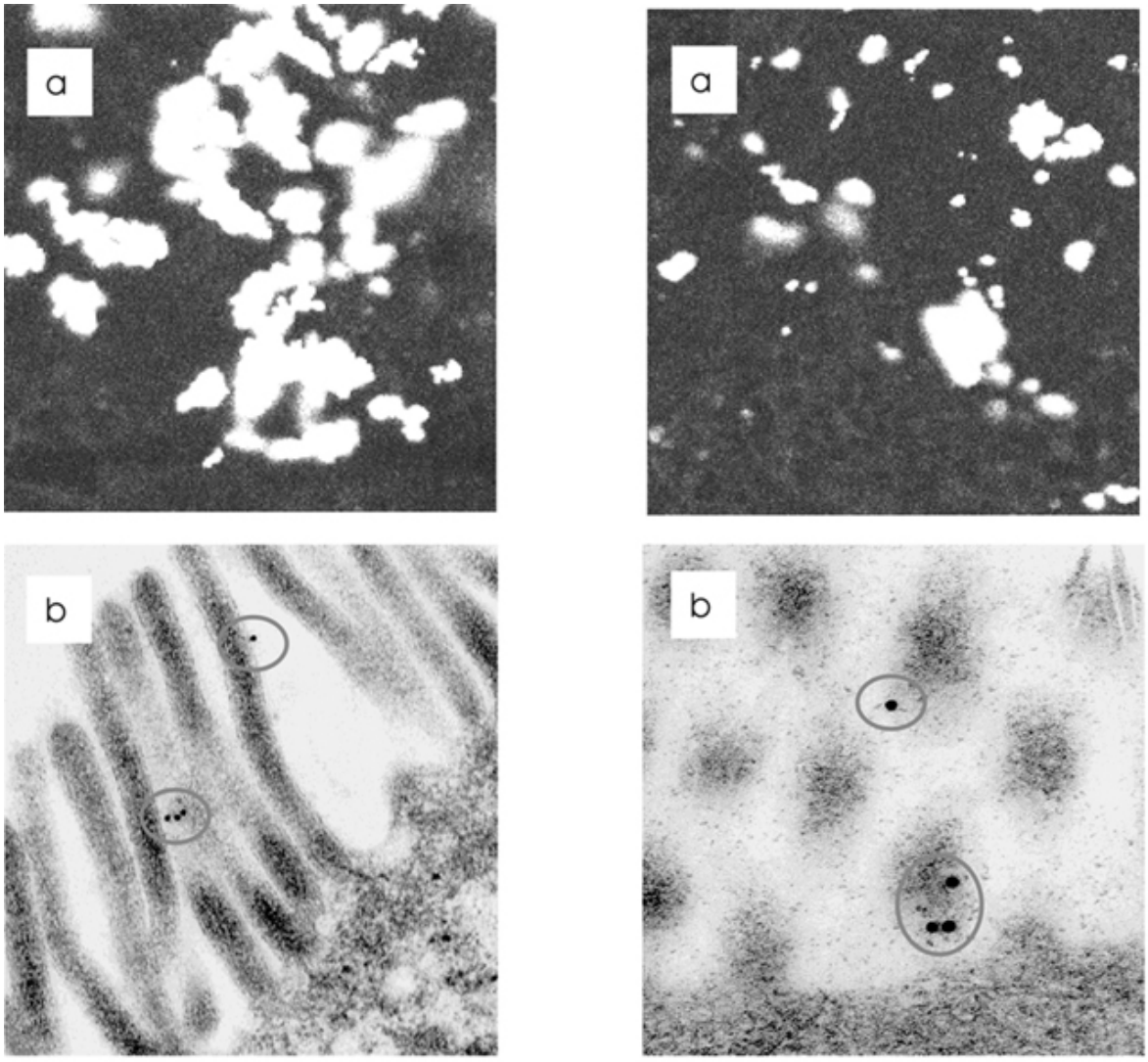

Figure 9: a) Confocal laser scanning micrographs of complex bioadhesion in Caco-2 cell culture (charged polymer-BSA)
b) TEM images of uncharged polymer-HSA complexes in Caco-2 cell culture

Identical experiments were performed with pure protein solutions (50 µg/ml) as negative control. For CLSM imaging the polymer complex counterpart was a fluorescently labeled BSA (FITC-BSA). As can be seen on the CLSM images (fig. 9a), a major increase in cell-associated protein amount was found compared to the control experiment, where no protein could be localized on or in the cells. Similar results were obtained using inclusion-like complexes consisting of an uncharged polymer and human serum albumin (HSA), prepared by LCST precipitation at 37°C. TEM investigations (fig. 9b) revealed that small protein amounts were located at the cell surfaces. It is worth noting, that the incubation

had no negative effect on the caco-2 cell monolayer, the transepithelial electrical resistance (TEER) did not change during these experiments.

The oral performance of these complexes were investigated in mice using tetanus toxoid (Ttx) as model antigen. Three doses of these complexes containing 5 LF of Ttx were orally administered in the course of three weeks (n=10). It is worth noting that the pH value of the stomach was not buffered in the in vivo experiments leaving gastric enzymes active. As can be seen in fig. 10 a significant increase of serum IgA and IgG titers could be achieved.

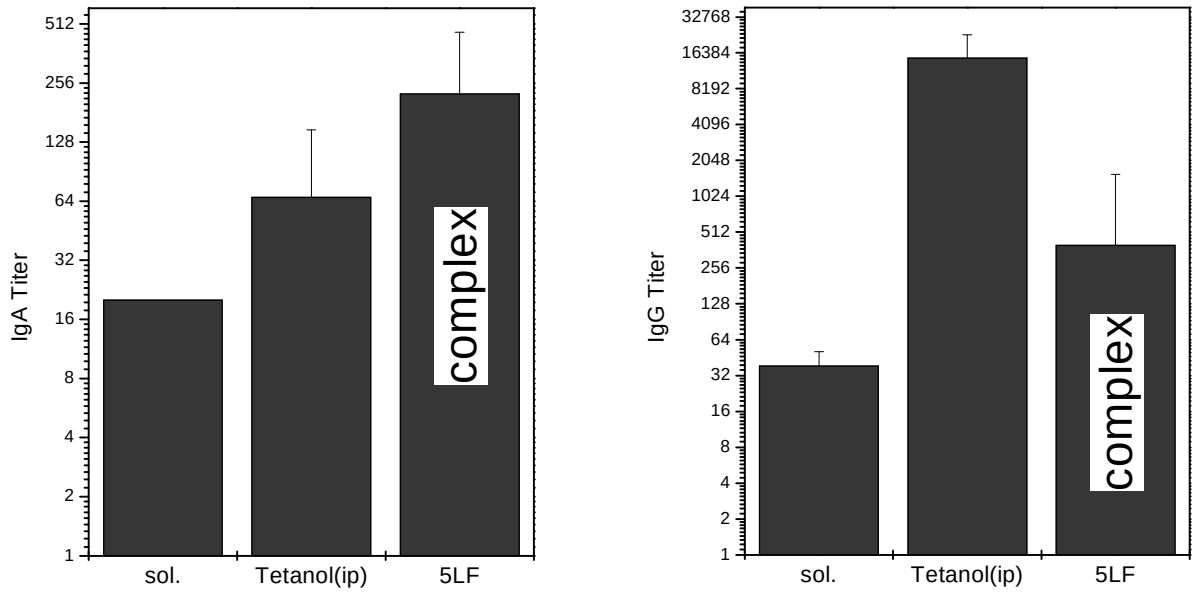

Figure 10: IgG and IgA titers after oral administration of different tetanus toxoid formulations

While no substantial titers were observable after oral administration of a Ttx solution (even under stomach buffering conditions), the well-documented increase especially of IgG after intraperitoneal application of alum adsorbed Ttx (Tetanol) was found. An about 4 to 6 times higher IgA titer was achieved with the colloids compared to Tetanol, indicating their increased mucosal activity in vaccination. Although these preliminary results have to be confirmed by further

experiments, they demonstrate the potential of colloidal vaccination with this novel polymeric carrier system.

8.5 Conclusions

A novel class of water-soluble comb PLGA, charged as well as uncharged, could be developed by reduction of PLGA chain lengths. The maximum chain lengths for water solubility should not exceed 5 PLGA units per chain. The polymers showed stimuli-sensitive properties enabling the formulation of protein drug delivery systems either by temperature trigger or by ionic interaction.

Spontaneous reversible formation of complexes with a number of relevant proteins was observed. In case of polyelectrolyte complexes with charged polymers a suitable candidate could be any protein if processed at a pH below its point of isocharge. Especially basic proteins, such as e.g. cytochrome C, will enable polyelectrolyte complexation at pH values near physiological conditions. Experimental data confirm, that under these conditions an unwanted reaction with plasma proteins such as albumin is unlikely to occur.

While colloid sizes, in the range from ca. 100 nm to several µm, were controllable by adjustment of concentrations, solution pH and ionic strengths, the release rates of the complexed proteins could be equally manipulated by pH value. Increased bioadhesion and initial oral vaccination data in mice indicate the considerable potential of this system in controlled drug delivery.

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List of Abbreviations

LA	lactide
GA	glycolide
PLA	poly(lactic acid) (poly(lactide))
PLGA	poly(lactic-co-glycolic acid) (poly(lactide-co-glycolide))
PVA	poly(vinyl alcohol)
PVB	poly(vinyl benzoate)
P(SB-VA)	poly(2-sulfobutyl-vinyl alcohol)
P(DEAE-VA)	poly(diethylaminoethyl-vinyl alcohol)
PEO	poly(ethylene oxide)
Dex	dextran
DSS	dextran sulfate (sodium salt)
PVA-g-PLA	poly(vinyl alcohol)-graft-poly(lactic acid)
PVA-g-PLGA	poly(vinyl alcohol)-graft-poly(lactic-co-gylcolic acid)
PVB-g-PLA	poly(vinyl benzoate)-graft-poly(lactic acid)
P(SB-VA)-g-PLGA	poly(2-sulfobutyl-vinyl alcohol)-graft-poly(lactic-co-gylcolic acid)
P(DEAE-VA)-g-PLGA	poly(diethylaminoethyl-vinyl alcohol)-graft-poly(lactic-co-gylcolic acid)
PEO-PLA$_n$	n=2: poly(ethylene oxide)-block-poly(lactic acid) n=4;8: poly(ethylene oxide)-star-poly(lactic acid)
PEO-PLGA$_n$	n=2: poly(ethylene oxide)-block-poly(lactic lactic-co-gylcolic acid) n=4;8: poly(ethylene oxide)-star-poly(lactic lactic-co-gylcolic acid)
Dex-PLA	dextran-graft-poly(lactic acid)

Dex-PLGA	dextran-graft-poly(lactic-co-gylcolic acid)
DSS-PLA	dextran sulfate-graft-poly(lactic acid)
DSS-PLGA	dextran sulfate-graft-poly(lactic-co-gylcolic acid)
BSA	bovine serum albumin
HSA	human serum albumin
CytC	cytochrome C
FITC	fluorescein isothiocyanate
Mw	weight average molecular weight
Mn	number average molecular weight
D	polydispersity (= Mw / Mn)
Tg	glass transition temperature
Tm	melting temperature
ΔHm	melt enthalpy
DP	degree of polymerization
DS	degree of substitution
DC	degree of crystallinity
CMC	critical micelle concentration
PI	point of isocharge
SEC	size exclusion chromatograpy (gel permeation chromatography)
SLS	static light scattering
DLS	dynamic light scattering
PCS	photon correlation spectroscopy

NMR	nuclear magnetic resonance
1D	one dimensional
2D	two dimensional
APT	attached proton test
COSY	correlated spectroscopy
ppm	parts per million
FT-IR	fourier transform infrared spectroscopy
DSC	differential scanning calorimetry
DMTA	dynamic mechanical thermal analysis
ITC	isothermal titration calorimetry (microcalorimetry)
SEM	scanning electron microscopy
FE-SEM	field emission scanning electron microscopy
TEM	transmission electron microscopy
CLSM	confocal laser scanning microscopy
EDX	energy dispersive x-ray analyis
WAXS	wide angle x-ray scattering
PAGE	poly(acryl amide) gel electrophoresis
EA	elemental analysis
ASES	aerosol solvent extraction system
MS (MP)	microspheres (microparticles)
NP	nanoparticles
PDS	parenteral depot system
DDS	drug delivery system

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