Bulk- and Surface-Erodable Polymers:

Effects of Polymer Structure on Physico-Chemical and Biological Properties

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Die vorliegende Arbeit entstand

auf Anregung und unter Leitung von

Herrn Prof. Dr. Thomas Kissel

am Institut für Pharmazeutische Technologie und Biopharmazie

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Table of Contents

Chapter 1

Introduction / Objectives

Objectives of this work

In this dissertation, degradation and biocompatibility of biodegradable polymers were investigated with a special emphasis on structure-property relationships.

Several attempts have been made to overcome shortcomings of the widely used PLGA based drug delivery systems such as bulk degradation causing an acidic microenvironment inside the degrading matrix and the difficult to control triphasic release profile. One promising approach are amphiphilic ABA triblock copolymers containing hydrophobic polyester A-blocks and hydrophilic polyether B-blocks. As reviewed in **Chapter 2,** these block co-polyesters are able to form physically cross-linked hydrogels generated by rapid swelling upon exposure to an aqueous environment providing an attractive alternative to chemically cross-linked systems since they allow incorporation of macromolecular drug substances under mild process conditions.

The idea of using co-polymers in drug delivery was enhanced by the generation of comb-like, branched polyesters with a hydrophilic amine-modified PVA backbone and short hydrophobic PLGA side chains. A particular advantage of these branched polyesters is their versatility of structural modifications. We postulated that the physico-chemical properties of these polymers could be custom tailored by controlling (1) the degree of amine-substitution, (2) the PLGA side chain length and (3) the number of PLGA side chains per PVA backbone molecule. To investigate that hypothesis, structural effects upon cytotoxicity *in vitro* were systematically investigated in **Chapter 3.** A systematic evaluation of the influence of the polymer composition on *in vitro* degradation behaviour is reported in **Chapter 4.**

While the yet investigated PLGA-based polymers were all subject to degradation upon hydrolytic cleavage of ester bonds, “biodegradation” can also occur by other mechanisms such as enzymatic or biocatalytic cleavage. Poly(ethylene carbonate) (PEC) has been shown to exhibit an *in vivo* surface degradation mechanism by superoxide anions produced by

adhering polymorphonuclear leucocytes and macrophages. We hypothesized, that the surface degradation-controlled release mechanism of PEC may provide the basis for “on demand” drug eluting stent coatings, releasing an incorporated drug predominantly at an inflamed implantation site upon direct contact with superoxide-releasing macrophages. Consequently, we investigated the feasibility of a PEC-based drug eluting stent and the physico-chemical, in vitro biological and in vitro release properties of PEC in **Chapter 5.**

Chapter 2

ABA-Triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins

Advanced Drug Delivery Reviews 54 (2002) 99-134.

1. Summary

Hydrogels are very attractive delivery systems for hydrophilic macromolecules such as proteins and DNA because they provide a protective environment and allow control of diffusion by adjusting cross-link densities. Physically cross-linked hydrogels generated by rapid swelling upon exposure to an aqueous environment can be obtained from ABA triblock copolymers containing hydrophobic polyester A-blocks and hydrophilic polyether B-blocks. They provide an attractive alternative to chemically cross-linked systems since they allow incorporation of macromolecular drug substances under mild process conditions. Moreover, they show controlled degradation behavior and excellent biocompatibility. In this review the synthesis and characterization of ABA triblock copolymers from polyester hard segments and poly(ethylene oxide), PEO, soft segments as well as their biological and degradation properties will be discussed. Their use as biodegradable drug delivery devices in the form of implants, micro- and nano-spheres has attracted considerable interest especially for proteins and may provide an alternative to poly(lactide-co-glycolide).

2. Synthesis and characterization of ABA triblock copolymers from poly(ethylene oxide) and biodegradable polyesters

2.1. Introduction

Early attempts to use “biomaterials” which allow replacement of diseased or defective limbs date back to the ancient Egyptians and Greeks. Since then the field has rapidly developed and with the advent of synthetic materials numerous applications have been identified [1]. Segmented block copolymers consisting of “hard” polyester A-blocks and “soft” poly(ethylene oxide), PEO, B-blocks have attracted the interest of material scientists because they allow a modification of physical and chemical properties, leading to an accelerated biodegradability. These were considerations that stimulated research into new block copolymers in the late 70’s and block copolymers of PEO and poly(terephthalate) for use in

surgery were the first example for this new concept to our knowledge [2]. Since then numerous block or graft copolymers of PEO and various polyesters were reported in the literature. These different polymers can be classified according to their structure as AB di-block, ABA or BAB tri-block, multi-block, star-block and graft copolymers as shown in figure 1.

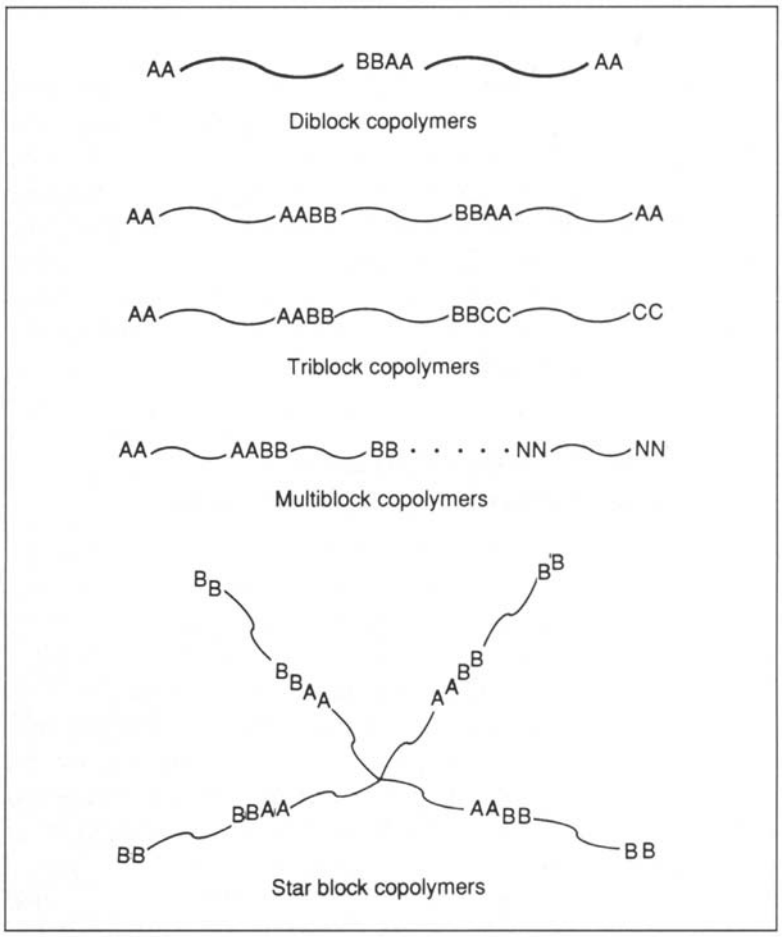

Figure 1: *Schematic presentation of the architecture of block copolymers from [103].*

In this review we limit our discussions to polyester/ether block copolymers of ABA and star-branched structures, where A designates a hydrophobic, biodegradable polyester block, and B consists of PEO. The lack of a universally accepted nomenclature of ABA polymers makes a retrospective analysis of literature data often difficult, if not impossible. Two systems have been proposed, namely the PELA nomenclature giving the molecular weight of the PEO

block and the average degree of polymerization of the A-blocks (e.g. PELA 6000/ 277) [3] and as a second approach adding the average degree of polymerization as subscript to the block designation (e.g. $PLA_{109}/PEO_{41}/PLA_{109}$) [4]. Since the data could often not be recalculated due to lack of information, we use the designation originally used in the literature.

AB-diblock- and multi-block copolymers possess micellar properties and have been studied as long-circulating carriers for hydrophobic drugs after intravenous injection. Their potential as drug delivery systems was reviewed recently [5, 6]. In almost all cases, the block copolymers were synthesized using a variety of different catalysts, but also catalyst free methods of synthesis have been described. The potential use of ABA triblock copolymers as drug delivery system for hydrophilic macromolecular drugs, such as peptides and proteins was recognized in the early 90's [7]. ABA triblock copolymers can be designed to exhibit rapid swelling upon contact with water, forming a physically cross-linked, biodegradable hydrogel. The advantage of this approach over chemical cross-linking of PEO is the ease of incorporation of sensitive proteins and the broad spectrum of existing technologies for device manufacturing, which will be discussed later in this review.

2.2. Synthesis of block copolymers from PEO and lactones

Homo- and copolymers of 6-hydroxycaprylic acid, lactic acid and glycolic acid are usually synthesized by the ring-opening polymerization of the cyclic monomers, e.g. ε-caprolactone, lactide and glycolide. In the last three decades, the block copolymers of poly(ethylene oxide) (PEO) and lactones have been investigated by many research groups. The terminal hydroxyl group of PEO can initiate successfully the polymerization of lactones in the presence of catalysts. Perret and coworkers were the first to our knowledge who prepared a series of block copolymers consisting of PEO and poly(ε-caprolactone), PCL, by anionic polymerization in THF using naphthalene-sodium complex as catalyst [8]. The group of Cerrai and coworkers

described block copolymers of PEO and PCL using a catalyst-free polymerization [9]. The polymerization of ε-caprolactone with low molecular weight PEO was carried out in bulk at 185°C with almost quantitative conversion. They argued that the first step of the reaction involved the addition of two ε-caprolactones to the terminal PEO hydroxyl groups yielding the corresponding bis-ε-hydroxy diester, through selective acyl-oxygen bond cleavage of the lactone ring, which then reacted further with ε-caprolactone according to the usual ring opening polymerization mechanism. Therefore, the copolymer formation proceeds, step by step, with the reaction between the hydroxyl function of the regenerated ε-hydroxycaproyl end unit and excess ε-caprolactone. Polymerization of ß-propiolactone and PEO was rather slowly and did not lead to quantitative conversion [10].

Synthesis of ABA-triblock copolymers of PEO B-blocks and lactic acid or glycolic acid A-blocks using Sb_2O_3 and phosphoric acid as catalysts was first described by Cohn et al. in 1987 [3, 11]. The copolymers were synthesized through the polycondensation of lactic acid or glycolic acid in the presence of PEO under nitrogen flow. ABA polymer compositions varied between 20 to 80 mol% poly(lactic acid), PLA, with PEO chains in the 600 –6000 molecular weight range. They designated this new family of biomaterials as PELA and proposed a nomenclature based on the molecular weight of PEO and the number average degree of polymerization of the PLA A-block, which was not universally accepted. They extensively investigated mechanical and thermal behavior as well as degradation and biocompatibility [12].

Tin catalysts were frequently used in the ring opening polymerization of lactones [13-23]. X.M.Deng et al. used stannous chloride as catalyst to synthesize successfully ABA triblock copolymers of PEO and PLA [13]. The polymerization was carried out in bulk at 170-200°C and yielded ABA polymers with a single peak in GPC analysis and narrow polydispersity Stannous octoate and metal oxides were used by Kricheldorf et al. as catalyst to prepare block

copolymer of PEO and lactide [14]. With metal oxides, such as GeO_2 and SnO_2 only low conversions of lactide were obtained, regardless of the reaction temperature, whereas Sb_2O_3 caused partial racemization of L-lactide and only SnO gave satisfactory results. However, with stannous octoate, racemization free ABA triblock copolymers was obtained with a high conversion [14]. Similar results were obtained by others [15, 20, 24]. Molecular weights of the ABA polymers corresponded to the feed ratios of monomer and initiator by an equation based on a simple chain reaction model. However, the GPC trace showed a shoulder indicating that some homopolymerization of lactide occurred. After fractionation, the shoulder peak was removed and a unimodal peak was obtained [15, 20].

Also aluminum triisopropoxide can be used as catalyst in the copolymerization of L-lactide or L-lactide/glycolide and PEO [25, 26]. The polymerization was carried out in bulk at 150°C and the conversion was over 90%.The GPC trace showed a narrow molecular weight distribution and unimodal peaks. The molecular weights of the block copolymer are in agreement with the molecular weight calculated from the molar ratio of the monomer and initiator as long as the molecular weight of the product is not too high. ^{1}H-NMR also demonstrated the block structure of the copolymers. Alkyl aluminum compound was also used in the copolymerization of PEO and lactide [27, 28].

Recently, rare earth metal alkoxides were used for the synthesis of ABA block copolymers in solution. Yttrium tris(2,6-di-tert-butylphenolate) was used by Feijen et al. for the polymerization of PEO and lactide in dichloromethane at 25°C [29, 30]. In the first step, the large 2,6-di-tert-butylphenoxy ligands are exchanged for the sterically less demanding alcohol ligands, then the alkoxide reacts with carbonyl group by formation of the ring opening product. This catalyst system is very effective to obtain narrow molecular weight distribution product with high conversion.

CaH_2 and Zn metal as well as lithium chloride were used heterogeneous catalyst system in the copolymerization of PEO and lactones and usually are more suitable for synthesizing low molecular weight block copolymers [31, 32]. Catalyst free polymerizations of lactones was always an ideal goal for polymers used as biomaterials. Cerrai et al. described the synthesis of ABA triblock copolymers of L-lactide and PEO without catalyst [33]. Their results demonstrated that their method is not competitive with catalyzed reactions due to a very long reaction time needed to get reasonable conversion of L-lactide.

Anionic polymerization was also used in the preparation of the block copolymer of PEO and lactone. Jedlinski et al. synthesized block copolymer of PEO and L-lactide through the anionic polymerization of L-lactide in the presence of sodium PEO alkoxide in THF at 25°C [34]. The polymerization proceeds fast and after 5 min. the lactide was almost entirely consumed. The obtained product exhibits a molecular weight higher than that of the prepolymer and a unimodal molecular weight distribution. Selective extraction experiments showed, that the composition did not change, contrary to what is observed in a homopolymer blend. A slight racemization was observed during the polymerization.

Kricheldorf and coworkers also reported the anionic polymerization in the preparation of the block copolymer of PEO and lactide [35]. In their experiments, they synthesized AB diblock and ABA triblock copolymers of PEO-methyl ether or PEO and lactide in toluene at mild temperatures of 50 or 80°C using KOt-Bu as catalyst. Both ^{1}HNMR and GPC could prove the quantitative reaction of the PEOs with L-lactide.

Star shape block copolymers of PEO and lactide were reported by several groups [28, 36]. These copolymers are prepared from multi-arm PEO, i.e. 4- or 8- arm PEO. Y.K.Choi et al. prepared 2 - to 8 - arm star shape block copolymers of PEO and L-lactide or ε-caprolactone using stannous octoate as catalyst. Using triethylene aluminum as catalyst, we synthesized 4 and 8-arm block copolymers with L-lactide or L-lactide/glycolide. The polymerization was

carried out in toluene at 70°C. GPC analysis showed an unimodal GPC trace and light scattering analysis showed a significant increase in molecular weight, corresponding to the ratio of monomer relative to multi-arm PEO in the feed. Polydispersity was comparable to that of the parent multi-arm PEOs. Connections of ester blocks to ether blocks could be verified by ^{1}H-NMR.

2.3. Mechanism of copolymerization from PEO and lactones

Although the mechanism of the polymerization of lactone with many metal compounds remains somewhat speculative, the coordination-insert mechanism is most generally accepted, especially in the case of metal alkoxides [14, 15, 20, 26] (figure 2).

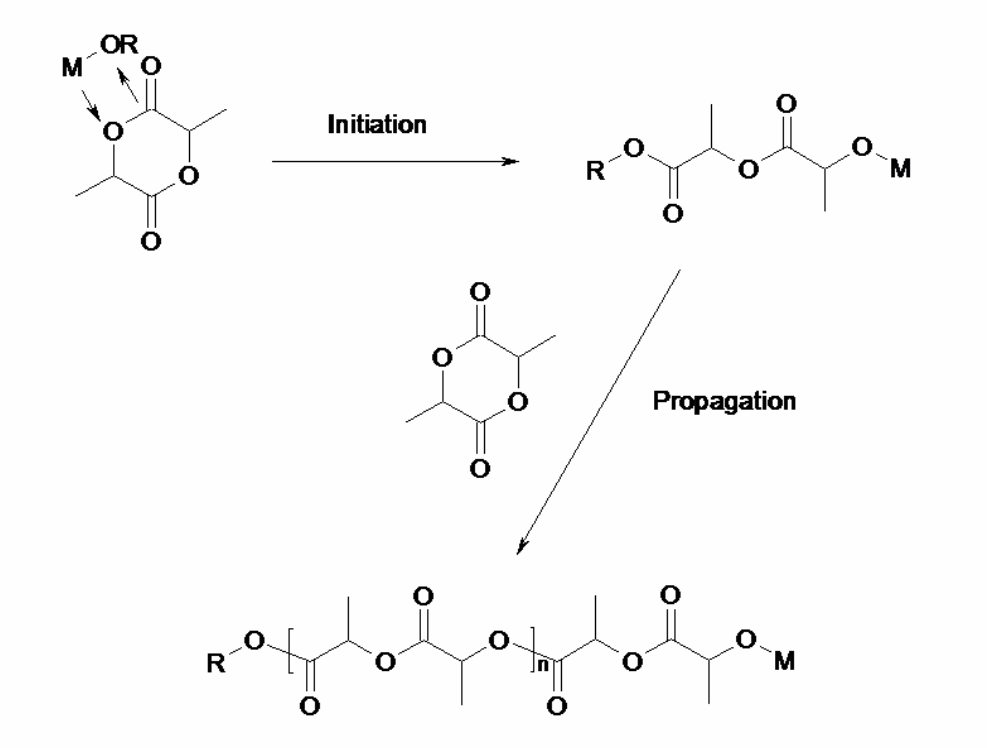

Figure 2: *Schematic representation of the Coordination-insert mechanism for the polyesters.*

In the presence of PEO molecules, the polymerization involves a transfer of metal alkoxide to metal PEOate [14, 26]. The ring-opening polymerization is initiated by this macro-initiator of the metal alkoxide as coordinated-insertion mechanism similar to low molecular weight metal

alkoxide catalysts. Stannous octoate, a salt of 2-ethylhexanoic acid, does not seem to fit into this scheme. Many investigations show that, most of terminal groups of the product of the ring-opening polymerization of lactones initiated by stannous octoate are hydroxyl groups, indicating that a hydroxyl groups, e.g. residual water or other impurities, participate in the initiation as co-catalyst. Macauley et al. studied the effect of hydroxyl and carboxylic acid substances on the polymerization in the presence of stannous octoate [16]. They found both, hydroxyl and carboxylic acid substances affecting the polymerization of lactide, but alcohol increased the PLLA polymerization rate and carboxylic acid decreased it.

Du et al. reported their detailed investigation on the mechanism of the copolymerization of PEO and lactones in the presence of stannous octoate [23]. Kinetic measurement and mechanistic studies suggest that the reactivity of the initiator, a hydroxyl group-bearing reagent, is an important parameter on the polymerization mechanism. In the case of primary and secondary alcohols, i.e. PEO and methyl lactate, it was found that when the initiator concentration exceeds the catalyst concentration, the number of the propagation chain formed exceeded the number of catalyst molecules. The chains were propagated through shifts of the catalysts from one chain to another. It was demonstrated that the cooperation of stannous octoate with the terminated hydroxyl group of PEO formed a metal alkoxide initiator. In situ ^{1}H-NMR was used to observe the mechanism of the copolymerization, PEO of molecular weight 1 000 was used for the kinetic study (figure 3). Their results show the hydroxyl terminal group of PEO is esterified, and the number of the methylene protons were in agreement with those calculated from the molecular weights of final products, indicating that the lactones are bound to the PEO chains. This evidence suggests that, both, stannous alkoxide and tin salts of carboxylic acids can initiate the ring opening polymerization, but the stannous alkoxide is more active than the salt. Thus, in the presence of alcohol, the initiation of ring opening polymerization of the lactones will be initiated by stannous alkoxide. In the

case of aluminum alkoxide catalyst, i.e. aluminum triisopropoxide, the transfer of the aluminium ion on terminal hydroxy group of PEO leads to the evaporation of isopropanol at high temperatures, resulting the formation of a macro-initiator [26].

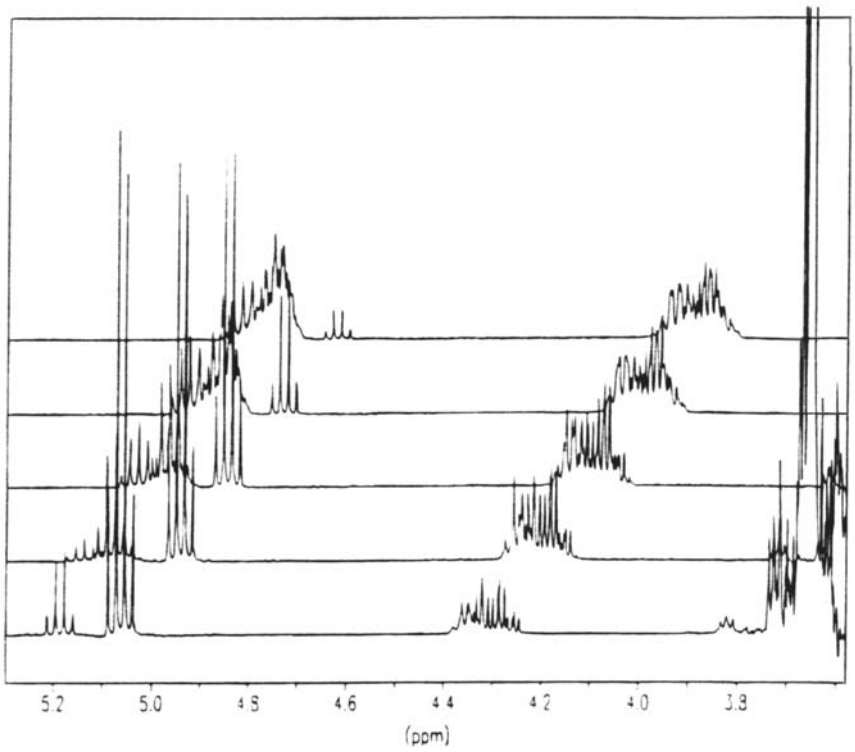

Figure 3: *Kinetic measurement of the copolymerization of PEG 1000 with lactide by ^{1}HNMR spectroscopy - Change of the peak at 4.3 ppm [23].*

The mechanism of the anionic polymerization of the lactones seems to be clearer than the coordination-insertion mechanism [27, 34]. It is considered that the anionic polymerization is a nucleophilic reaction in which the initiator attacks the carbonyl group directly (figure 4).

In most cases of anionic polymerizations side reactions were observed [27, 35]. Deprotonation was found in the case of KOt-Bu as initiator. Kricheldorf and coworkers reported in their later work, that they used KOt-Bu as catalyst to prepare L-lactide block copolymer in presence of PEO [35]. They found, that ^{1}H-NMR shows the existence of more

hydroxy terminated groups than ester groups and 10 to 20 % racemization was also observed, indicative of a chain transfer reaction with monomer via deprotonation.

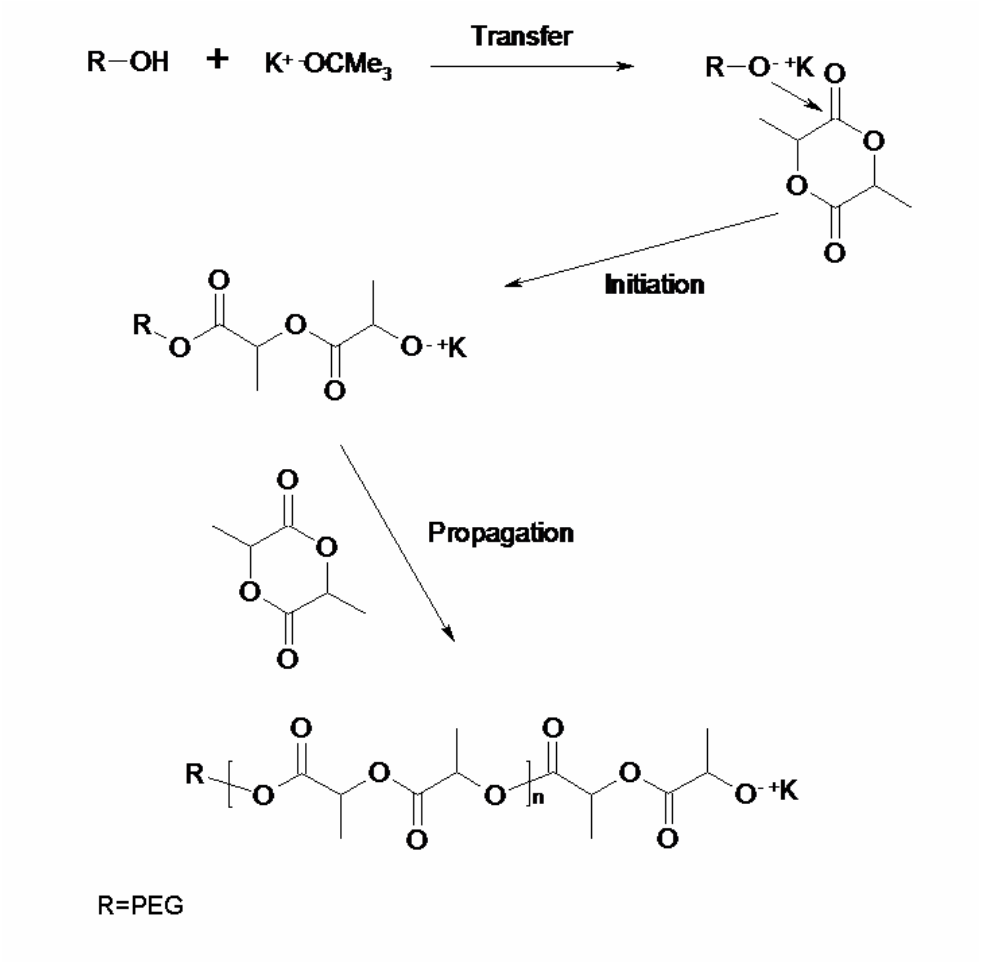

Figure 4: *Schematic diagram of the mechanism of anionic polymerization for polyesters.*

2.4. Physical properties of block copolymers from PEO and lactones

Introduction of a hydrophilic PEO chain into hydrophobic polylactones leads to a new family of biomaterials with properties differing from the corresponding homopolymers with respect to their physicochemical and biological behavior. For drug delivery systems, biocompatibility and biodegradability are important prerequisites which are closely linked to their

physicochemical properties. There are numerous reports in the literature on the characterization of the block copolymer of PEO and lactones.

2.4.1. Microphase separation and Crystallinity

Due to the incompatibility of polyether and polyester blocks, microphase separation in the block copolymers was to be expected depending on their composition. Cohn et al. used differential scanning calorimetry (DSC) to investigate the thermal property of PLLA-PEO-PLLA triblock copolymers [3]. For matrices comprising PEO chains with molecular weight below 3400, no soft-segment (PEO) crystallinity was detected. When long hard segments (PLLA) were present, essentially monophasic, semicrystalline polymers were obtained with PLLA blocks melting around 130°C. Polymers with longer soft segments exhibited a two-phase matrix, with both components being able to crystallize. But the thermal history strongly affected the morphology of the block copolymers, especially when both segments are long enough to crystallize.

Using both DSC and small angle X-ray scattering (SAXS) to characterize ABA triblock copolymers of PEO and PLLA as well as PLLGA, Li et al. demonstrated a microphase separation. The average size of the domains was estimated to be in the range of 100 to 140 angstroms based on SAXS.

DSC analysis showed the endothermic peak of either PEO or PLLA when its content was not below 40% (PEO) or 30%(PLLA), these melting peaks were strongly affected by the composition, which indicated that the microphase separation leads to the crystallization of either PEO or PLLA segment but there was a certain degree of phase compatibility which reduced the crystallinity of both PEO and PLLA in the block copolymers. In contrast to the ABA block copolymer of PEO and PLLA, those of PEO and Poly(L-lactide-co-glycolide)(PLGA) showed no marked melting peaks for both PLGA and PEO segments. By introduction of glycolide into PLLA segments, the crystallinity of A-block was destroyed.

The amorphous PLGA and PEO segments showed an increased phase compatibility, resulting in an elimination of the melting peaks (figure 5). But SAXS analysis indicated that a microphase separation exists in this amorphous copolymer.

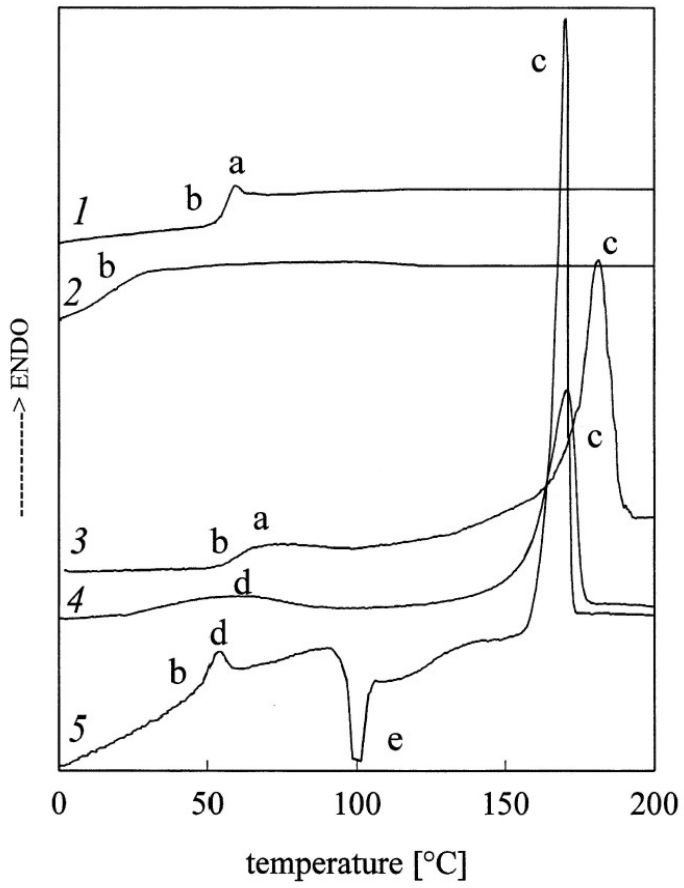

Figure 5: *DSC thermograms of PLA homopolymers, PLA/PEO block copolymers and blends. (1)PDLLA; (2)PDLLA-PEO-PDLLA; (3) PLLA; (4) Blend of PLLA-PEO-PLLA and PLLA; (5) PLLA-PEO-PLLA; (a) relaxation peaks of PDLLA and PLLA homopolymers; (b) Tg of the PLA block in the copolymer and/or homopolymer; (c) Tm of the PLA block in the copolymer and/or homopolymer; (d) Tm of the PLA block in the copolymer and/or a blend; (e) partial crystallization of LA units [18].*

Deng et al. also found that, the block copolymers of PEO with Mw > 4000 g/mol and D,L-lactide, showed fusion endotherms around 35°C which was assigned to the crystallinity of the soft segments, but no glass transition could be detected. The glass transition temperature of the copolymers and the melting point of PEO with molecular weight over 4 000 seem to overlap at the same temperature range. Two copolymers comprising PEO 1500 and 800 had

glass transition at 27°C and 22°C, respectively, in which PEO segment failed to crystallize and no fusion endotherms appeared. The glass transition of PEO was also found at –54 to –9.5°C depending on the molecular weights and composition. DSC and transmission electron microscopy (TEM) was combined to characterize the block copolymers of PEO and LLA or DLLA [18]. DSC results showed that compared to the melting point at 63 or 180°C for the parent homopolymer of PEO or PLLA respectively and amorphous PDLLA with only a glass transition point of 56.3°C, block copolymers showed modified properties. A block copolymer consisting of 44%mol PEO (MW 6110) and 56%mol LLA exhibited melting endotherms at 43.3 and 169.3°C, A decrease in melting points compared with their parent homopolymers indicated a partial phase compatibility, in agreement with the literature [26]. For a shorter PEO chain (MW 3090), no melting endotherm of PEO segment was found. This might be due to an increase compatibility of the shorter PEO chain with within dominant PLLA blocks and insufficient phase separation or because of the amorphous structure of PEO domain. Electron microscopy results showed the lamellar structure of semicrystalline of PEO in accordance its melting endotherm.

A similar morphology also exists in the block copolymer of PEO and ε-caprolactone as well as and δ-propiolactone. An et al. studied the thermal behavior of the triblock copolymers of PEO and PCL using DSC, wide angle X-ray diffraction (WAXD) and small-angle X-ray scattering (SAXS) methods [37]. They observed the crystallinity for both PEO and PCL segments. The crystallization and melting behavior of the PCL-PEO-PCL block copolymers can be categorized into three groups. The first one occurs when the central B-block, PEO, is shorter than the flanking PCL blocks, in which the crystallization of PCL blocks is dominant. The second group, which has a two or three times larger center blocks compared to PCL, shows that both PEO and PCL blocks crystallize. In the third group crystallization of PEO becomes dominant due to the shorter A blocks.

Star shape block copolymers show significant differences in the physical property in comparison with that of linear block copolymers [28, 36]. Due to the three dimensional branched molecular architecture, the interaction between the molecules of the star block copolymers is reduced. Therefore, it is not surprising that these polymers show lower glass transition and melting temperatures in DSC analysis than their linear counterparts [28]. In the case of star-block copolymer of 8-arm PEO and L-lactide, nearly no crystallinity of L-lactide segment was found by DSC analysis.

2.4.2. Hydrophilicity and swelling behavior

Hydrophilic PEO segments will change the physicochemical properties of hydrophobic and biodegradable PLA or PLG segments especially with respect their hydrophilicity and swelling [25, 26]. The block copolymers containing hydrophilic microphases are expected to show an increase interaction with water [26]. When polymeric matrix is immersed in water, the water content in the matrix increases rapidly, and an initial equilibrium is reached after a few hours (figure 6). The introduction of PEO segments is responsible for this significant increase in water uptake. Depending on the PEO content in the block copolymers, the water uptake could be adjusted. Compared to the block copolymer of PEO and lactide, the water uptake of the block copolymer of PEO and lactide/glycolide is slightly increased, as one would expect from theoretical considerations. Following the rapid initial water-uptake is a slow increase in the water content in the polymeric matrix, accompanying the erosion of the matrix, this biphasic water uptake was found by Pitt et al. [38]. They thought that this biphasic water uptake reflected the contribution of two processes: rapid diffusion of water into the initially miscible PEO and PLA blocks; then a slower rate of hydration possibly due to phase separation and hydrolytic cleavage of the PLA blocks.

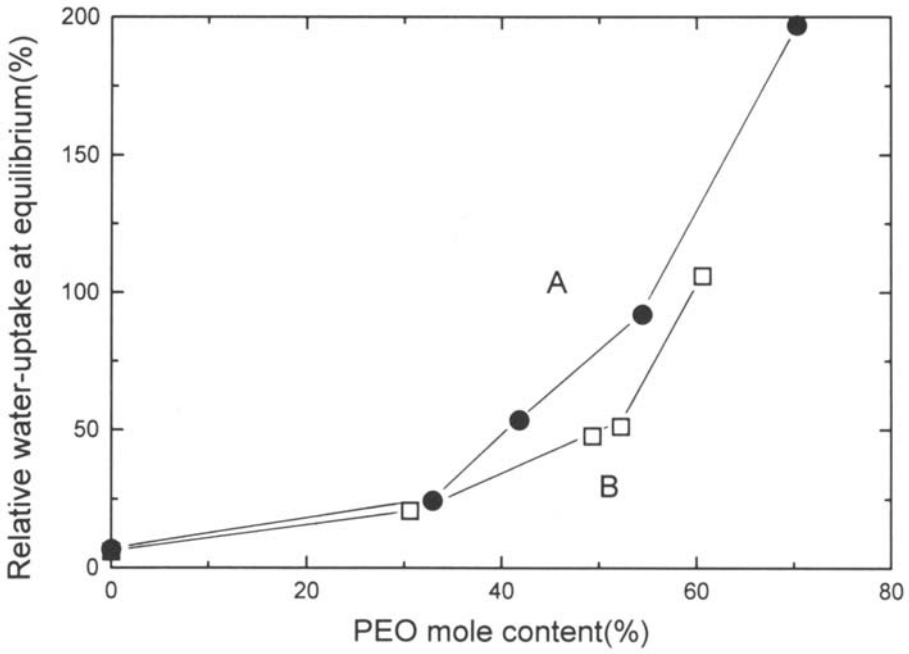

Figure 6: *Water Uptake of ABA triblock copolymers and the random copolymer of LLA and GA. (A) ABA copolymer of PEG and LLA/GA; (B) ABA triblock copolymer of PEG and LLA [26].*

2.5. Conclusions

Considerable effort has focused on the combining hydrophilic and biocompatible PEO and bioerodible polyester to give a copolymer whose characteristics may be varied from hydrophilic to hydrophobic and from non-degradable to degradable, depending on its precise composition.

The preparation of these polymers is similar to that of the preparation of the homopolyester. Ring-opening polymerization of lactones has been widely used in the preparation the block copolymers of PEO and polyester in the presence of poly(ethylene oxide) using various metallic oxides, -alkoxides or -carboxylates. The copolymerization proceeds according to a “coordination-insertion” or anionic mechanism in most cases. Few other methods e.g. catalyst-free and polycondensation, have been also used but they could not compete with the methods described above. The combination of hydrophilic PEO and hydrophobic polyester segments leads to a class of biomaterials which show differences in the physicochemical

properties compared to their parent homopolymers due to either micro-phase separation or compatibility. High hydrophilicity of the block copolymers leads to a potential in the delivery of hydrophilic macromolecules e.g. peptide and protein as well as DNA.

3. Degradation and biological properties of ABA triblock copolymers from polyester A-blocks and PEO B-blocks

Acceleration of degradation properties for relatively slow degrading polyesters was one of the driving forces to study ABA polymers. Although drug delivery systems based on these polymers have attracted considerable interest in the past three decades, their suitability for the application of acid sensitive proteins or peptides is limited due to their degradation behavior, as will be discussed in section 4.4.

An improved degradation pattern has been achieved by inserting hydrophilic, non-degradable poly(oxyethylene), (PEO), soft segments into a degradable PLA, PLGA or poly(ε-caprolactone) (PCL) chain. Degradation of these polymers can proceed either by hydrolytic cleavage of the polyester A-blocks or by enzymatic degradation catalyzed by esterases.

3.1. Degradation of ABA triblock copolymers based on PEO and polyesters

Possible enzymatic effects on the degradation of PELA 3400/126 were ruled out by Cohn et al. who observed no acceleration of degradation rates after addition of carboxylic ester hydrolase [12, 39]. More detailed studies have not been reported and similar to PLGA mainly random chain cleavage of the A-block segments need to be taken into account.

Compositional parameters, such as the ratio of A and B blocks and average A- and B-block chain lengths affect physico-chemical properties such as crystallinity, hydrophilicity and swelling, which are known to be of importance for the degradation of biomaterials.The incorporation of PEO as a hydrophilic component into hydrophobic A blocks led to a shorter induction period prior to the onset of erosion of the resulting ABA block copolymer compared to PLLA and PLA homopolymer [7, 38]. An accelerated weight loss (erosion) rate of

$PLLA_{43}$-PEO_{41}-$PLLA_{43}$ and $PLLA_{109}$-PEO_{41}-$PLLA_{109}$, compared to PLLA mainly resulted from the presence of PEO segments according to Li et al. [31, 40].

Pitt and coworkers investigated relatively short (total M_n ranging from 3200 to 10200) PLA-PEO-PLA copolymers synthesized from D,L-lactic acid and found the rate of chain scission to be independent of the composition of the ABA block copolymers and additionally, no significant differences have been detected for the rate of chain scission when compared to PLA homopolymers. Thus, they concluded that the shorter induction period prior to the onset of erosion is not due to a higher rate of chain scission but arises because of greater solubility of the PEO – PLA oligomers and a greater rate of diffusional loss in the hydrated copolymer samples [38]. This conclusion in turn renders the swelling properties of the ABA triblock copolymers to be a critical factor for their degradation behaviour.

Since crystallinity and swelling properties are closely related, crystallinity deserves a more detailed look. Crystalline regions are known to be a physical barrier for water uptake into polymer specimens, whereas amorphous regions are able to facilitate water uptake. In a series of studies evaluating different PLLA-PEO-PLLA polymers Vert and coworkers found, that highly crystalline $PLLA_{109}$-PEO_{41}-$PLLA_{109}$ absorbed small amounts of water, whereas slightly crystalline $PLLA_{43}$-PEO_{41}-$PLLA_{43}$ with a relatively higher PEO content attained a water content of about 60% [4]. They further stated, that $PLLA_x$-PEO_y-$PLLA_x$ copolymers with $DP_{PEO}/DP_{PLLA} > 4$ appeared soluble in water, while those with $DP_{PEO}/DP_{PLLA} < 4$ led to turbid mixtures of swollen copolymer in water. If the ratio of DP_{PEO}/DP_{PLLA} became < 1, no swelling was detected [41].

Increasing the relative EO content resulted in an increased hydrophilicity facilitating the diffusion of water and thus enhancing erosion of the polymer according to Cerrai et al. [42]. In another study, they investigated the degradation of PLLA-PEO-PLLA and PCL-PEO-PCL ABA block copolymers by measuring the intrinsic viscosity [η] of polymer specimens over

time. They detected decreasing [η] values with increasing copolymer hydrophilicity due to increasing relative EO contents. From this findings they concluded a faster rate of chain scission with increasing relative EO contents being consistent with their results obtained earlier [42], allowing modulation of the degradation rate of these materials by variation of their composition [43]. However, these findings are in disagreement with the statements of Shah et al. [38]. Although the hydrophilicity of the ABA triblock copolymers seems to be primarily determined by the relative EO content, an increased hydrophilicity of the A blocks is reflected by an increased erosion of the polymer specimen. This can be rationalized by assuming the degree of hydrophilicity as PCL < PLA < PLGA and comparing the degradation rates detected for the different copolymers by different groups. The rate of erosion tends to be the higher the more hydrophilic the A blocks are when looking at constant relative EO contents [7, 12, 31, 38, 39, 44, 45].

The degradation behavior of PLLGA-PEO-PLLGA and poly(L-lactide-co-glycolide), PLGA, has been studied with respect to molecular weight loss and polymer erosion. Implants were prepared by either compression moulding or extrusion using a laboratory ram extruder. The analysis of the pH inside ABA rods using electron paramagnetic resonance spectroscopy, gave a pH of 5.2 after incubation with a subsequent increase to pH 6.0 during the first day, approaching the pH of the medium after nearly 33 d. Contrary to PLG rods, acidic degradation products did not accumulate inside the ABA rods, thus making the incorporation of proteins or peptides being sensitive to an acidic environment possible [44].

Another interesting point to consider when investigating in vitro degradation is the influence of the buffer medium. Cohn and Younes investigated the influence of different pH values, namely pH 5, 7.5 and 9, on the degradation of PELA 3400/126 and PELA 1500/45 copolymers in vitro. The copolymer matrices degraded faster in alkaline buffer than in

physiological or acidic media. Thus, they suggested a base-sensitive ester bond-cleavage [12, 39].

The influence of pH and ionic strength of the buffer on degradation kinetics of microspheres prepared from PLLGA-PEO-PLLGA (molar composition of LLA/GA/EO 60:10:30, M_n 14100) was evaluated by Bittner et al. demonstrating that both mass loss and molecular weight decay were accelerated in alkaline and acidic pH [45], thus showing differences in pH impact on degradation of PELA and PLLGA-PEO-PLLGA copolymers.

The results of the degradation study in two differently buffered media, 300 and 600 mOsm respectively, were not significantly different suggesting, that the ionic strength of the surrounding medium did not influence the degradation of the copolymer [45].

Regarding the influence of temperature, the degradation kinetics have been found to be the faster, the higher the temperature, as expected [12, 39].

Recently, Witt et al. studied the influence of different shapes of parenteral delivery systems (PDS), namely extruded rods, tablets, films and microspheres with respect to molecular weight, mass, polymer composition and shape and microstructure of the PDS on its erosion. For each device the onset time of bulk erosion (t_{on}) and the apparent rate of mass loss (k_{app}) were calculated. In the case of PLLGA, the t_{on} was 16.2 days for microspheres, 19.2 days for films and 30.1 days for cylindrical implants and tablets. The k_{app} was 0.04 $days^{-1}$ for microspheres, 0.09 $days^{-1}$ for films, 0.11 $days^{-1}$ for implants and 0.10 $days^{-1}$ for tablets. The degradation rates were in the same range irrespective of the geometry and the micrographs of eroding PDS demonstrated pore formation; therefore, a complex pore diffusion mechanism controlled the erosion of PLLGA devices. In contrast, PDS based on PLLGA-PEO-PLLGA ABA triblock copolymers showed swelling, followed by a parallel process of molecular weight degradation and polymer erosion, independent of the geometry. In summary, the insertion of a hydrophilic B-block led to an erosion controlled by the degradation of ABA

copolymers (figure 7), whereas for PLGA a complex pore diffusion of degradation products controlled the rate of bulk erosion (figure 8) [46].

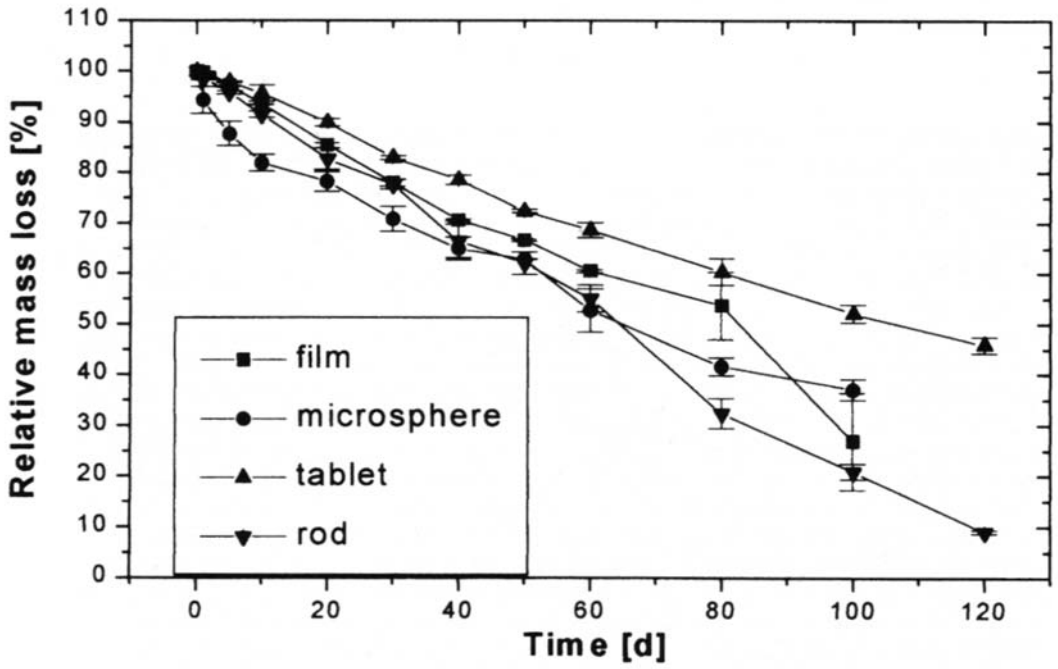

Figure 7: *Erosion of an PLGA-PEO-PLGA ABA triblock copolymer (ABA 2) as a function of the device (each sample was measured in triplicate) [46].*

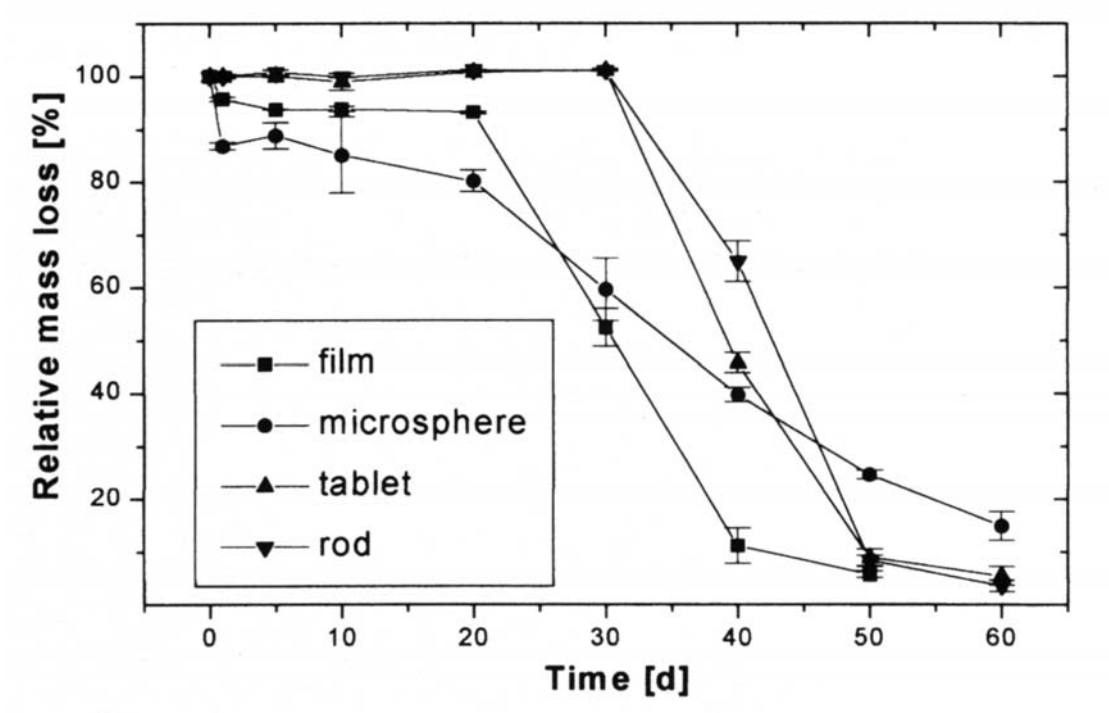

Figure 8: *Erosion of PLGA as a function of the device geometry [46].*

The postulated degradation mechanism is somewhat controversial, especially regarding the preferred cleavage site for hydrolysis. Water soluble PEO-PLA degradation products were detected in the buffer medium using ^{1}H-NMR spectroscopy, suggesting that chain scission occurred statistically in the A blocks, and that EO-LA and LA-LA linkages seem to possess the same hydrolytic lability. [39]. Using ^{1}H- and ^{13}C-NMR spectroscopy, chain scission both at the LA-EO and LA-LA sites were observed [15].

The group of Vert studied the degradation of $PLLA_{43}$-PEO_{41}-$PLLA_{43}$ and $PLLA_{109}$-PEO_{41}-$PLLA_{109}$. They found evidence for a hydrolytic degradation by random chain scission. PEO blocks attached to very short PLLA blocks were released especially in later stages of degradation, resulting in an increasing LLA/EO ratio in the residual material [31]. Pitt et al. investigated copolymers with relatively short PEO segments (M_n 1000 and 2000 g/mol). They determined the degradation behavior over a period of 40 days. Using ^{1}H-NMR analysis, increasing LLA/EO ratios were detected even in the initial period of degradation contrary to the findings of Hu and Liu. This could be due to the relatively short PEO segments diffusing out of the residual specimen from the beginning of degradation [38].

A preferential cleavage of PLLA-PEO-PLLA triblock copolymers in the vicinity of the PLLA/PEO interface was postulated in another study using ^{1}H-NMR analysis. In the initial phase of degradation a rapidly decreasing PEO content was found suggesting that primarily ester-ether bonds were cleaved and thus, the release of PEO segments has been facilitated [7].

A biphasic degradation profile has been observed for PLLA-PEO-PLLA copolymer films. The pattern has been characterized by a rapid initial loss in number average molecular weight of the tested copolymers followed by a second phase featuring a slower number average molecular weight decay. In both stages M_n decay was closely paralleled by polymer mass loss. In comparison, the results obtained for PLLGA-PEO-PLLGA copolymers showed a significantly accelerated number average molecular weight decay and a less pronounced

biphasic behaviour. The M_n decay was shown to be closely paralleled by polymer mass loss as well. When comparing the mass loss rates of PLLA-PEO-PLLA copolymers and PLLGA-PEO-PLLGA copolymers, they found the latter to erode substantially faster. After a time period of 40 days, the PLLA-PEO-PLLA copolymer lost 27% of weight, while the PLLGA-PEO-PLLGA copolymer lost 40% of weight. This mass loss was shown to be further accelerated by increasing the molar ratio of glycolic acid. Thus, Kissel and coworkers suggested a different phase model for the swollen PLLGA-PEO-PLLGA matrix with the PLLGA phase being completely in an amorphous rubbery state as compared to the swollen PLLA-PEO-PLLA copolymers, where the PLLA phase is said to consist of a partially crystalline core surrounded by an amorphous, rubbery shell (figure 9) [7].

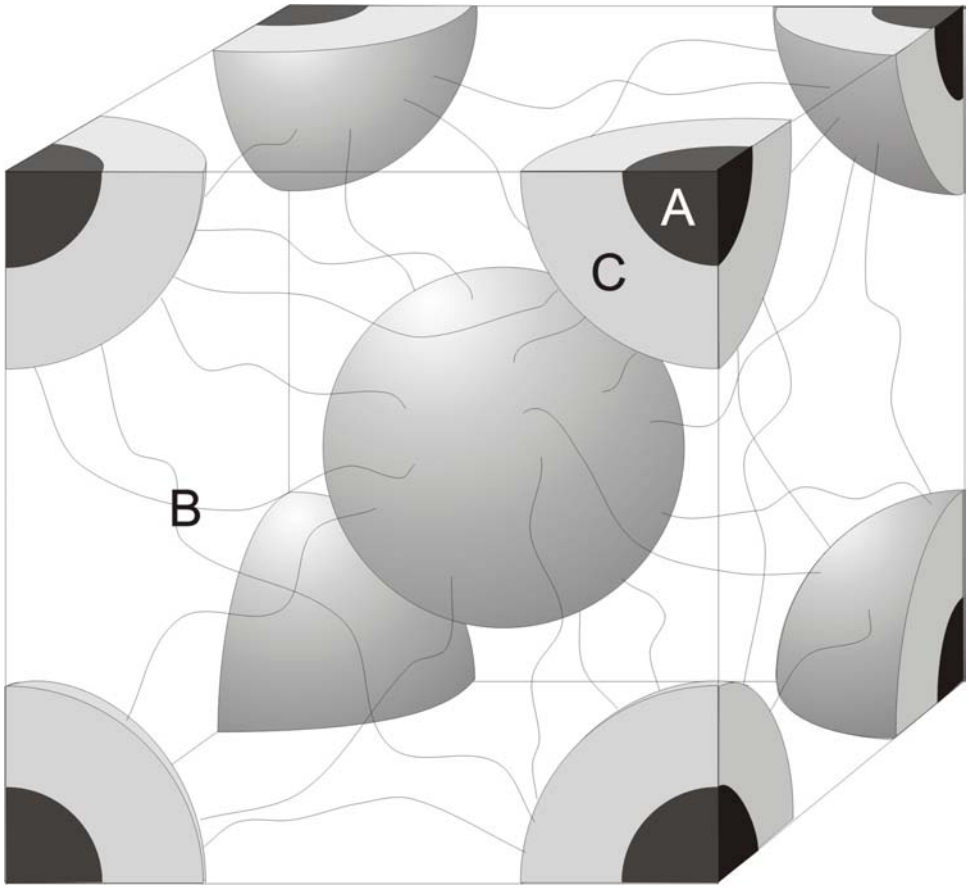

Figure 9: *Schematic model of the in vitro degradation of a PLA-PEO-PLA triblock copolymer taking the microphase structure into account (in PLGA-PEO-PLGA, even A would be replaced by the rubbery C – phase); A: crystalline PLA core in PLA-PEO-PLA, B: swollen PEO phase, C: amorphous, rubbery PLA phase in PLA-PEO-PLA [7].*

When looking at SEC chromatograms of hydrolytically degrading PLLA and PLLGA homo- and copolymers, bimodal molecular weight distributions have been frequently reported. [40, 47-49]. Comparable results have been described by Vert and coworkers [31] and Hu and Liu [15, 50] for PLLA-PEO-PLLA.

In principle, three explanations can be put forward for generation of bimodal molecular mass distribution in ABA polymer degradation. Firstly, the degradation rate of the polymer is faster inside the matrix than at the surface, causing bimodal SEC profiles due to the heterogeneous degradation mechanism [47, 48]. Secondly, the degradation of initially amorphous but crystallizing polymers causes a narrow polydispersity with an additional peak from low molecular fragments. This narrow polydispersity was caused by degradation-induced crystalline domains [40, 48, 49]. Thirdly, the degradation of initially crystalline polymers results in bimodal or even multimodal SEC profiles due to the selective degradation in amorphous zones and at the edges of crystalline zones [40].

Vert and coworkers studied the degradation of four differently composed triblock copolymers, the number average molecular weight of the PEO B block ranging from 4800 to 15650 and the LLA/EO ratios ranging from 1 to 5 over a time period of 25 weeks. They concluded that case two and three seem to be applicable. The third theory was said to be the best applicable to the degradation profile of a copolymer composed of longer PLLA blocks when compared to the PEO blocks. These PLLA blocks being initially long enough to yield well developed crystalline structures lead to a preferential degradation in the remaining amorphous zones as well as the edges of crystalline ones resulting in a bimodal molecular weight distribution [31].

It appears that the degradation properties of ABA triblock copolymers are still incompletely understood. The complicated phase behavior and swelling properties clearly influence the degradation rate. A generally accepted structure-function (degradation) relationship has not emerged so far. In order to be able to compare results from different work groups, more

standardized test procedures (e.g. ISO 10993-13 [51]) and a clearer, quantitative and more descriptive way of expressing the degradation would be desirable for future studies. The comparability of the results greatly suffers from a different nomenclature used by each individual group of researchers as mentioned in section 2.1.

As general rules an increased degradation rate of poly(ester-ether-ester) triblock copolymers can be obtained by either increasing the $DP_{PEO}/DP_{PLA/PCL}$ ratio and thus facilitating faster and increased water-uptake or by introducing randomly copolymerized glycolic acid into PLLA A blocks resulting in a better phase compatibility, less crystallinity and hence a more rapid swelling of the resulting copolymer.

3.2. Biocompatibility of ABA Polymers

The fundamental understanding of cellular and tissue responses, which account for the biocompatibility of materials intended to be used in close contact to biological systems is important for the design of new polymeric drug delivery systems. Thus, new biomaterials, such as ABA polymers, were investigated extensively both, under in vitro (see Table 1) and in vivo (see Table 2) conditions according to the guidelines of the International Standardization Organisation (ISO) [52].

3.3. In Vitro – Cytotoxicity/Biocompatibility of ABA Polymers

Different PLLA-PEO-PLLA and PCL-PEO-PCL triblock copolymers have been investigated in a series of studies by the group of Cerrai et al. under in vitro conditions.

In vitro biocompatibility of PLLA-PEO-PLLA block copolymers, obtained by bulk ring opening polymerization of PEO and L-lactide in absence of catalysts at 120-140°C, was tested in a cell culture assay. Two series of ABA with different molecular weight, relative length of blocks and hydrophilicity were evaluated for their cytotoxicity in 3T3 mouse fibroblasts using different endpoints, namely Neutral Red uptake for cell viability and the MTT assay for

mitochondrial activity. Semi-quantitative assessment combined with statistical analysis showed generally good biocompatibility for the copolymers synthesized from PEO 35000 with only slight differences compared to the negative control phosphate buffered saline. Negative results were obtained from cytotoxicity testing determining cell proliferation and cell adhesion tests on films, suggesting that fibroblasts were neither activated by nor adhering to ABA surfaces.

Furthermore, hemocompatibility was studied measuring the activation of plasma prekallikrein to kallikrein. The results showed lower activation compared to borosilicate glass (positive control) as well as PLLA homopolymers and demonstrated good hemocompatibility. The cytotoxicity properties of ABA polymers obtained from PEO 3000 were inferior to those from PEO 35000, pointing to structural effects on cytotoxicity properties. These findings were attributed to a racemization of L-lactide during synthesis, resulting in non-stereoregular amorphous PLA sequences [42], but also differences in degradation rates and hence concentration of cleavage products could be responsible for this difference in cytotoxicity.

In a second study with similar design PCL-PEO-PCL copolymers were investigated. In addition, the hemocompatibility was tested by the contact activation of thrombocytes, measuring the release of platelet factor 4 and ß-thromboglobulin. By calculating the ratio of ßTG and PF4 and comparison of the ratios to the values obtained from pure blood, silicone and glass, a slight platelet activation for the ABA was demonstrated. In conclusion, the authors suggested that ABA triblock copolymers were generally cyto- and hemocompatible, thus making them suitable as biomaterials [53].

Also cell culture tests of PLLA-PEO-PLLA and PCL-PEO-PCL regarding their possible use in the field of cardiovascular devices were reported. Human umbilical vein endothelial cells (HUVEC) metabolism was studied in the presence of both, polymer films and their hydrolytic breakdown products. The release of prostacyclin (PGI_2) and angiotensin II (A_{II}) was measured

at different times for ABA of different compositions and hydrophilicity. Block copolymers of both series did not alter the PGI_2 and A_{II} release of HUVEC cell cultures after prolonged exposition (10 days). Similar results were found also after a prolonged contact between HUVEC and hydrolytic breakdown products. However, the ABA triblock copolymers with long PLLA chains significantly stimulated the release of either PGI_2 or A_{II} at the earlier time points, namely 3 and 5 days. Stimulation was closely related to the amount of degradation products released and possibly due to active metabolism of the degradation products by HUVEC leveling off at day 10 because of metabolic adjustment. This finding might point towards enzymatic interactions between cells and degradation products of ABA [54]. The breakdown products of PLLA-PEO-PLLA and PCL-PEO-PCL were measured by HPLC analysis and the concentrations of 6-hydroxyhexanoic acid and lactic acid were monitored for three ABA copolymers during 3-8-week experiments. The experiments were carried out both in the presence and absence of 3T3 mouse fibroblast cells. When the fibroblast cell populations were present in the same wells together with the biodegradable copolymers, signs of cellular metabolism of the degraded monomers could be detected. Results of LDH measurements showed no cytotoxic effects of the copolymers. In fact, the LDH activity detected in the presence of polymer samples was even lower than the negative control results [43].

The biological response of differently composed PLLGA-PEO-PLLGA ABA triblock copolymers has been intensively investigated in a series of studies under in vitro and in vivo conditions. The results of an in vitro testing of a set of ABA triblock copolymers were compared to values obtained from commercially available poly(D,L- lactide-co-glycolide) (PLGA), tin stabilized PVC and PE as reference materials in different cell culture models using L929 mouse fibroblasts. The in vitro cytotoxicity studies demonstrated that the ABA polymers were well tolerated by fibroblasts in cell culture [55].

Table 1: *In Vitro Biocompatibility studies of ABA Polymers in literature.*

Ref.	Probe	total M_w	PEO M_w	A:B block ratio (B=1)	notation used by author	in vitro test sytem [1]	results
[42]	PLLA-PEO-PLLA	7300	3000	1,4	LA-8	cytotoxicity: 3T3 mouse fibroblasts, extracts from copolymer in PBS (5 days), Neutral Red uptake, Kenacid blue R binding, MTT hemocompatibility: activation of plasma prekallikrein (PKK) to kallikrein	cytotoxicity: not assessed hemocompatibility: not assessed
		6300	3000	1.1	LA-13		
		5900	3000	1.0	LA-9		
		5500	3000	0.8	LA-10		cytotoxicity: more cytotoxic than the copolymers obtained from PEO 35000 hemocompatibility: not assessed
		4700	3000	0.6	LA-11		
		3700	3000	0.2	LA-12		
		128000	35000	2.6	LA-3		cytotoxicity: comparable cytotoxicity results as the negative control PBS after 72 hrs hemocompatibility: PKK activation mostly lower than positive control (borosilicate glass) and PLA homopolymer
		83000	35000	1.4	LA-5		
		66000	35000	0.9	LA-6		
		54000	35000	0.5	LA-7		
		43000	35000	0.2	LA-4		
[53]	PCL-PEO-PCL	10800	9200	0.2	C13	cytotoxicity: 3T3 mouse fibroblasts, extracts from copolymer in PBS (5 days) and extracts from copolymer in PBS (121°C, 1h) Neutral Red uptake, Kenacid blue R binding, MTT, hemocompatibility: activation of plasma prekallikrein (PKK) to kallikrein	cytotoxicity: not assessed moderate hemocompatibility
		13500	9200	0.5	C14		
		34500	20000	0.4	C20		cytotoxicity: not assessed highest hemocompatibility
		95200	20000	3.8	C16		
		40700	35000	0.2	C24		cytotoxicity: comparable cytotoxicity results as the negative control PBS after 24 and 72 hrs moderate hemocompatibility
		53800	35000	0.5	C26		
		67300	35000	0.9	C25		
		116700	35000	2.3	C23		

Ref.	Probe	total M_w	PEO M_w	A:B block ratio (B=1)	notation used by author	in vitro test sytem [1]	results
[55]	PLGA-PEO-PLGA	29000	10000	1.9	ABA1	cells: L929 mouse fibroblasts extracts of copolymers prepared in culture medium and sesame oil (24 hrs at 37°C) cytotoxicity: MTT assay	not assessed
		20300	1000	19.3	ABA2		cytotoxicity: generally low (except ABA2 possibly due to residual monomer)
		15900	4000	3.0	ABA3		
		22000	10000	1.2	ABA4		
		19100	10000	0.9	ABA5		

[1] shortened for better overview

3.4. In Vivo – Biocompatibility of ABA Polymers

Younes et al. determined the biological response of a series of PLA/PEO block copolymers under in vivo conditions. The studies compared the tissue reaction elicited by various PLA/PEO ABA polymers to that evoked by PLA homopolymer after intramuscular implantation in rabbits. Evaluation of tissue reaction showed a non-specific foreign body, granulomatous reaction with chronic inflammation being apparent for all samples tested. The granulomatous reaction was comparable to the reaction observed with PLA [12].

In another study conducted, films of three PLLGA-PEO-PLLGA ABA triblock copolymers and one random PLGA copolymer were investigated regarding the influence of different polymer compositions and molecular weights on the tissue reaction and appearance of toxic degradation products in the cage implant system in rats. The inflammatory tissue reaction was followed over a 21-day implantation period by monitoring the leukocyte concentration, the extracellular acid, and alkaline phosphatase activities in a quantitative manner. Size and density of adherent macrophages and foreign body giant cells on the film surfaces were determined. The ABA and PLGA implants caused only a minimal inflammatory reaction, as characterized by a low concentration of leukocytes during the implantation period when compared to empty cage controls. The content of PEO had an influence on the density of the

adherent cells on the surface of the polymer film. An increase in PEO content and molecular weight decreased the cellular density during the implantation period. The results demonstrated, that the ABA block copolymers and PLLGA copolymer are equally well tolerated in the cage implant test system [56].

Furthermore, three PLLGA-PEO-PLLGA copolymers were formulated as microspheres and administered by intramuscular injection to rats (figure 10). The influence of different polymer compositions and molecular weights on the tissue reactions by histological analysis of the injection site was analyzed in comparison to PLGA. The tissue reaction was evaluated over a 56-day implantation period in rats characterizing the following factors: inflammation, necrosis, damage to the surrounding tissue, foreign body reaction, collagen deposition and fibrous capsule formation surrounding the microparticles at the injection site. Throughout the implantation period, all polymers showed a normal foreign body reaction and healing response. The foreign body reaction of all three ABA triblock copolymers was mainly a granulation tissue type of healing response with the presence of macrophages, fibroblasts and foreign body giant cells. New small blood vessels were detected. Neither necrosis nor significant muscle damage could be identified in the histology slides examined. Following their results, they suggested, that microparticles prepared of ABA triblock copolymers can be considered as a biocompatible delivery system [57].

Generally, the ABA triblock polymers reviewed show acceptable biocompatibility in all studies, although a trend can be seen, that biocompatibility is increased with increasing the $DP_{PEO}/DP_{PLA/PCL}$ ratio and thus increasing hydrophilicity. Also, the shorter the A block, the less acidic degradation products leaking from the polymer occur and thus the less toxic the copolymer. These two facts combined with an accelerated degradation rate obtained in the same way might lead to the conclusion, that reducing the polyester chain length and increasing the PEO chain length results in the perfect copolymers. But it has to be kept in

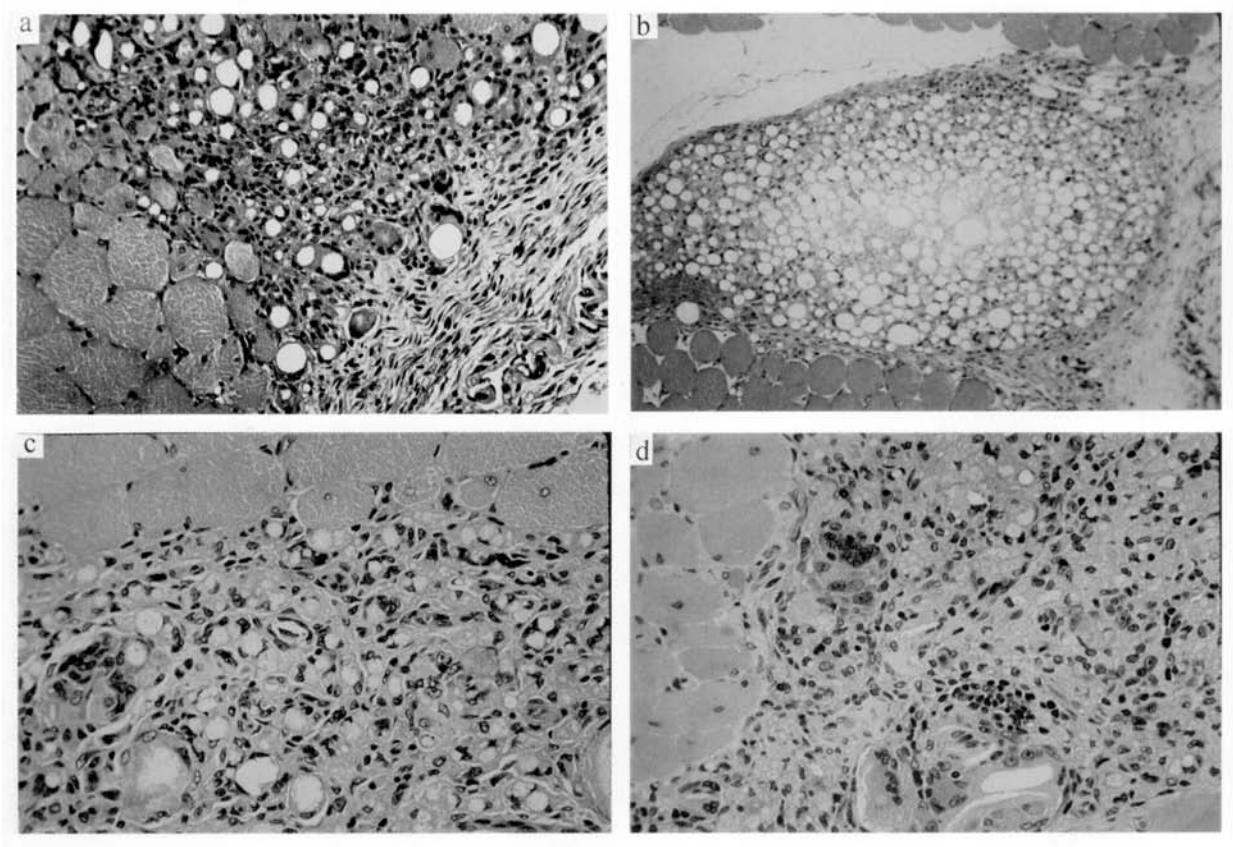

Figure 10: *Histology of PLGA-PEO-PLGA ABA (ABA 2) microparticle implantation sites in rats . (a) 6 days implant site, original magnification 63 x , tissue reaction characterized by macrophages, foreign body giant cells (FBGC) and fibroblasts, (b) 10 days implant site, original magnification 40 x , injection site enclosed in a collagenous capsule, (c) 21 days implant site, original magnification 100 x , changing size and morphology of microparticles indicative of an onset of erosion, (d) 35 days implant site, original magnification 100 x , high number of macrophages and fibroblasts due to extensive degradation, only few microparticles left [57].*

mind, that the physical characteristics change as well, and when changing the polymer composition in the said way, copolymers become brittle and waxy being no more suitable for the preparation of implants, nano- and microparticles. Also, for the use of polymers as vaccine delivery systems, “bioincompatibility” might be wanted to a certain amount thus resulting in an adjuvant effect.

Table 2: *In Vivo Biocompatibility of ABA polymers in literature.*

Ref.	Probe	total M_w	PEO M_w	A:B block ratio (B=1)	species	device	results
[12]	PLLA-PEO-PLLA	26000	6000	3.3	male and female rabbits weighing 1.5 – 2.0 kg	intramuscular implants, sterilized by gamma irradiation	marked dispersion of polymer particles, associated with acute inflammation after 12 days
		12400	3400	2.6		intramuscular implants, sterilized by ethylene oxide	non-specific, foreign body, granulomatous reaction with chronic inflammation after 7, 12, 17 and 22 days
		4700	1500	2.1		intramuscular implants, sterilized by ethylene oxide	non-specific, foreign body, granulomatous reaction with chronic inflammation after 7, 12, 17 and 22 days
[57]	PLGA-PEO-PLGA	26000	1000	25	male and female Sprague-Dawley rats weighing 300 g	intramuscularly injected microparticles prepared by solvent evaporation method	initial acute but localized inflammatory response on day 1, no irreversible changes to surrounding muscle tissue, implantation sites gradually replaced by collagenous tissue up to day 56, no major differences between different copolymer compositions detected
		18000	4000	3.5			
		31000	10000	2.1			
[56]	PLGA-PEO-PLGA	26000	1000	25	female Sprague-Dawley rats weighing 250-300 g	solvent-casted films, cage-implant sytem	exudate analysis led to lower leukocyte levels than empty control, lowest macrophage adhesion at day 4
		18000	4000	3.5			exudate analysis led to lower leukocyte levels than empty control
		31000	10000	2.1			exudate analysis led to lower leukocyte levels than empty control, highest macrophage adhesion at day 4

4. Parenteral Delivery Systems based on ABA Polymers

4.1. Introduction

The application of ABA polymers for controlled release of bioactive materials emerged when shortcomings of polyesters, such as poly(lactic-co-glycolic acid), PLGA, with respect to protein delivery became apparent and required biodegradable polymers which did not affect

protein stability and release properties. Apart from these polyesters also other biomaterials, such as poly(ortho esters) and poly(anhydrides) have been utilized. A recent review deals with various aspects of different biodegradable polymers as platform for controlled drug delivery [58]. Not only PLA and PLGA, but also poly(ε–caprolactone), PCL, [9] and ß-propiolactone [10] were used to generate ABA triblock copolymers with improved degradation properties. Again, modification of the biomaterials' properties was the main objective of these investigations. As outlined in section 2, not only compositions, but also different block-copolymer structures were realized using various synthetic methods.

BAB Triblock copolymers display thermo-sensitive properties, which are of interest for injectable protein depot systems. Details of this novel platform for drug delivery will be discussed elsewhere.

4.2. Classification of Parenteral Depot Systems

Parenteral Depot Systems, PDS, are devices which release a bioactive substance over several days up to several months at a constant rate. After subcutaneous or intramuscular administration, the drug substances are directly reaching the tissue of interest or the general circulation and drug concentration is maintained ideally in an infusion-like fashion over a prolonged period of time. Several PDS containing peptide hormones have become commercially available. Apart from LHRH agonists [59], somatostatin derivatives [60] have been incorporated into PDS either in form of implants or microspheres.

Implants are cylindrical devices of 1mm diameter and 10-20 mm length which are deposited into the subcutaneous tissue using a hollow needle (trocar). One advantage of implants is the possibility of their removal by a small surgical procedure, if adverse events necessitate discontinuation of therapy. Also other geometries for implants, such as tablets or films have been realized experimentally.

PDS in form of microspheres or microparticles are by definition spherical devices with diameters in the range of 1-100 µm to allow subcutaneous or intramuscular injection in suspension using conventional syringes. Microspheres are monolithic devices in which the drug is either dissolved as "solid solution" in the polymeric matrix or dispersed as "solid dispersion". Microcapsules consisting of a drug core coated with a thin rate-controlling polymeric film can be classified as reservoir systems. The latter micro-morphology would be very attractive from theoretical considerations since constant zero order release rates are expected for those devices. In the case of hydrophilic macromolecular drug substances, such as peptides and proteins, diffusion rates through films of PLGA are too low and the permeability of the coating changes as a function of degradation. This then could lead to a undesirable drug dumping when the rate-controlling coating disintegrates prematurely. Therefore, mainly monolithic devices of a solid dispersion morphology have been utilized in conjunction with biodegradable polymers. Microspheres have been preferred over implants due to the possibility to use conventional injection techniques and to administer higher doses of PDS (200-800 mg).

Apart from continuous release profiles also discontinuous or "pulsatile" release patterns have been investigated, especially in conjunction with antigen or vaccine delivery systems. In this case, the degradation of the polymeric matrix is used to release an antigen dose at a predetermined time point. For bacterial toxoid vaccines the conventional immunization protocol consisting of three injections at 0,1 and 12 months can be accomplished by a single injection containing a mixture of PLGA-microspheres which degrade after 1 and 12 months. Advances in this field have been reviewed recently [61].

More recently also formulations have been described where the formation of a polymeric depot occurs spontaneously in the muscle after injection of a liquid drug suspension or solution. An "in situ forming" drug delivery system (Atrigel™) for leuprorelin based on a

formulation containing PLGA 75/25 dissolved in N-methyl-2-pyrrolidone with 3% w/w leuprolide acetate suppressed serum testosterone for ca. 3 months in animal studies. A relatively simple manufacturing process and the ease of injection are positive features while the long-term safety of the solvents needs to be demonstrated [62].

4.3. Overview on Manufacturing techniques for PDS

Various techniques for manufacturing PDS from biodegradable microspheres have been described in the literature [63]. It is estimated that more than 2000 patents were issued covering manufacturing of microspheres and implants. No attempt will be made to comprehensively review this topic. As will become apparent from the discussion below, ABA-triblock copolymers can be treated in a similar way as PLGA to manufacture PDS. Therefore, some of the more common manufacturing methods for PDS utilized on an industrial scale are briefly described here.

Implants were usually prepared by melt extrusion using commercially available single- or twin-screw extruders. The drug substance and polymers are introduced as solids, heated above the glass transition temperature of the polymer, intensively mixed and then extruded through a nozzle of appropriate size. After cooling the rods are cut to the specified length and packaged. Although an aseptic manufacturing of implants is technically feasible, gamma-irradiation has been used to sterilize the implants in the final packaging.

On a laboratory scale also ram-extruders and compression molding were used for the preparation of implants since screw-extruders require larger amounts (> 100 g) of often expensive drug substances and polymers. Injection molding can also be used for preparing implants but the yields are even lower than in the case of melt extrusion.

For the preparation of microspheres mainly five methods have been used for large-scale production. These techniques can be classified into three categories, namely physico-chemical methods, phase separation methods and emulsion techniques. The polymers are dissolved in

an appropriate volatile organic solvent, e.g. dichloromethane, ethylacetate, and the drug substance is added in solid form to yield a solution or a suspension. Most peptides and proteins are insoluble in these solvents. Therefore, suspensions need to be prepared from sterilized drug substance in the case of aseptic manufacturing. Alternatively, drug can be subjected to microencapsulation as aqueous solution which then is homogenized to form an water-in-oil (W/O) emulsion. The latter method has some advantage for handling proteins and is more straightforward for aseptic manufacturing, since sterile-filtration can be employed to introduce the drug substance into the process.

In the case of physico-chemical microencapsulation methods, the particle/droplet formation occurs in the gas phase. A prototype of this technique is the spray-drying method, where the solvent of the drug/polymer suspension is evaporated by a stream of warm air or drying gas. The microspheres are collected from the drying gas using a cyclone. Spray formation can be performed using different atomizers, such as nozzles, rotary and ultra-sonic atomizers. Particle size and size distribution is mainly determined by the atomizing method and the viscosity of the feed suspension. Spray-drying is a well established method for microencapsulation and can be performed under aseptic conditions [64].

A recent variation of the spray-drying technique is the “cryogenic microencapsulation method”, where the drying/hardening step is not performed by a drying gas, but by lyophilisation of spray-droplets collected at the interface between frozen ethanol and liquid nitrogen [65].

The phase separation technique is performed in solution by adding a non-solvent for the polymer to the above described drug solution or suspension. Usually silicon oil is used to induce the formation of a liquid polymer-enriched phase (coacervate) under intensive stirring, which then encapsulates the drug particles. The “embryonic” microspheres are soft and require removal of solvent and silicon oil by dilution with a large volume of hexane to

become solid. The microspheres are collected by filtration and require rigorous drying to remove residual solvents [66].

The emulsion techniques rely on emulsification as principle for the formation of spherical droplets from which the volatile solvent is removed by evaporation under stirring. Lipophilic drug substances such as contraceptive steroids were manufactured using a simple W/O emulsion technique [67]. Hydrophilic substances can be either introduced as solids or as emulsion (W/O) which is added to an excess of a second water-phase leading to the transient formation of a W/O/W double emulsion. Microencapsulation and hardening occurs by a complicated process involving phase separation and solvent evaporation. Residual water and solvents are removed by lyophilization [63].

All the microencapsulation techniques described above have been scaled up to production size and are currently used to produce PDS based on PLGA on a commercial scale. Since ABA triblock copolymers possess different physico-chemical properties compared to PLGA, in particular solubility in solvents, glass transition temperatures, swelling behavior, etc., adaptation techniques is anything but straightforward.

4.4. Release mechanism of proteins and peptides from PLGA devices

Numerous studies have been carried out to characterize the release behavior of peptides and proteins prepared from PLGA devices. It is generally accepted that at least two release phases can be distinguished. In the initial phase drug release is governed by diffusion of the peptide or proteins through a interconnecting network of aqueous pores while at later stages erosion of the polymeric matrix plays a more prominent role. Pore diffusion is affected by parameters such as drug loading, particle size of the drug, distribution of drug in the matrix and geometry of the device as well as physico-chemical properties such as molecular weight, solubility and distribution coefficient of the drug substance itself.

As outlined in section 3, degradation of polyesters is thought to occur by non-enzymatic random chain scission by hydrolysis leading to a decrease in molecular weight of the polymeric matrix. When the cleavage products become water-soluble, mass loss or erosion of the PDS is observed. Superimposing both, pore diffusion and polymer erosion can lead to more continuous release profiles or to a more or less pronounced lag phase depending on the erosion properties of the polymer.

In the case of peptides and proteins additional factors, namely osmotic effects, swelling of the devices, protein adsorption and ionic interactions e.g. such between polymer terminal carboxylic acid groups and basic amino acids have to be taken into account to explain the release properties of these delivery systems [60].

A particular feature of PLGA degradation is the phenomenon of autocatalysis which leads to a non-heterogeneous degradation of devices. Implants of PLGA have been shown to degrade from the “inside-out” leading to hollow structures after prolonged incubation in buffer media [68, 69]. The degradation of PLGA is thought to be accelerated by oligomers and cleavage products due to the increasing number of carboxylic end groups and a concomitant drop of the pH in the degrading PLGA matrix. Since degradation products can diffuse more rapidly from the surface of the device, a pH gradient should exist within the degrading matrix. Experimental evidence on the spatial and temporal distribution of pH in PLGA was provided using confocal microscopy yielding minimal pH levels of 1.5 in the core area of microspheres [70]. Similar results were obtained earlier with EPR spectroscopy and a pH-sensitive probe coupled to albumin [71].

This acidic microenvironment is known to be deleterious to many proteins [72]. Insufficient peptide and protein stability under acidic conditions can lead to incomplete release of the drug substance from the degrading PDS, mostly by non-specific adsorption of the compound to the matrix material, formation of non-covalently bound aggregates or covalent aggregation [73,

74]. Different approaches for overcoming the deleterious effects of the acidic microclimate such as coencapsulation of basic additives (e.g. $Mg(OH)_2$ or $ZnCO_3$) [109,110] or sugars (e.g. trehalose or gamma – hydroxypropyl cyclodextrin) [111] were used to protect proteins or peptides. Attempts to stabilize proteins by addition of excipients were reviewed recently and demonstrated that generally applicable guidelines cannot be provided yet [74].

Consequently, new biodegradable polymers which overcome the problems described for PLGA are clearly needed. ABA triblock copolymers from biodegradable PLGA (A) blocks and a hydrophilic PEO (B) block could be of general interest in this context, due to their physico-chemical properties, such as microdomain formation, higher hydrophilicity, faster swelling and accelerated degradation. The use of ABA polymers for different PDS will be discussed below.

4.5. Implants/devices from ABA Polymers

Several implants for parenteral use have become commercially available containing LHRH agonists, such as goserelin (Zoladex ™) or buserelin (Depot-Profact ™) in PLGA. Depending on the indication, controlled release of the peptides occurs over a time-span of 1-3 months. Implants for therapeutically relevant proteins based on PLGA have not been reported to our knowledge, most likely for the reasons discussed above.

On the other hand have environmentally interactive monolithic devices made with hydrophilic polymers been first proposed by Hopfenberg [75], and in this context also poly(ethylene oxide), PEO, was considered. Compression-molded tablets from PEO with a relatively high molecular weight of 600'000 to 4'000'000 g/mol containing etofylline as model compound were investigated under in vitro conditions. Drug release from high Mw PEO was controlled by swelling, whereas release from low Mw PEO was related to polymer dissolution [76]. These findings illustrate two problems. Firstly, for controlled release of non-crosslinked PEO relatively high molecular weights are necessary and secondly, that these high Mw preclude

their use in parenteral drug delivery systems, since high Mw PEO is not degraded and eliminated from the body.

Also crosslinked hydrogels from a crystalline-rubbery poly(ethylene oxide) network have been used for studying the controlled release of prostaglandin E as early as 1980 [77]. Water-insoluble ABA triblock polymers can be considered as devices which form a physically crosslinked structure upon exposure to water as will become apparent from the discussion below.

The melting temperature of PEO (ca. 60° C) is significantly reduced or abolished in ABA polymers depending on PEO content and molecular weight, yielding PDS which become viscous liquids at moderately elevated temperatures (50-60°C). By thorough mixing with drug substances at this temperature biodegradable polymeric paste formulations are obtained, which can be used to administer drug substances during surgery ("surgical pastes") for local delivery with antibiotic, cytotoxic or anaesthetic activity. For example, paclitaxel, a potent inhibitor of micro-tubuli formation, was mixed into melted poly(DL-lactide)-b-poly(ethylene glycol)-b-(DL-lactide) (PDLLA-PEO-PDLLA) copolymers and blends of low molecular weight poly(DL-lactic acid) and poly(ε-caprolactone) (PDLLA/PCL) to obtain such paste formulations. These pastes are administered at tumor resection sites where a controlled release of a cytostatic agent may potentially destroy remaining tumor cells and prevent local recurrence of disease. The release of paclitaxel from ABA paste formulations with 20% loading into PBS albumin buffer was controlled by PEO content over a period of up to 2 months by combined diffusion and polymer erosion mechanism. The efficacy of these pastes in inhibiting growth was evaluated in a tumor mouse model placing the paste s.c. to tumor sites. After 16 days, the reduction in tumor weight was measured. Both the paclitaxel loaded ABA and 90:10 PDLLA/PCL blend formulations significantly inhibited tumor growth in mice. The pastes with faster in vitro release rates resulted in greater efficacy in inhibiting

tumor growth. The results showed that biodegradable polymeric surgical pastes are promising formulations for the local delivery of paclitaxel to inhibit tumor growth [78].

PEO is also a widely used plasticizer and facilitates processing of polymers by melt extrusion or compression moulding. In ABA polymers PEO acts as an intramolecular plasticizer reducing thermal processing conditions to become acceptable for handling of proteins. ABA and PLGA were used to prepare implants by either compression moulding or extrusion using a laboratory ram extruder. Insertion of an elastoplastic B-block did not lower the processing temperature, but the entanglement of the polymer chains was significantly reduced as can be seen from the diameters of the extruded rods, allowing processing at 60- 80°C [44].

The swelling behavior upon incubation was studied using ^{1}H-NMR spectroscopy, demonstrating that protons in the PEO B-blocks of the swollen ABA copolymers were mobile, while those of the A-blocks remained rigid during incubation. The analysis of the pH inside ABA rods using electron paramagnetic resonance, EPR, yielded a pH of 5.2 after incubation with a subsequent increase to pH 6.0 during the first day, approaching the pH of the medium after nearly 33 d. Acidic degradation products did not accumulate inside the ABA rods. These findings are in marked contrast to PLGA where a rapid decrease of the pH in the degrading matrix to pH values as low as pH 1.5 were noted. Degradation and erosion started immediately and concomitantly upon incubation. By contrast, PLG rods showed the typical sigmoid erosion profile with a clear lag phase. The influence of device geometry was insignificant for PLGA [44].

An analysis of the swelling of tablets containing 5-fluorouracil and ABA polymers consisting of short PCL blocks with a molecular composition $PCL_6PEO_{90}PCL_6$ was carried out by Martini and coworkers [79]. In this case a non-zero order release kinetic was observed which was attributed to a gradual increase of the gel layer thickness caused by a lack of synchronization of the hydration and erosion process. Since no data on degradation and

erosion of the devices were provided, a direct comparison with PLGA A-blocks is difficult, but hydration and swelling properties are mainly controlled by the ratio of A/B blocks.

Also compressed tablets were used to study the release of vitamin B_{12} and tetracycline from ABA polymers with low, medium and high hydrophilicity. For both drug substances fairly rapid in vitro release rates were seen at low (1-2% loading), which argues more against the device manufacture than the polymers [80].

An interesting case history for the use of PLA implants in comparison to ABA polymers is delivery systems for bone morphogenetic proteins (BMP). Different homopolymers of PLA with molecular weights of 105,000, 21,000, 3300, 650 and 160 g/mol were evaluated as carriers for BMP. Devices consisting of 4 mg of water-soluble, semi-purified BMP and 100 mg of PLA were implanted into the dorsal muscles of mice. Implants from PLA105000, PLA21000, PLA3300, and PLA160 failed to induce new bone formation. PLA105000, PLA21000, and PLA3300 elicited foreign- body reactions or chronic inflammation (or both), and PLA160 produced tissue necrosis. Only PLA650/BMP implants induced cartilage formation within one week and induced bone with hematopoietic marrow at three weeks post implantation. PLA650/BMP composites were completely absorbed and replaced by new bone. These results highlight the narrow balance between polymer degradation, protein release and local tissue reaction for achieving pharmacologically active devices [81].

Comparing PLA650 with an ABA polymer (PLA_{650}-PEO_{200}-PLA_{650}) in mice, three weeks after implantation, the ABA/BMP composites were completely absorbed and replaced by newly induced bone with hematopoietic marrow. The composites induced twice as much bone as implants of BMP and a PLA_{650} homopolymer [82]. To improve the mechanical properties of these polymers, the authors modified the composition of the ABA polymers. From screening studies in mice using rh-BMP-2 the authors found that A-block sizes of 6500 and B-blocks of 3000 g/mol produced the highest amount of bone formation three weeks post

implantation [83]. These data suggest that molecular weight and ratio of PLA/ PEO are essential factors determining the efficacy of a BMP delivery system based on ABA block copolymers. After implantation, ABA pellets might have absorbed tissue fluids and become swollen, resulting in bone formation that exceeded the size of the original implants. This swelling was thought to be a potentially beneficial property, given the intended practical application of the polymer in the repair of bone defects [84].

One shortcoming of ABA polymers is the relatively slow degradation of residual amounts of the A blocks under in vitro and in vivo conditions [44, 56]. Therefore, Saito et al. explored copolymerization of PLA with p-dioxanone (DX) as a way to accelerate the degradation of the A block, in analogy to [26]. A molecular weight of 9500 g/mol and a molar ratio of PLA:DX:PEO = 45:17:38 was selected for further studies. Indeed a complete erosion was observed after 12 d of incubation with PBS at 37°C. PLA-DX PEO/rhBMP-2 implants induced ectopic new bone formation more effectively than collagen in a time- and dose - dependent manner when tested in vivo. These implants were shown to induce repair of large bone defects in the rat ilia. The authors conclude that ABA polymers represent an advance in the technology for the enhancement of bone repair [85].

The use of pre-swollen hydrogels from ABA polymers has recently been suggested by Molina et al.. Both, fibrinogen and BSA were incorporated into the swelling hydrogel. While for fibrinogen almost linear release was noted, BSA release was more comparable to a matrix-diffusion mechanism. The reason for this release behavior could be phase separation because of incompatibility between PEO segments and fibrinogen. More work is necessary to elucidate this interesting release mechanism [86].

In summary, implants from ABA polymers are advantageous for PDS containing sensitive proteins both with respect to degradation and protein stability and merit further investigations. While some guidelines have emerged regarding the design of those delivery systems, more

work is necessary to establish the relation between ABA structure on one hand and functional properties, such as degradation rate and biocompatibility on the other hand. The potential of ABA polymers for “in situ” forming implants has not yet been fully explored.

4.6. Microspheres from ABA Polymers as delivery systems for proteins

As discussed in section 4.3 various techniques have been developed to encapsulate bioactive materials. Although biodegradable ABA polymers have been considered mainly for hydrophilic macromolecular drugs, such as peptides, proteins and oligo/polynucleotides, it should be noted, that “lipophilic” ABA triblock copolymers consisting of poly(ε–caprolactone), PCL, and PLA have been used for the microencapsulation of levonorgestrel using a O/W emulsion-solvent evaporation process. Constant release rates of 0.4- 1.0 µg/d/mg from microspheres were maintained for up to 6 months. In vitro release could be adjusted by copolymer composition and an increased erosion compensated declining drug diffusion from the PCL phase. These microspheres have shown potential as long-acting contraceptives for family planning [87].

To the best of our knowledge, the first report on microencapsulation of hydrophilic macromolecules using ABA triblock copolymers consisting of PLGA A-blocks and a hydrophilic PEO B-block appeared in 1994 [88]. Microspheres prepared from these biodegradable polyesters show striking differences in their release behavior for the macromolecular model compounds, such as FITC-dextrans (molecular weight 4000-500,000 g/mol), when compared to poly(DL-lactide-co-glycolide) as demonstrated in figure 11.

While the release from PLG is biphasic and almost independent of the molecular mass of FITC-dextran, ABA polymers show a continuous and molecular mass-dependent release, reminiscent of cross-linked hydrogels. The release of bovine serum albumin from microspheres prepared from different ABA polymers demonstrated that molecular weight, ratio of A/B blocks and lactyl/glycolyl ratio in the A block affected the degradation, leading

to a continuous and complete release of BSA from microspheres. By manipulation of the PEO content in vitro release rates could be adjusted in the range of 10 to 30 days [7].

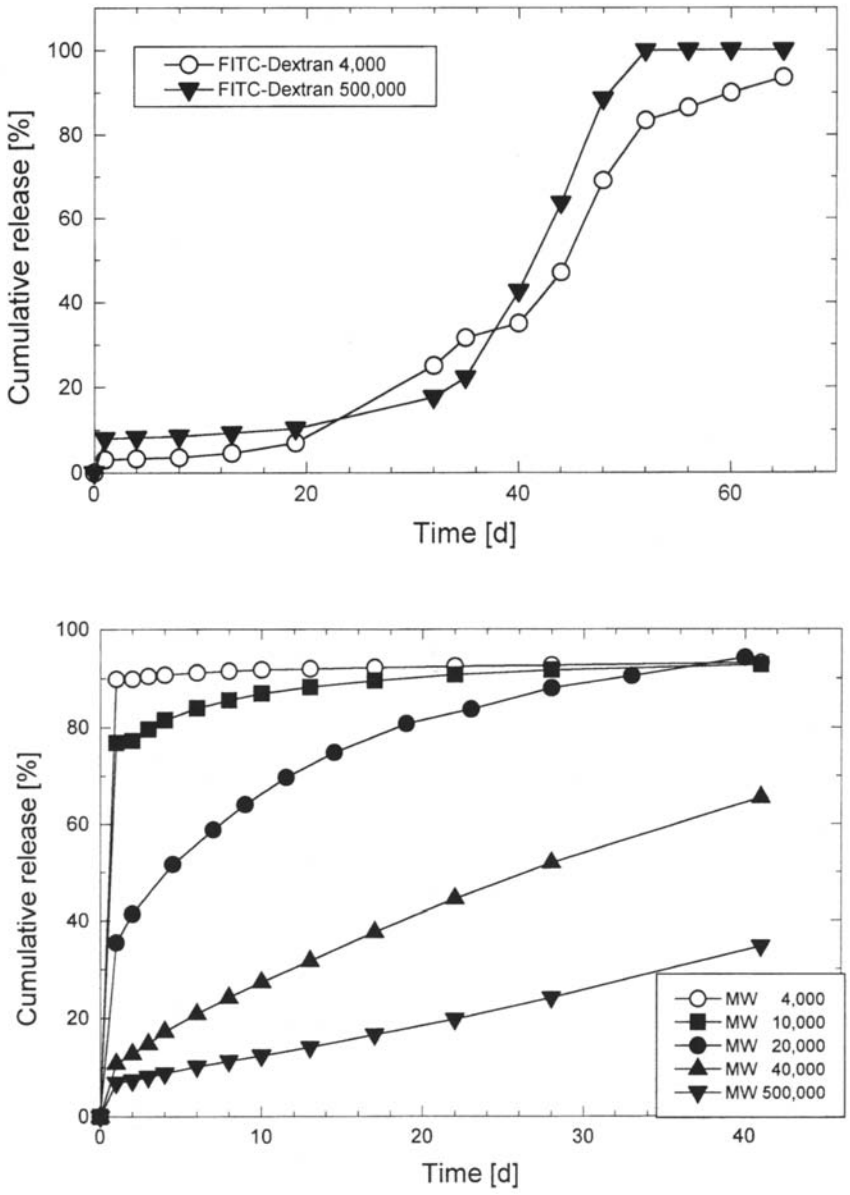

Figure 11: *Release of FITC-Dextran of different molecular weights from PLGA and PLA-PEO-PLA microspheres under in vitro conditions [89].*

Several model proteins were then microencapsulated using a modified W/O/W double emulsion method and an ABA triblock copolymer with a molecular weight of ca. 30,000 g/mol and a PEO B-block 10,000 g/mol. The molar composition determined by NMR spectroscopy was LPLA:GA:PEO = 48:14:38. Such diverse proteins as cytochrome c (13

kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and tetanus toxoid (150 kDa) could be microencapsulated with very high efficiency and release was continuous and complete over 20-50 days as demonstrated in figure 12. We attribute this change in the release behavior compared with PLGA to a combined mechanism of swelling and erosion, leading to a hydrogel-like structure, also observed by scanning electron microscopy (figure 13) [89].

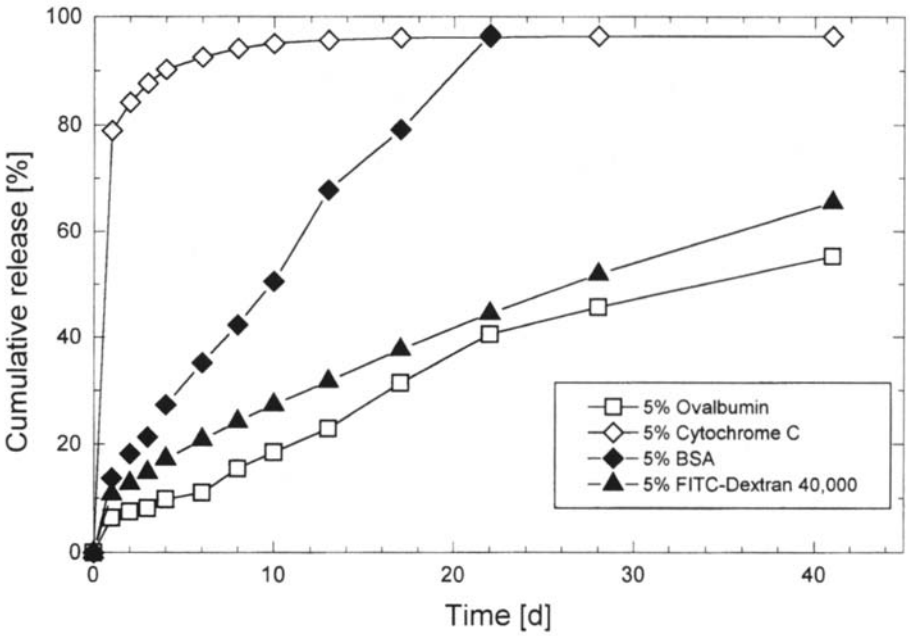

Figure 12: *In vitro release of proteins from ABA microspheres [89].*

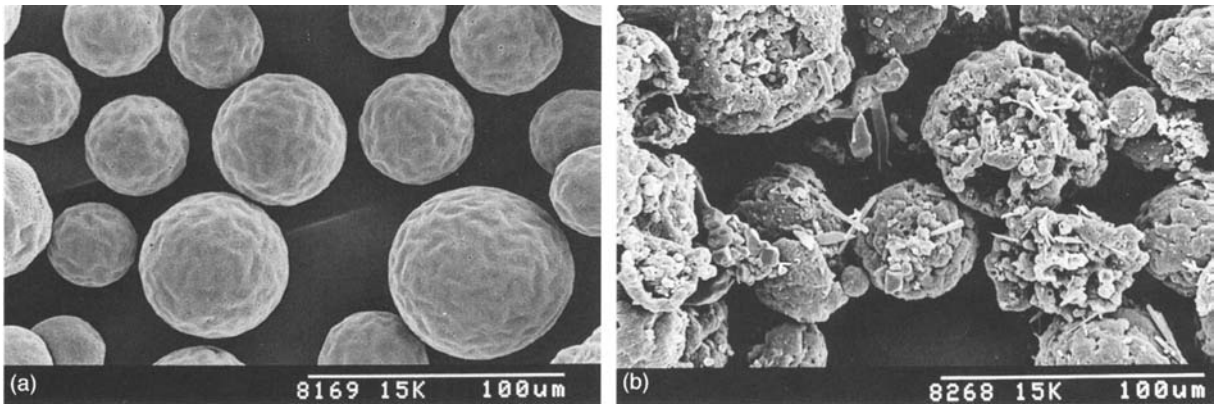

Figure 13: *Scanning electron micrographs of ABA Microspheres from [89].*

Also low molecular weight drugs, namely lidocaine and propranolol hydrochloride were encapsulated into PDLLA-PEO-PDLLA using different microencapsulation techniques. In vitro release rates from microspheres were generally higher for the ABA polymers than for PLA and hydrophilic propranolol hydrochloride was released more rapidly than lipophilic lidocaine base at the same drug loading. The o/w emulsion technique for hydrophobic drugs was preferred over phase separation and W/O/W double emulsion, because it provided appropriate porosity. Unfortunately a direct comparison with other studies is not possible, because no data on degradation and erosion were provided, but it demonstrates that not only diffusion of hydrophilic molecules through aqueous pores, but also distribution phenomena changing upon hydration of the ABA microspheres need to be considered [90].

Recombinant human erythropoietin (EPO), a 30,400 g/mol glycoprotein consisting of 165 amino acids, is the main regulator and growth factor of red blood cell production. EPO is an interesting candidate for a parenteral depot system due to its rapid clearance from circulation and instability. EPO is used for the treatment of renal anemia and related conditions. Also EPO is known to be sensitive to aggregation and degradation posing many problems for the formulation of a parenteral depot system . Biodegradable microspheres containing EPO were prepared from ABA triblock copolymers using a modified double- emulsion process (W/O/W). The encapsulation efficiency for EPO, ranging from 72% to 99% was quite acceptable. ABA polymers with a molecular weight of ca. 18,500 g/mol and a composition of LA:GA:PEO=51:16:33 were found to produce acceptable drug bursts. The formation of high molecular weight EPO aggregates, however, was higher than in PLGA microparticles, leading to an incomplete release of EPO from ABA microspheres. Using different excipients with known protein stabilizing properties, such as serum albumin (BSA), poly(L-histidine), poly-(L-arginine) or a combinations with dextran 40, the EPO aggregate content was significantly reduced, allowing a low ,continuous release of EPO from microspheres for up to 2 weeks

under in-vitro conditions. Comparing in vitro release profiles from ABA microspheres of EPO to those of FITC-dextran 40, a non-aggregating hydrophilic macromolecule of similar molecular weight, a very similar pattern is observed in the initial release phase, while EPO release was leveling off at later time points. These findings suggest, that EPO aggregation caused a discontinuation of the release process [91]. It was interesting to note that blends of PLGA and PEO also did not generate continuous EPO release profiles, but rather induced EPO aggregation [108].

LPLG-PEO-LPLG triblock copolymers (35% PEO; Mw 30,000 g/mol) in combination with 5% serum albumin yielded both, an acceptable level of EPO aggregates and a continuous release profile under in-vitro conditions for up to 2 weeks. The formation of EPO aggregates at later time points is possibly induced by acidic cleavage products of the biodegradable polymer or, more probable, by the presence of PEO itself and requires further optimization of the ABA polymer composition [91].

To further elucidate the effect of ABA composition on degradation and erosion properties as well as the pH, microspheres were prepared using W/O/W double emulsion technique. Degradation and mass loss occurred faster in the ABA polymers than in PLGA of comparable molecular weights. Erosion commenced immediately after incubation in different buffer media. Both pH and ionic strength of buffer media affected mass loss (erosion) and degradation of ABA polymers, while there was no effect on PLGA, in accordance with data from the literature [45]. The release properties of ABA (35% PEO; Mw 30,000 g/mol) and PLGA microspheres in relation to polymer degradation were then studied under in vitro conditions. The release of BSA from ABA microspheres paralleled the faster swelling and erosion rates compared to PLGA. This was also confirmed by electron paramagnetic resonance, EPR, measurements with spin labeled albumin, whereby an influx of buffer medium into the ABA microspheres was already observed within a few minutes. In contrast,

PLG microspheres revealed a burst release without any erosion. This study demonstrated that the environmental conditions affect the degradation and erosion of ABA microspheres in the same way as the release of the model protein, showing rapid exchange of protons with the surrounding fluid. This behavior lead to a more favorable degradation and release profile compared to PLGA [45].

As mentioned earlier, EPO was found to be a protein reacting very sensitive to aggregating influences. The formation of EPO aggregates was not caused by the microencapsulation process or the pH change during degradation, but rather by the presence of PEO itself. PEO can be used to precipitate proteins [112] and hence may induce aggregation of sensitive proteins such as EPO. In a further attempt to generate a viable EPO delivery system, we studied star-block copolymers consisting of L-lactide, glycolide and branched multi-arm poly(ethylene oxide) [28]. While mass loss and molecular weight degradation were of comparable order in the initial phase, loss of branched PEO seemed to be reduced compared to non-branched ABA polymers [92].

Microspheres containing EPO or FITC-dextran were prepared using a W/O/W encapsulation method and both ABA (35% PEO; Mw 30,000 g/mol) and star-block AB polymers. In the case of ABA polymers, a higher PEO content yielded smaller microparticles, a lower encapsulation efficiency, and a higher initial drug release both in the case of EPO and FITC-dextran. The investigation of technical parameters on microsphere formation showed that the process temperature plays an important role. Microsphere formation at +1°C produced higher drug loadings without increasing the amount of residual dichloromethane inside the particles. Other parameters such as the homogenization of the primary W/O emulsion and of the W/O/W double- emulsion have less impact on microsphere characteristics. Star-block AB polymers also showed potential for the preparation of drug loaded microspheres. EPO loaded microspheres could be produced under optimized conditions. However, the amount of EPO

aggregates was comparable to ABA polymers, and a continuous release of the protein from these star-shaped polymers could not be achieved [93]. This investigation underscores the fact that our understanding of protein stability and protein release from biodegradable delivery systems is still insufficient to permit predictions. Clearly, more work is necessary to gain more insight into these complex processes.

Controlled release vaccine delivery systems are another research area where new approaches and new biomaterials with improved properties enhancing protein stability in devices might be of critical importance [61]. The microencapsulation of antigens using biodegradable polymers allows either continuous or pulsatile release patterns. As biodegradable polymers mainly PLGA has been utilized, since these materials are known to be biocompatible and non-toxic. Also additional adjuvant effects, increased shelf-life and avoidance of a cold chain are issues for parenteral vaccine delivery systems. Using different microparticles that release antigens in a pulsatile pattern at predetermined time points one hopes to induce protective immunity by a single administration of the vaccine delivery system. Tetanus toxoid (TT), a protein mixture of ca 150,000 g/mol was used to investigate protein stability during microencapsulation, in vitro release and storage of TT microparticles. TT is known to be sensitive to changes in pH conditions (pH 5) and to thermal stress. TT microparticles can be prepared by a W/O/W double emulsion technique with satisfactory encapsulation efficiencies in good yields. In accordance with other investigators we observed an adjuvant effect of TT microspheres in mice upon s.c. administration leading to a long-lasting antibody response. The issue of an ideal release pattern remains open, since a boosting of the antibody titers during the bioerosion of the TT microspheres was not observed, possibly due to inactivation of TT in the degrading microspheres [94].

We then used TT as a model antigen to the induction of antibody titers in mice using ABA (35% PEO; Mw 30,000 g/mol) polymers in comparison to PLGA. While the release of TT

from PLG microspheres was found to be biphasic, ABA polymers showed a continuous, almost linear release of TT reaching 80% release after 30 days. Immunization of mice demonstrated, that tetanus toxoid is also released under in-vivo conditions, leading to a prolonged antibody response very similar to PLGA. Although there is some controversy in the literature regarding the optimal immunization scheme, we believe that the lack of a booster effect seen with PLGA microspheres containing TT can be explained by inactivation of the protein inside degrading microspheres. Although protein degradation of TT is reduced in ABA polymers compared to PLGA, it should be noted that more than 50% of immune reactivity is lost during this experiment [94]. Further stabilization of TT and similar antigens under in vitro and in vivo conditions are necessary to make the single shot vaccine a realistic goal.

In a further study, we extended this concept to even more sensitive viral antigens. A monovalent influenza split vaccine was microencapsulated in PLGA and ABA (35% PEO; Mw 30,000 g/mol) polymers using a W/O/W double emulsion technique [95]. To stabilize the antigen, influenza vaccine was also co-encapsulated with liposomes. Antigen release from microspheres was determined in vitro using a hemagglutinin-specific ELISA. PLGA-microspheres with liposomes released immunoreactive hemagglutinin in a pulsatile manner, a preferred feature for the development of a single dose vaccine delivery system. Influenza hemagglutinin specific IgG and neutralizing antibody responses were studied in BALB/c mice following subcutaneous injection of different microsphere preparations. PLGA-microspheres elicited a significantly higher primary IgG response compared to non-encapsulated antigen. ABA- microspheres seemed to be less immunogenic than PLGA-microspheres based on the IgG antibody response. However, similar levels of neutralizing antibodies were observed after eight weeks with both polymers. Entrapment of the antigen in liposomes prior to microencapsulation did not further enhance the immune response. The immuno-potentating

effect of the antigen-loaded microspheres was prominently enhanced when they were given as suspension in fluid antigen, suggesting that free antigen may serve as priming and microencapsulated antigen as booster dose. Eight weeks after a single subcutaneous immunization with PLGA or ABA- microspheres neutralizing antibodies were as high as those obtained after two subcutaneous administrations of fluid vaccine four weeks apart [95]. The microencapsulation of influenza split vaccines may have potential for a single dose vaccine delivery system with adjuvant properties. Both ABA and PLGA seem to be suitable polymers for this purpose.

Also other bacterial antigens were studied, such as outer membrane proteins from leptospira interrogans. ABA polymers consisting of LPLA A-blocks and PEO (11.5 mol%; 58,900 g/mol) designated as PELA were used to encapsulate extracts of outer membrane protein (OMP) using a W/O/W double emulsion technique. A small volume of internal W_1 phase and intermediate volumes of organic phase were favorable to achieve microspheres with a size of 1-2 µm and high antigen encapsulation efficiency (70-80%). In vitro OMP release profiles from ABA microspheres consist of a small burst release (20%) followed by a gradual release phase up to 60% after 30 days [96]. Using the same approach also outer membrane protein (OMP) from Vibrio cholera was microencapsulated into ABA polymers with a suitable size for oral vaccination (0.5-5 µm). High loading efficiency (about 60%) and a low level of residual solvent (lower than 20ppm) were obtained. A higher OMP loading efficiency was achieved by adding NaCl or adjusting the pH at the isoelectric point of OMP in the external water phase. In vitro release rates were similar as described above [97]. Data on antigen integrity or antibody titers in animals or control experiments with PLGA were not provided, rendering a comparison of these results with other antigens or ABA polymers impossible. The effect of ABA composition in the range of 0-30% mol PEO and corresponding molecular weights from 60,000 to 18,000 g/mol on in vitro degradation of the polymer and release of

human serum albumin from microspheres prepared by a W/O/W double emulsion method was reported by Li et al.[98]. They confirm for DLPLA-PEO-DLPLA, that similar degradation rates and BSA release rates are observed as with LPLA-PEO-LPLA [7].

In summary, ABA microspheres as parenteral protein depot systems have clear advantages over PLGA regarding drug loading, control of protein release rate and protein compatibility with the ABA polymer. A very crucial issue is the protein stability in the polymeric matrix during release conditions. It seems that quantitative predictions for therapeutically relevant proteins are not (yet) possible, mainly because of a lack of basic knowledge and models. More interdisciplinary work on protein stability in PDS would be desirable to fully exploit the potential of these protein delivery systems.

4.7. Nanoparticles from ABA Polymers for drug targeting

While microspheres and implants are deposited at their subcutaneous or intramuscular injection or implantation site and release the drug substance in a controlled manner to the systemic circulation, the concept of injectable nanoparticles differs in several aspects.

Nanospheres or nanoparticles are delivery systems of similar morphology as the microparticles discussed above, but with a much smaller particle size <1 µm = 1000 nm. These nano-carriers are smaller than typical blood cells, such as erythrocytes or lymphocytes (ca. 7-10 µm) and should, therefore, allow injection into the blood stream. After intravenous injection, they achieve site or tissue specific delivery by exploiting physiological clearance mechanisms in the body. Release of the drug substance then occurs after uptake of the nanoparticles inside the cells of the target tissue. Numerous applications can be conceived, such as a dose reduction as a consequence of targeting, more favorable pharmacokinetic properties of the delivery system, protection of proteins against enzymatic degradation, reduction of toxic side effects in non-target tissue etc. Unfortunately most particulate delivery systems are eliminated by the reticuloendothelial system (RES) within minutes irrespective of

their chemical composition after intravenous injection. These particles accumulate in macrophages of liver and spleen by phagocytosis. Targeting to macrophages of the RES may be desirable in some clinical situations, where parasites or microorganisms “hide” inside macrophages, e.g. leishmaniasis, but for reaching non-RES targets, such as tumor cells, avoidance of recognition by the RES is considered to be a major obstacle to passive drug targeting [5].

Uptake of nanospheres and liposomes is caused by the adsorption of blood components to the particle surface. These so-called “opsonins” promote phagocytosis, whereas dysopsonins suppress particle uptake. Components of the complements system (C3 and C4) as well as immunoglobulins are well-known opsonins. To achieve long circulation time in blood and consequently localization in non-RES tissues, surface modification of nanospheres using PEO has been studied intensively.

ABA triblock copolymers (Mw 29 000-147 000) containing LPLA A-blocks attached to central PEO B-blocks of two molecular weights (Mw 6600, 20 000) were used to prepare ^{3}H-progesterone nanoparticles with an average diameter in the range of 152-377 nm. The effects of the polymer characteristics on the pharmacokinetics of the nanoparticles and the biodistribution of the nanoparticles were studied in rats after intravenous injection at a 1 mg/kg dose. Circulation-times were affected by the total Mw of ABA polymer, the PLA/PEO ratio, the PEO Mw and the polydispersity index Mw/Mn. Highest circulation times of ca. 7 h were obtained with ABA (Mw 83,000 g/mol; 10% PEO 6000) and a broad polydispersity index. The $t_{1/2}$ in circulation of PLA nanospheres was ca. 4 h, which suggests only a modest improvement by a factor less than 2x. The Purification of ABA polymers was important for the controlling of the drug burst. ABA nanoparticles were taken up by liver and spleen to a significantly reduced extend ca. 20% versus ca. 45% for PLA, but it should also be noted that the mass balance was only ca 50-70% and non-stealth PLA should have given higher

accumulation in RES. The authors explain these results by the avoidance of adsorption of opsonins to ABA nanoparticles as a result of the orientation of PEO loops on the surface of the particles [99].

In this study, the nanoparticles of ABA polymers synthesized by ring opening polymerization in bulk with subsequent fractionation were prepared by a solvent evaporation method employing ultrasonication [20]. Drug entrapment efficiencies were around 70% and the weight-averaged mean diameters of the nanoparticles were < 335 nm. Drug release increased with PEO/LPLA ratio and was relatively rapid under in vitro conditions. The polydispersity index of ABA polymers were unusually high at ca. 12 and required fractionation. ABA with high polydispersity showed slower release rates. Degradation under in vitro conditions is in the 10-15% range after 24 h, probably not contributing to the release process in a significant manner [100].

In a fundamental study, De Jaeghere compared different approaches to stabilize nanoparticles by either physically adsorbed or covalently bound PEO coatings. Nanospheres were produced from various combinations of PLA and AB or ABA polymers by the salting-out process and purified by the cross-flow filtration technique. The amount of PEO at the nanoparticle surface, as well as the residual amount of emulsifier poly(vinyl alcohol) were assessed. Phagocytosis of the different types of PEO-stabilized nanoparticles was compared by flow cytometry after incubation with human monocytes in serum and in plasma under in vitro conditions. The influence of the PEO molecular weight and surface density on the particle uptake was especially marked for the diblock and triblock copolymer formulations, with a decrease in uptake of up to 65% with one of the diblock copolymer formulations. Nanoparticles made of triblock copolymer with short PEO chains at their surface in the postulated "loop conformation" proved to be as resistant to cellular uptake as nanoparticles made of diblock copolymers with PEO chains in the "brush conformation" [101].

Using the same polymers and technology, redispersion of nanoparticles after freeze-drying under various conditions was assessed. The surface of the nanoparticles was characterized and classified in terms of "brush" and "loop" conformations. Upon freeze-drying, it appeared that the presence of PEO at the nanoparticle surface could severely impair the redispersibility of the particles, especially in the PEO-grafted systems. This effect was shown to be related to the amount and molecular weight of PEO in the various formulations. In most cases, particle aggregation was prevented by use of trehalose as lyoprotective agent. Increasing the concentration of particles in the suspension was shown to induce much less damage to the nanoparticles, and freezing the suspension at a very low temperature (-196 °C) was found to further improve the lyoprotective effect. Most of the lyoprotected nanoparticles remained stable for at least 12 weeks at 4 and -25 degrees C. The authors conclude that production and preservation of freeze-dried PLA-PEO-AB diblock and ABA triblock copolymer nanoparticles is feasible under optimized lyoprotective conditions [102].

Also ABA triblock copolymer based on PCL as hydrophobic A-blocks were studied. Core-shell type nanoparticles from PCL-PEO-PCL polymers were prepared by a dialysis technique. According to the amphiphilic characters, these block copolymer can self-associate at certain concentration and their critical association concentration (CAC) was determined by fluorescence probe technique. CAC values were in the order of 0.0030 g/l and decreased with increasing of PCL chain length. Particle size of nanoparticles was 32.3+/-17.3 nm with a monomodal and narrow distribution. Particle size, drug loading, and drug release rate of nanoparticles were changed by the initial solvents and the molecular weight of ABA. The release kinetics of the lipophilic model drug clonazepam were affected by Mw of the A-block and drug loading and were dominated by diffusion mechanism. Degradation of nanoparticles was relatively slow with 17% in 30 days.

In summary, one can conclude that the preparation of nanospheres from ABA polymers is

feasible and that small particle sizes can be attained using different technologies. Stabilization of the nanospheres by lyophilization seems to be a promising approach to obtain commercially viable products. Loading of the ABA nanospheres with lipophilic drug substances seems to be possible, data on more attractive compounds and hydrophilic molecules are yet to be developed. Release under in vitro conditions is relatively fast, but probably sufficient for targeting purposes. Avoidance of RES has been demonstrated both on a cellular level and in animal studies, but more work is necessary to further characterize the biological properties of these colloidal carrier systems. From the information available one can conclude that in nanospheres from ABA polymers loops of PEO are associated with the particle surface, an approach which has considerable potential for long-circulating nano-carriers.

4.8. Release Mechanism of Proteins from ABA Polymers

Block or graft copolymers containing hydrophilic and hydrophobic domains allow control of drug transport in and from these devices by the size and shape of the domains and thermodynamic interactions between hydrophilic and hydrophobic components. Using simple free volume-based theories, mean field theory and percolation theory the diffusion in heterogeneous polymer networks can be described [103]. While these theoretical models have been very useful to assess the crosslinked hydrogels, the complicated polymer-polymer phase behavior [104] and changes in the heterogeneous network caused by simultaneous degradation made theoretical predictions of drug release from biodegradable ABA device a challenging task.

Chemically crosslinked PLA-b-PEO-b-PLA prepared by photopolymerization allowed an analysis of the effects of degradation rate on the network mesh size, solute diffusivity and drug release profiles of lysozyme and BSA from those devices [105]. Relationships developed were used to predict the effects of cross-linking density and hydrolytic degradation rate on the

controlled release of proteins from these hydrogels. Theoretical drug release profiles, obtained using scaling laws, agree qualitatively with experimental observations and show a decrease in the protein release rate with an increase in protein size, extent of macromer functionalization, and macromer concentration. Quantitative predictions of release behavior were possible under certain experimental conditions. In these chemically crosslinked hydrogels the PLA block size is well defined and the number of hydrolytically labile ester bonds can be calculated. For physically crosslinked hydrogels the change in crosslink density as a function of degradation is more difficult to estimate and hence to our knowledge a predictive theoretical model has not been put forward. From a phenomenological point of view, however, release rates of solutes from photochemically and physically crosslinked devices show clear similarities [7, 86].

The morphology of ABA triblock copolymers from PLGA and PEO is also not very well documented and requires further work. Films prepared by solvent casting showed for instance a preferential orientation of the PLGA to the surface in the dry state, as documented by XPS and SSIMS analysis [106]. After exposure to water, swelling and a rearrangement occurs leading to a preferential orientation of PEO loops to the surface of the device as documented by phagocytosis experiments [101]. The swelling kinetics of ABA polymers can be very rapid and systematic studies of the morphology in the dry state as a function of ABA composition demonstrate that PLA block length is the critical parameter [4, 41].

Biodegradable ABA triblock copolymers are potential material for the controlled release of protein or other macromolecular drug substances. The release of these substances is controlled both by their diffusion from the polymer and the erosion of the matrix. The high hydrophilicity of the PEO block leads to a higher water content in the matrix, leading to a physically crosslinked hydrogel structure. Continuous release profiles of proteins with a lower

initial burst were obtained, compared to conventional poly(lactic-co-glycolic acid) microspheres [7, 88].

4.9. Protein interactions with ABA Polymers

The interactions between ABA polymers and proteins are not very well documented and more data would be desirable. From a swollen hydrogel structure one would expect less deleterious conditions than in PLGA matrices, because rapid rehydration of the protein, less interactions with the hydrophobic PLGA matrix and avoidance of an acidic microenvironment should occur. The microenvironment of an encapsulated model protein during the release from biodegradable microparticles (MP) made from three different polymers, namely poly(lactide-co-glycolide) (PLG) and ABA-triblock polymers containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic PEO B blocks with an A:B ratio of 90:10 (ABA10) and 70:30 (ABA30) was recently reported. Microspheres loaded with spin labeled albumin were prepared by a W/O/W technique and characterized non-invasively using 2 GHz EPR spectroscopy [71]. Water penetrated rapidly into all microspheres in the range of few minutes. A burst release was observed for PLGA microspheres. The release from ABA triblock copolymers continued for over 14 days despite the rapid solubilisation of the protein inside the microparticles. The initial microviscosity of the protein environment inside the ABA particles after exposure to buffer was 2 mm^2/s and increased with time. A gradual decrease of the pH to a value of 3.5 was observed within the microspheres. The results clearly demonstrate that ABA-block polymers are superior to PLGA allowing a controlled release of proteins from swollen microspheres. It would be interesting to study also proteins which have shown some aggregation in ABA devices, such as EPO or BMP-2 to delineate the aggregation mechanism in more detail.

For nanoparticles from ABA polymers used for drug targeting, the adsorption of plasma proteins is an important consideration to achieve long-circulating carriers in blood. The

surface properties can influence the biodistribution of those carriers. Polymer composition and the production method of spherical ABA carriers on the in vitro plasma protein adsorption were investigated using two-dimensional electrophoresis (2-DE) [107]. Both, polymer composition and, also the encapsulation technique, influenced the interactions with plasma proteins significantly. For example, the relative ratio of several apolipoproteins in the plasma protein adsorption patterns were distinctly higher with the spray-dried spheres compared to the W/O/W- technique. Possibly, polyvinyl alcohol used as stabilizer in the W/O/W-technique influences the surface properties relevant for protein adsorption. The plasma protein adsorption on particles composed of ABA copolymers was drastically reduced when compared to microspheres made from PLGA. The adsorption patterns of ABA-particles were dominated by albumin adsorption. These plasma protein adsorption patterns detected on the different microspheres are likely to affect their in vivo performance as parenteral drug delivery systems, but again more work is necessary to systematically explore the relation between surface properties of ABA nanospheres and polymer composition on one hand and effects on biodistribution on the other hand.

5. Conclusions and perspectives

ABA triblock copolymers from hydrophobic PLGA A-blocks and hydrophilic PEO B-blocks have succeeded in making a remarkable transition from a biomaterial mainly considered for surgical applications to valuable component of different drug delivery systems potentially useful for the controlled or site-specific administration of therapeutics such as proteins, oligonucleotides and DNA. Intensive studies are underway to optimize and explore the possibilities offered by these new materials.

The main advantage of ABA polymers over PLGA is their rapid swelling upon exposure to water, providing a friendly environment for sensitive macromolecular drugs. The release of proteins from ABA polymers differs clearly from that seen with PLGA and can be best

explained by a physically crosslinked hydrogel morphology. More work is necessary to establish predictive models describing drug diffusion and hydrogel erosion in a quantitative fashion.

The compatibility and stability of proteins with ABA polymers is another area which has not sufficiently been explored. Data describing the stability model proteins such as BSA and others in ABA devices during manufacturing, storage and release under in vitro and in vivo conditions are somewhat contradictory to results obtained with therapeutically relevant proteins, e.g. BMP-2, EPO or TT. Although in the majority of cases stabilizing additives will be necessary, the BMP-2 case history demonstrates that optimization of the ABA structure may be successful in overcoming stability issues for protein delivery systems. This aspect merits further attention and systematic studies delineating the degradation and/or aggregation mechanism of relevant proteins in biodegradable ABA devices as a function of polymer properties would be desirable to fully exploit the potential of these new materials.

With respect to manufacturing techniques for parenterally administered dosage forms, several trends can be distinguished. While devices and implants were successfully prepared using conventional techniques, such as compression molding and melt extrusion, in situ forming devices have not been studied in great detail. This mode of application was used successfully to deliver BMP-2 in animal studies. In situ forming drug depots are formed when the liquid, viscous polymer/drug solution or mixture comes into contact with physiological fluid.

The microencapsulation method preferred for proteins seems to be the W/O/W double emulsion technique, but also other methods such as spray-drying and phase separation were shown to be suitable for the encapsulation of a wide variety of compounds. Due to lower glass transition temperatures of ABA polymers compared to PLGA some adaptations are necessary. Since many proteins and peptides are sensitive to γ–irradiation, manufacturing under aseptic conditions will be necessary. This technological issue has not been studied in detail so far.

Modulation of the biodistribution of drug substances using ABA nanospheres has recently been investigated and holds some promise. The encapsulation of proteins into ABA nanospheres is a challenging task yet to be accomplished. Also more data on biodistribution and clearance of these carriers would be desirable to evaluate their potential in relation to AB block-copolymer nanoparticles or coatings.

In summary, there is considerable potential in using physically crosslinked hydrogels for protein drug delivery. Some ground-rules have been established since the late 80's but clearly more work is necessary to translate this theoretically attractive concept into therapeutically useful products.

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Chapter 3

Branched Polyesters based on Poly[vinyl-3-(dialkylamino)alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide): Effects of polymer structure on cytotoxicity

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1. Summary

Branched polyesters of the general structure Poly[vinyl-3-(dialkylamino)alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide) have shown potential for nano- and micro-scale drug delivery systems. For further optimization of this polymer class their cytotoxicity needs to be characterized establishing structure-toxicity relationships. Effects of type and degree of amine substitution as well as molecular weight on cytotoxicity were evaluated in L929 mouse fibroblasts using a MTT assay whereas interactions with cell membranes were quantified by LDH release and caspase 3/7-activation. Finally, direct cell-polymer contact assays were conducted. Ungrafted amine-modified polymer backbone yielded IC_{50} values in the range of 0.05 - 10 mg/ml. Generally higher toxicities were observed with an increasing degree of amine substitution. Amine substituents could be ranked as diethylaminopropylamine (DEAPA) < diethylaminoethylamine (DEAEA) < dimethylaminopropylamine (DMAPA) but the degree of amine substitution was more dominant than the type of amine function. Membrane interactions seem to cause necrotic cell reactions in a dose dependent manner for highly charged Amine-PVA backbones. To attenuate cytotoxic effects DEAPA-PVA backbones were grafted with biodegradable PLGA side chains at molecular ratios of 1:10 and 1:20. Cytotoxicity of extracts of these polymers was significantly lower compared to ungrafted polymers possibly caused by shielding of polycationic backbone with hydrophobic PLGA side chains. P(33)-20, a polymer containing a sufficiently high degree of amine substitution could serve as a lead candidate for further investigations. In conclusion, structure-toxicity relationships could be established and shielding the polycationic backbone using PLGA side chains seems to be a promising strategy meriting further investigations.

2. Introduction

Drug delivery systems based on polymeric biomaterials have received considerable interest as parenteral depots [1, 2] and as non-viral gene delivery systems [3]. Most frequently biodegradable polyesters such as poly(lactic-co-glycolic acid), PLGA, have been used due to their excellent track record with respect to biocompatibility and lack of toxicity [4]. Linear PLGA was shown to degrade by non-enzymatic hydrolysis of ester bonds generating an acidic microenvironment for encapsulated drug substances [5] which is detrimental for sensitive peptides, proteins, RNA and DNA [6, 7]. New biomaterials which allow the design of degradation and release properties would be of considerable interest.

Branched polyesters have been shown to possess physico-chemical properties vastly differing from their linear counter-parts, such as solution behavior, melt rheology, mechanical properties, and crystallinity [8]. Their potential for drug delivery has not been exploited to a large extend although degradation and release properties are known to be influenced by the polymer architecture. The influence of polymer architecture and drug delivery has recently been reviewed [9]. Biodegradable, branched poly(vinyl alcohol), (PVA), grafted with poly(lactic-co-glycolic acid) (PLGA) side chains were shown to offer advantageous properties for drug delivery, recently reviewed in [10].

Modification of the branched structure with pendant amine functionalities led to positively charged biodegradable polyesters [11], which have successfully been used for the nano-encapsulation of DNA potentially useful for gene delivery system with surprisingly high transfection efficiency [12, 13]. Polymer-insulin nanocomplexes for transmucosal protein delivery [14-16] and nanoparticles intended for pulmonary delivery of drugs [17-19] are further applications of this polymer class. In addition, amine-modified PVA backbone without grafted PLGA chains was investigated as polycationic, non-viral gene delivery vehicle [20].

A particular advantage of branched polyesters is their versatility of structural modifications allowing the modification of solubility, drug loading and degradation by variation of degree of substitution and type of amine functionalities as well as length of PLGA chains grafted to the PVA backbone. Preliminary degradation data demonstrated that degradation times could be designed to range from less than 10 days to more than three weeks depending on charge density and degree of branching with PLGA [21].

A systematic evaluation of the structure-function relationships regarding cytotoxicity and biocompatibility has not been carried out so far. An ideal drug carrier should be non-toxic and biocompatible; however, polycations are known to cause cytotoxic reactions [22]. Here the cytotoxicity of a series of amine-modified PVA backbones and the corresponding branched polyesters was investigated in cell culture models under *in vitro* conditions following ISO 10993 guidelines [23, 24] with a special emphasis on elucidating the relationship between polymer structure and biological properties.

Cell viability based upon metabolic activity was monitored using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay frequently applied for screening of polymer cytotoxicity [25]. Polymer-cell interactions were quantified by the release of the cytosolic enzyme lactate dehydrogenase (LDH) since polycations are thought to interact with negatively charged cell membranes through electrostatic interactions [26]. In addition, caspase-3/7 activity was measured to evaluate apoptotic cell reactions. Moreover, a direct contact assay was conducted with spin-coated films of water insoluble amine-PVA-g-PLGA to assess changes in cell morphology and proliferation.

3. Materials and Methods

3.1. Materials

Diethylaminoethylamine (DEAEA) (purum, >98%), Diethylaminopropylamine (DEAPA) (purum, >98%), Dimethylaminopropylamine (DMAPA) (purum, >98%), carbonyl di-imidazole (purum, ~97%), N-methyl-pyrrolidone (NMP) (absolute) and poly(vinyl alcohol) (PVA) (MW 15 kDa; degree of polymerization 300 (P=300); degree of hydrolysis 86-89%) were purchased from Fluka GmbH (Deisenhofen, Germany) and used as received. D,L-lactide (S-grade) and glycolide (S and A-grade) (Boehringer Ingelheim, Germany) were recrystallized twice from ethyl-acetate. Tetrahydrofuran (THF) (BASF, Ludwigshafen, Germany) was dried over sodium and distilled under nitrogen before use. Polyethylenimine (Polymin™ water free, 99%, MW 25 kDa) was a gift of BASF (Germany). All other chemicals were used as received without further purification.

3.2. Polymer Synthesis

Synthesis and characterization of the polymers were described in detail elsewhere [11]. Briefly, amine-modified polymer backbones were synthesized by activation of di-amine derivatives using carbonyl di-imidazole (CDI) in THF. Activated CDI components were then added in different ratios to PVA in NMP at 80°C. Amine-modified PVA backbones were purified by ultrafiltration (cut off 1000 g/mol) substituting the solvent by demineralized water. Concentrated solutions were frozen at -20°C and lyophilized. Table 1 gives an overview of the characteristics of these polymers. Amine-modified branched polyesters were obtained by grafting D,L-lactide and glycolide (1:1) onto the backbone in stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups: monomer units) by polymerization in bulk at 150°C. The product was dissolved in acetone and precipitated in water: isopropanol (1:1) mixtures. The source-based IUPAC nomenclature for e.g. DEAPA-modified polymers is

Poly(vinyl 3-(diethyl amino) propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly (D,L-lactide-co-glycolide).

Table 1: *Physicochemical properties of Amine-PVA polymers used in this study*

Polymer	Molecular weight (kDa)[a]	Molecular weight per Charge Ratio[b]	IC_{50} (mg/ml)[c]
M(7)	15.3	2269	> 10
M(13)	15.8	1217	> 10
M(21)	16.8	787	7.59 ± 2.71
M(32)	17.9	554	0.413 ± 0.066
M(69)	22.4	324	0.0235 ± 0.0012
E(6)	15.4	2560	> 10
E(12)	16.0	1366	> 10
E(20)	16.9	842	> 10
E(33)	18.4	564	1.163 ± 0.221
E(70)	23.6	338	0.0182 ± 0.0016
P(6)	15.4	2454	> 10
P(12)	16.1	1342	> 10
P(18)	16.7	943	> 10
P(33)	18.4	564	2.582 ± 0.688
P(68)	23.9	351	0.0193 ± 0.0014
PEI	25.0	43	0.0116 ± 0.0007
PVA	14.7	0	> 10

[a] Molecular weight: as calculated from ^{1}H-NMR (PEI as reported from manufacturer)
[b] Molecular weight per Charge ratio : Determined as the molecular weight per charge
[c] IC_{50}: calculated from MTT assay as described in the materials and methods section

As abbreviation the type of amine substitution (P=DEAPA, M=DMAPA, E=DEAEA) was adopted followed in parenthesis by the total average number of amine functions per PVA. For graft copolymers, a suffix indicating the poly (D,L-lactide-co-glycolide) (PLGA) side chain length (y) was used, for example E(12)-10. Data for amine-modified branched polyesters are summarized in table 2.

Table 2: *Physicochemical properties of Amine-PVAL-g-PLGA polymers used in this study*

Polymer	M_n[a] (kDa)	M_w [a](kDa)	SCL[b]	Degradation half-life (days)[c]	IC_{50} (cm^2/ml)[d]
M(7)-10	177	248	12.4	18	> 6
M(13)-10	325	632	12.4	9	> 6
M(69)-10	132	155	7.2	n.d.	n.d.
P(6)-10	196	263	11.2	> 21	> 6
P(26)-10	364	548	12.0	5	> 6
P(33)-10	195	367	9.4	1	4.121 ± 0.045
P(6)-20	250	336	18.7	> 21	> 6
P(33)-20	375	712	17.2	13	> 6
P(68)-20	470	1203	14.1	5	> 6
PVC (tin stabilized)	250[e]		-	-	0.2467 ± 0.0106
PE	n.d.		-	-	> 6

[a] M_n, M_w: as measured by GPC-MALLS
[b] SCL: side chain length as calculated from ^{1}H-NMR
[c] Degradation half-life: time at which 50% mass loss of a polymer film occurred (extrapolated from plot, n=3)
[d] IC_{50}: calculated from MTT assay as described in the materials and methods section
[e] as reported by manufacturer (no further information about M_n or M_w available)

3.3. Polymer Film Preparation

Polymer films were cast from a 5% (w/v) solution in dichloromethane using Teflon™ moulds. After 72 h of drying at a temperature of 4°C the samples were recovered and cut into stripes using a razor blade. Residual solvents were then removed in vacuum at room temperature until constant weights were obtained.

To determine the in-vitro degradation profiles, weighed film samples (ca. 30 mg, n=3) were placed in 10 ml of phosphate buffered saline (PBS, pH 7.4, 0.15 M) and kept at 37°C in an incubator.

3.4. Polymer mass loss

Degradation of water insoluble amine-modified graft polyesters was measured gravimetrically after incubation of polymer films (approx. 30 mg, n=3) in 10 ml of phosphate buffered saline (pH 7.4, 0.15 M, 37°C) over 21 days as described previously [21].

3.5. Cell Culture

Cell culture experiments were carried out using L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) according to [27]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) and 2 mM glutamine at 37°C, 8,5% CO_2, and 95% relative humidity.

3.6. Extract Preparation

Test solutions from water-soluble amine-modified PVA backbones were prepared by dissolving polymer directly in serum supplemented DMEM at a concentration of 10 mg/ml. To investigate water insoluble amine-modified graft polyesters according to the ISO 10993 guideline [24], films with a surface area of 24 cm^2 and a thickness of 200 µm were prepared by solvent casting from dichloromethane. Incubation with 4 ml serum supplemented DMEM was carried out at 37°C for 24 hours. To determine the effect of PLGA-grafting upon cell viability, equimolar amounts of M(69)-10 calculated as the theoretical amine-modified PVA backbone content were immersed in ultra pure water and hydrolyzed at 121°C, 2 bar for 5 hours. Lost water was replaced before mixing with equal parts of double concentrated serum supplemented DMEM. All test solutions were adjusted to pH 7.4 and 270 mOsmol. The solution was filtered using a 0.22 µm syringe filter (Schleicher & Schuell, Dassel, Germany) prior to use.

3.7. Cell Viability

Cell viability was evaluated using the MTT assay as described previously [28]. Briefly, L929 cells were seeded into 96-well microtiter plates (Nunclon™, Nunc, Germany) at a density of 8000 cells/well. After 24 h the culture medium was replaced with serial dilutions of polymer/ polymer extract stock solutions in antibiotic-free DMEM (n = 8). After an incubation period of 24 h, 20 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Deisenhofen, Germany) (5 mg/ml in PBS) were added. After an incubation time of 4 h unreacted dye was removed by aspiration and the purple formazan product was dissolved in 200µl/well dimethylsulfoxide (Merck, Darmstadt, Germany) and quantitated by a plate reader (Titertek Plus MS 212, ICN, Germany) at wavelengths of 570 and 690 nm. Relative cell viability [%] related to control wells containing cell culture medium without polymer was calculated by [A] test / [A] control x 100. As suggested by ISO 10993 [23], tin-stabilized poly(vinyl chloride) and polyethylene were used as positive and negative controls, respectively. Additionally, PEI 25 kDa, a polycationic polymer widely used as gene transfer agent, and unmodified PVA 15 kDa were used as positive and negative controls, respectively, as these polymers are water soluble. The IC_{50} was calculated as polymer concentration which inhibits growth of 50% of cells relative to non-treated control cells according to [29]. The results of optical density measurements were fitted logistically by the Levenberg-Marquardt method of least square minimization for non-linear equations under the default conditions using Origin (OriginLab Software, Northampton, USA) by the following equation:

$Y = Y_0 + (Y_m-Y_0) / (1 + (C/C_0))$, where C_0 is the IC_{50} dose, Y is the optical density in a well containing a particular polymer/ extract concentration C. Y_0 and Y_m are the optical density corresponding to 0% viability and 100% viability, respectively.

3.8. Lactate Dehydrogenase Assay

Membrane damage resulting in necrotic cell death was measured using a commercially available lactate dehydrogenase (LDH) assay kit (CytoTox-ONE™, Promega GmbH, Mannheim, Germany) [30]. L929 cells were seeded into 96-well opaque microtiter plates (Nunclon™, Nunc, Germany) at a density of 12 000 cells/well. After 24 h the culture medium was replaced with 100 µl/ well of serial dilutions of polymer stock solutions in antibiotic-free DMEM (n = 3). After an incubation period of 6 h LDH activity was measured in cell culture supernatants using a LS 50B spectrofluorometer (Perkin-Elmer, Überlingen, Germany) with 560 nm excitation and 600 nm emission wavelengths. Results were presented as the percent of the maximum LDH release, which was determined by complete lysis of cells using a 9% (w/V) solution of Triton™ X-100 in water (positive control). Untreated cells were used as negative control.

3.9. Caspase-3/7 Activity Assay

Apoptotic activity was measured using the Apo-ONE™ homogenous caspase-3/7 assay (Promega GmbH) [31]. In brief, L929 cells were seeded into 96-well opaque microtiter plates (Nunclon™, Nunc, Germany) at a density of 12 000 cells/well. After 24 h the culture medium was replaced with 100 µl/ well of serial dilutions of polymer stock solutions in antibiotic-free DMEM (n = 3). After an incubation period of 6 h cells were lysed, and caspase-3/7 activity was measured by cleavage of the caspase-3/7 substrate rhodamine 110 (bis-(N-benzyloxycarbonyl-L-aspartyl- L-glutamyl-L-valyl-L-aspartic acid amide)), (Z-DEVD-R110). Samples were measured on a LS 50B spectrofluorometer (Perkin-Elmer) with 499 nm excitation and 521 nm emission wavelengths. Results were presented as relative fluorescence units (RFU) in comparison to untreated control cells.

3.10. Direct contact assay

To allow assessment of L929 mouse fibroblasts proliferation and morphology in direct contact with surfaces covered by amine-modified graft polyesters, thin films were prepared by spin-coating polymer solutions in dichloromethane onto glass cover slips. After drying in vacuum over night, coated glass cover slips were treated with isopropanol for disinfection, placed in 12 well tissue culture plates and pre-incubated with serum supplemented cell culture medium for 4 h. L929 mouse fibroblasts were seeded at a density of 35 000 cells /well and observed as described previously [22]. Pictures were taken using a SLR camera (Pentax MZ-7, Asahi Pentax, Japan). Cell proliferation and morphology were compared qualitatively to cells cultured on uncoated glass cover slips, which were used as negative control.

3.11. Statistical Analysis

All data were obtained at least in triplicate and presented as means ± standard deviation. One-way ANOVA at a significance level of $P \leq 0.05$ with Dunnett's post test was performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, USA). To compare results within one group, Tukey-Kramer post tests were applied.

4. Results and Discussion

The main factors influencing cytotoxicity of polycations were reported to be (i) molecular weight, (ii) charge density and the type of cationic functionality, (iii) structure and sequence (block, random, linear, branched), and (iv) conformational flexibility [26, 32, 33]. Since all polymers were derived from the same PVA backbone, aspect (iii) will not be considered further.

4.1. Effects of Amine-modified PVA backbones on cell viability measured by MTT assay

Amine-modified PVA backbones affected the metabolic activity of L929 cells during the incubation period of 24 h in a concentration dependent manner ranging from 0-10 mg/ml as

shown in figures 1-3. Corresponding IC_{50} values are summarized in table 1. As compared to unmodified PVA, cell viability at a concentration of 1 mg/ml was significantly reduced by all polymers except P(6) and P(12). However, in comparison to a frequently used polycationic polymer, namely PEI 25 kDa, cell viability remained significantly higher after treatment with all polymers with the exception of highly substituted derivatives E(70), P(68) and M(69). As a result, polymers comprising less than 21 amino groups per backbone molecule showed very low cytotoxicity irrespective of the amine functionality used. M(21) and P(33) showed moderate toxicity with IC_{50} values of 7.59 ± 2.71 mg/ml and 2.582 ± 0.688 mg/ml, respectively. M(32), E(33) and amine-PVAs comprising more than 33 amino groups yielded IC_{50} values not significantly different from PEI 25 kDa.

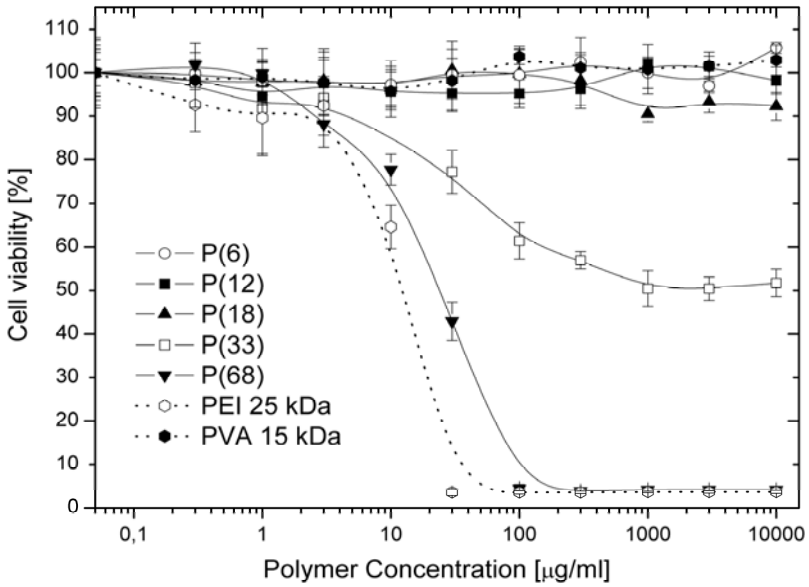

Figure 1: *Cell viability measured by MTT assay as a function of degree of amine modification after 24 hours of incubation with serial dilutions of DEAPA-PVA as compared to PVA 15 kDa (negative control) and PEI 25 kDa (positive control).*

Thus, water-soluble, amine-modified PVA polymers showed a cytotoxicity profile in accordance with other polycations. Higher molecular weights resulting from a higher degree of amine substitution led to a higher charge density, and this caused an increased level of

cytotoxicity. As an example, the IC_{50} values of DMAPA-PVA increased in the following order: M(69) < M(32) < M(21) < M(13), with molecular weight and charge density inversely correlated to the IC_{50} values. These results are in agreement with the suggestion of Ryser, who postulated a three-point attachment as being necessary to induce a biological response on cell membranes and hypothesized that the activity of a polymer will decrease when the space between reactive amine groups is increased in the primary structure [34].

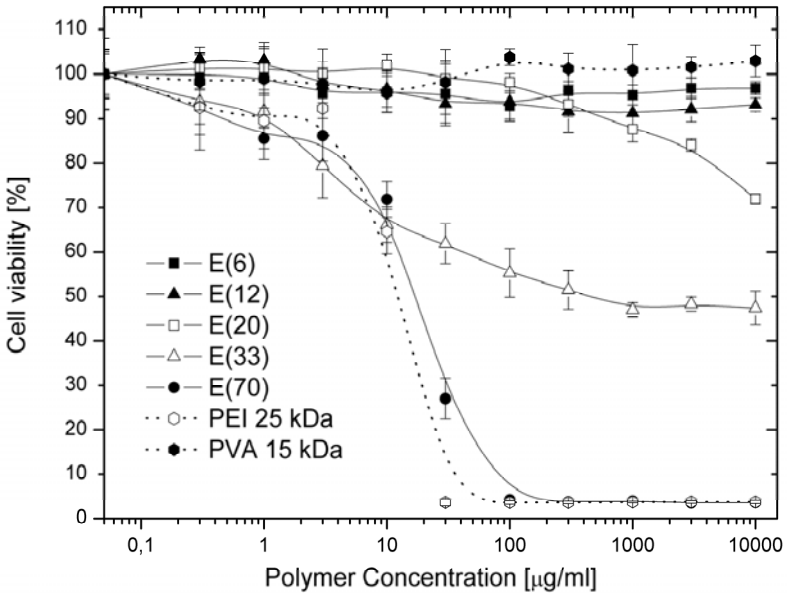

Figure 2: *Cell viability measured by MTT assay as a function of degree of amine modification after 24 hours of incubation with serial dilutions of DEAEA-PVA as compared to PVA 15 kDa (negative control) and PEI 25 kDa (positive control).*

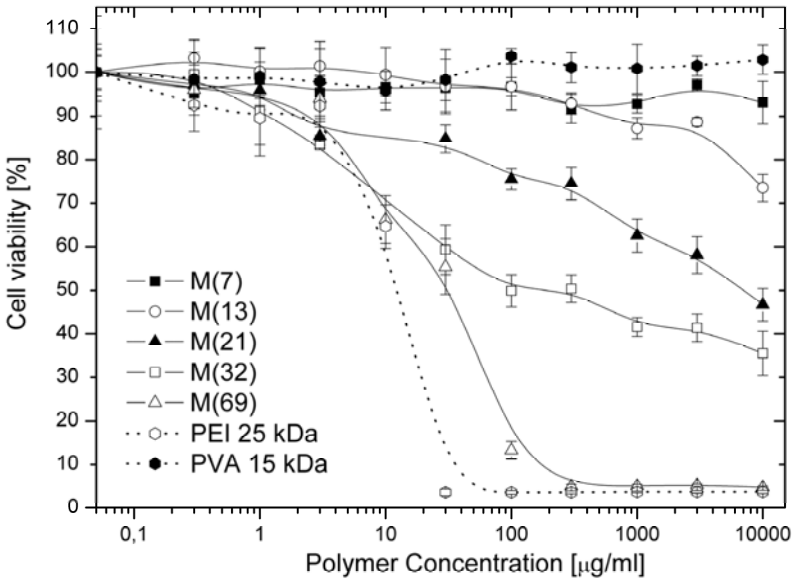

Figure 3: *Cell viability measured by MTT assay as a function of degree of amine modification after 24 hours of incubation with serial dilutions of DMAPA-PVA as compared to PVA 15 kDa (negative control) and PEI 25 kDa (positive control).*

Structure-toxicity relationships could also be visualized in a plot of logarithmic IC_{50} values versus the molecular weight/charge ratio, as shown in figure 4. A linear relationship could be observed. In addition, this plot clearly shows the dependency of the type of cationic (amine) functionality and its effect on cytotoxicity. Steeper curves indicating better cytocompatibility were observed in the following order: DEAPA-PVA > DEAEA-PVA > DMAPA-PVA. One possible explanation of differences in cytotoxicity caused by the different amine modifications could be different pK_a-values leading to unequally protonated polymers at testing conditions of pH 7.

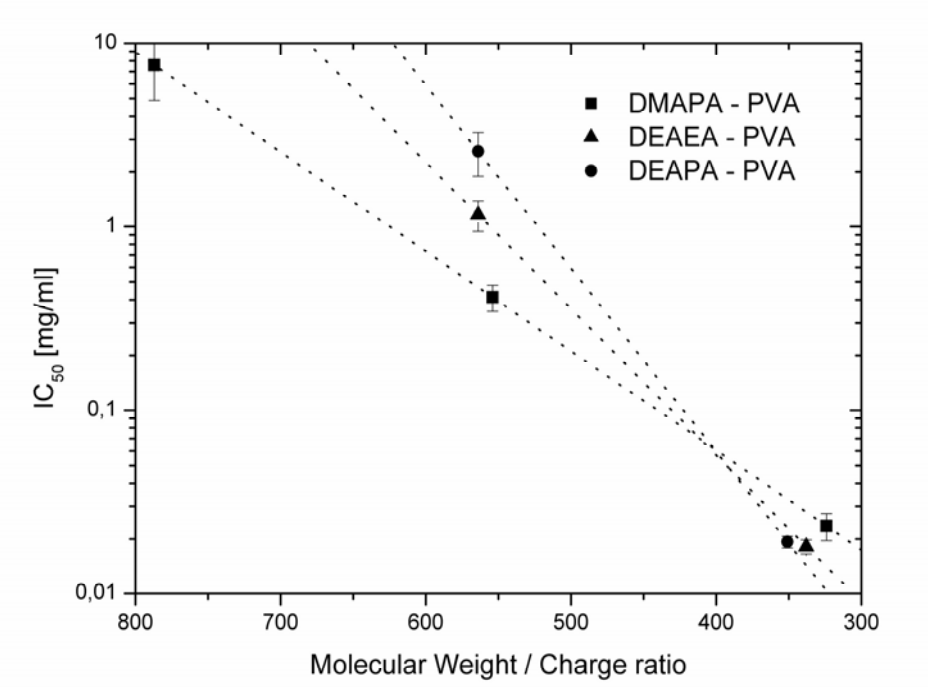

Figure 4: *Correlation of Molecular Weight / Charge ratio (determined as the molecular weight per charge) and IC_{50} values of Amine-PVA polymers. Dotted lines are interpolations to show slope of the curves.*

From the structural point of view, the functionalities directly protecting the amino groups (methyl-/ethyl-) and the spacer length (ethyl-/propyl-) are thought to influence cytotoxicity. A higher steric hindrance of ethyl-protected amino groups as compared to methyl-protected amino groups is suggested to cause relatively lower cytotoxicity. It is known, that a high conformational flexibility influences cytocompatibility negatively [35]. Although a lower flexibility of positive charges due to a shorter spacer was expected to result in lower cytotoxicity, the opposite was the case. The propyl spacer of DEAPA caused less toxicity than the ethyl spacer of DEAEA. However, even longer spacers (butyl-/hexyl-) could not reduce cytotoxicity significantly [20]. Therefore, to reduce cytotoxicity of amine-modified PVA polymers, amine functionalities comprising larger substituents shielding positive charges are favorable, while spacer length is of minor importance.

4.2. Mechanistic investigation of cytotoxic activity of amine-modified PVA polymers

To clarify the mechanism of cytotoxic activity of amine-modified PVA backbones, serial dilutions of M(69), E(70) and P(68), which have been found to inhibit cell proliferation significantly, were incubated with L929 mouse fibroblasts. Extra-cellular LDH concentrations were measured and compared to LDH release induced by serial dilutions of M(7), a polymer having been shown to have very little effects upon cell proliferation (Fig. 5).

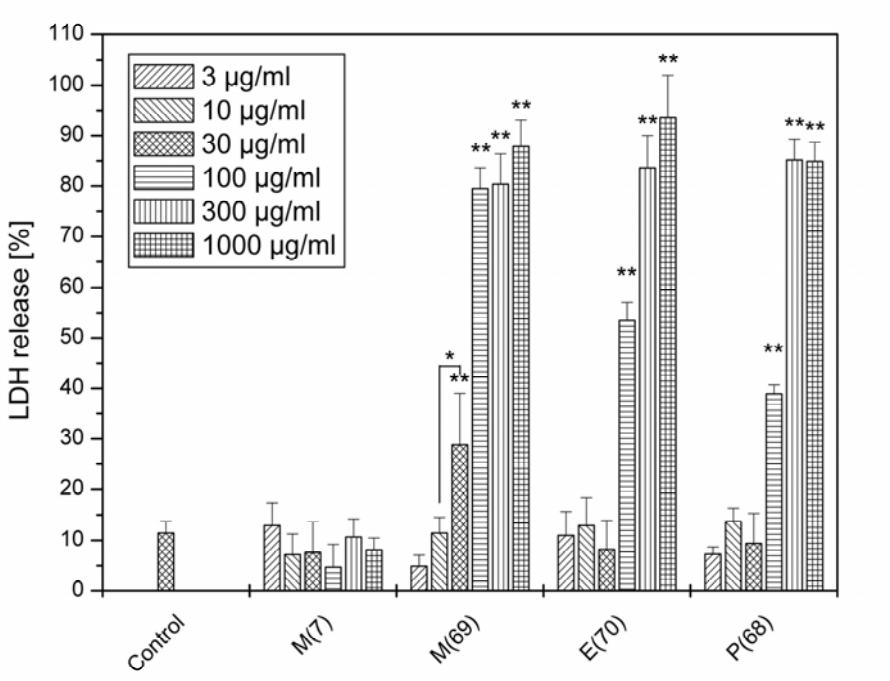

Figure 5: *Membrane toxicity of different concentrations of Amine-PVA polymers diluted in cell culture medium measured as LDH-release from L929 mouse fibroblasts after 6 hrs incubation (n=3, 100% = Triton™ X-100, 9% (w/V), Control = untreated vehicle cell culture).*

LDH release induced by cell treatment with M(7) did not differ significantly from values obtained from untreated control cells in all concentrations tested. As hypothesized, incubation of cells with dilutions of M(69), E(70) and P(68) led to a concentration dependent membrane damage resulting in LDH release significantly higher than untreated control cells for concentrations above 100 µg/ml. These results were in good agreement with Singh et al., who proposed a membrane destabilizing and concomitantly destructive effect of polycations

resulting from an interaction of positive charges (polymer) and negative charges (membrane) [35]. In accordance with cytotoxicity results from the MTT assay, membrane damaging effects increased with higher degrees of amine-substitution and increasing polymer concentrations. In contrast to cell viability testing, this assay also allowed discrimination between different types of amine functionalities even below a molecular weight/charge ratio of 400. At 100 µg/ml, dilutions of P(68), E(70) and M(69) led to 38,9 ± 4.1%, 53.5 ± 3.6% and 79.5 ± 4.1% LDH release, respectively. This could be explained by the fact, that this assay measures cell damage at an earlier point of polymer-cell interaction before actual cell death.

To gain further insight into the nature of cellular reactions and to distinguish between necrosis and apoptosis, M(7) which did not increase LDH levels at 1 mg/ml and M(69) which led to a LDH release of nearly 100% at 1 mg/ml, were studied with respect to the activation of caspases 3 and 7. In both, extrinsic and intrinsic pathway of apoptosis, caspases 3 and 7 have been found to play a major role as “death-effector caspases” mediating apoptosis [36] and thus, allowing a sensitive detection of ongoing apoptotic activity. In agreement with results from LDH assay, caspase 3 and 7-activity in cells treated with 1 mg/ml dilutions of M(7) and M(69) did not differ significantly from untreated control cells as shown in figure 6. Therefore, an induction of apoptosis could be excluded and the predominant type of cell death induced by highly charged amine-PVA backbones is necrosis presumably induced by polycationic membrane damage. Similar results were obtained for other polycations [22].

Using amine-modified PVA backbones, a series of polycations with IC_{50} values ranging from less than 0.05 mg/ml to more than 10 mg/ml could be generated. Compared to often used polycations of similar molecular weight such as PEI 25 kDa (0.0116 ± 0.0007 mg/ml) or poly(L-lysine) 36.6 kDa (0.038 mg/ml) [22], this result can be considered as a substantial

improvement since cytocompatibility is one of the key factors limiting success of polycationic drug delivery vehicles [37].

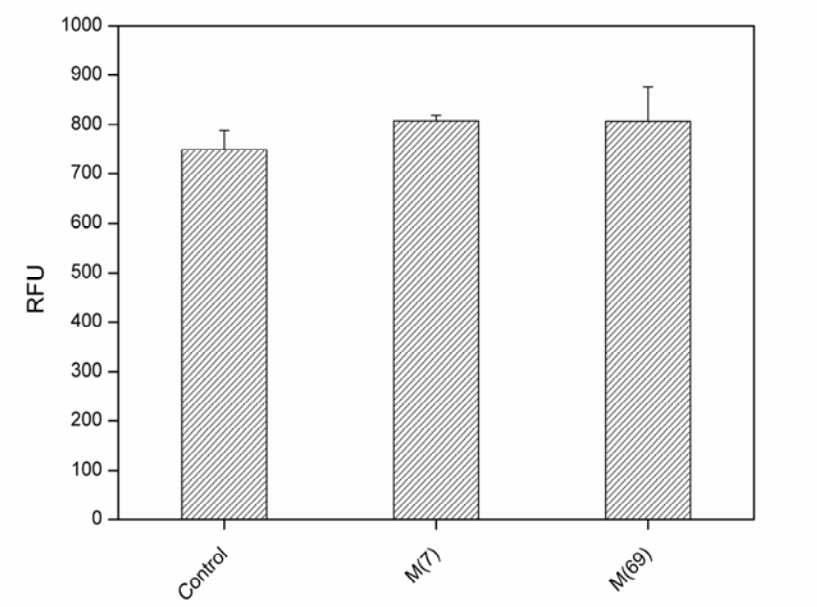

Figure 6: *Apoptosis (Caspase 3/7)-Assay of DMAPA-PVA treated L929 mouse fibroblast cells versus untreated vehicle control (n=3). Polymers were diluted in cell culture medium at 1 mg/ml and cells were incubated for 6 hours.*

In conclusion, a general trend towards higher cytotoxicity with an increased degree of amine substitution and an amine function in the order DEAPA < DEAEA < DMAPA was observed, whereby the degree of amine substitution was more dominant than the type of amine function. For cytocompatibility purposes, an optimal amine-modified PVA backbone should consist of amine functionalities according to the description in the previous section and an average total number of amine modifications of less than 33.

Collectively our data generated with amine-modified PVA backbones demonstrate that the cytocompatibility is largely determined by the cationic charge density and the degree of substitution. High degrees of substitution are thought to be beneficial for complexation of oppositely charged drug candidates [15, 20]. On the other hand this feature compromises the cytocompatibility. In an attempt to modulate the cytotoxicity of polycations we speculated

that the rate with which these molecules are presented to surrounding tissues could be of importance for the observed toxic effects. By conversion of the hydrophilic backbone into a water insoluble yet biodegradable it was assumed that the cytocompatibility of the resulting polymers could be improved in two ways, firstly by shielding the positive charges and thus reducing the deleterious attachment to cell membranes, and secondly, by modulating acute cytotoxicity due to water-insolubility of the non-degraded amphiphilic polymers.

Therefore, poly(lactic-co-glycolic acid) (PLGA) with a ratio of LA:GA of 50:50 was grafted onto the hydrophilic amine-modified PVA backbone due to its well documented biodegradability and biocompatibility [4, 38, 39].

A series of DEAPA-PVA backbones was generated by grafting PLGA side chains consisting of either 10 or 20 repeating units to the polymers to obtain comb-like-structured copolymers. In addition, a highly-charged DMAPA-PVA-g-PLGA polymer, M(69)-10 was synthesized to explore the limits of this approach.

4.3. Effects of PLGA grafted amine-modified-PVAs on cell viability

All polymer extracts tested showed cytotoxicity too low to calculate IC_{50} values by logistic curve fitting. Thus, maximum extract concentrations were compared with equal extract concentrations from PE (negative control) and tin-stabilized PVC (positive control). As compared with PE, cell viability was reduced by all polymers except P(6)-20 and P(33)-20. However, all polymers were significantly less toxic, than the positive control, except P(33)-10. Comparing P(33)-10 (97.1 ± 1.2 % viability reduction) with P(33)-20 (9.9 ± 5.7 % viability reduction) demonstrated that not the degree of amine substitution, but rather the PLGA side chain length was the major factor influencing cytotoxicity. Longer PLGA side chains drastically reduced cytotoxicity. This observation seems to be associated either with stronger shielding effects of longer PLGA chains or more likely with polymer degradation.

4.4. Effects of polymer degradation on cytotoxicity

Since PLGA side chains are thought to be cleavable by non-enzymatic hydrolysis, the release of water-soluble cleavage products was to be expected. Therefore, degradation half-lives of DEAPA-PVA-g-PLGA polymers [12] were compared with cytotoxicity results (Fig. 7) showing a clear trend.

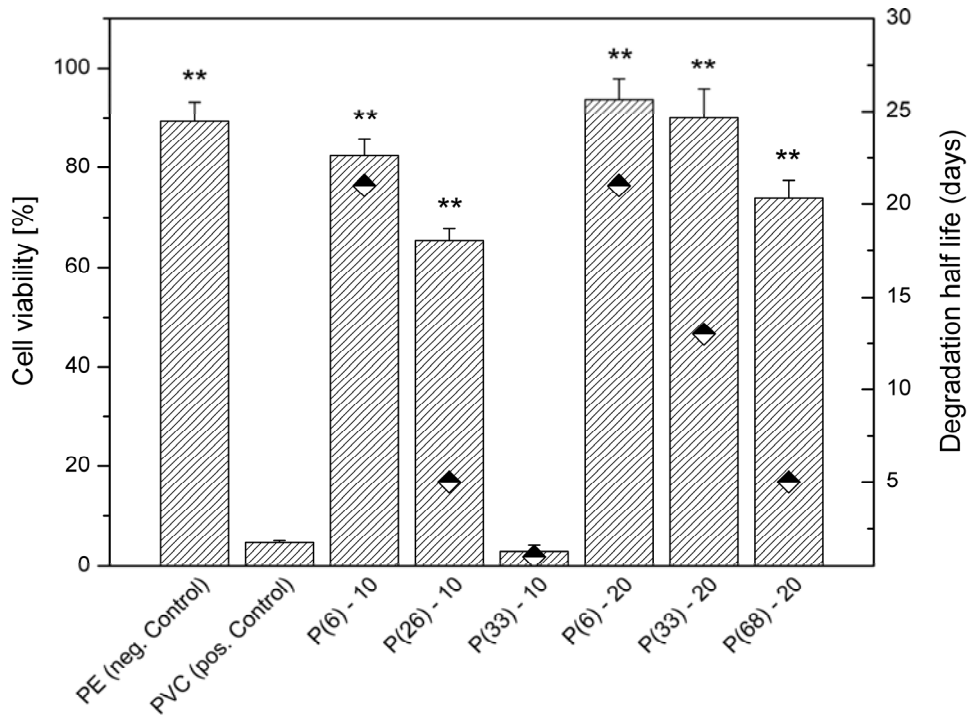

Figure 7: *PLGA shielding effect upon cell viability as a function of PLGA-side chain length and degree of amine substitution (6 cm^2/ml of a film of each polymer were incubated with cell culture medium for 24 hours. Mouse fibroblasts were cultured with these solutions for 24 hours and cell viability was measured by MTT assay (n=8)). PE and tin-stabilized PVC were used as negative and positive control, respectively. Stars represent significance levels vs. positive control. Diamond-shaped data points represent degradation half-life in vitro.*

As expected shorter degradation half-lives affected cell viability in a negative sense, suggesting that polymer erosion is an important factor influencing cell viability under *in vitro* conditions. This effect was attributed to oligomeric degradation products of PLGA chains and especially the amine-modified PVA-backbone. The amine-PVA backbone is known to be water-soluble even with short PLGA chains still attached to it [11]. Accumulation of cleavage

products in the closed *in vitro* system seems to induce a cytotoxic response similar to the unmodified amine-PVA backbone. Similar observations for PLGA extracts obtained at 37°C and 70°C are found in the literature [38]. PVA with a molecular weight of 15,000 kDa was chosen as backbone to allow renal elimination as documented in [40, 41]. Detailed studies of toxicity under *in vivo* conditions are in preparation. First promising results from nasally administered amine-modified PVA-g-PLLA-insulin complexes in rats have been published recently [14] where no signs of epithelial damage could be detected at the administration site.

While incubation of polymer films with serum gives a good estimate of leachables and rapidly degrading components of the polymers, additional experiments were carried out to investigate the cytotoxicity after complete hydrolysis. The cytotoxicity of ungrafted M(69) backbone was compared with PLGA-grafted M(69). Equimolar amounts of M(69)-10 based on backbone content were used to prepare extracts. Resulting cytotoxicity profiles are shown in figure 8.

Even after treatment under these harsh conditions, M(69)-10 retained a favorable cytocompatibility profile with 45.9 ± 1.1 % viable cells at the highest concentration tested as compared to 5.1 ± 0.4 % cell viability after treatment with unmodified M(69). This result can either be explained by the retention of the shielding effect due to incomplete degradation of PLGA side chains or more likely cleavage of the amine substituents from the PVA backbone.

As postulated above, the length of PLGA side chains clearly influenced cytotoxicity and degradation rate of these polymers. Within polymers of similar side chain length the degree of amine substitution is the critical factor for the cytocompatibility. Therefore, the PLGA side chain length controlled the exposure of cells to the amine-modified PVA backbone and reduced cytotoxicity, correspondingly.

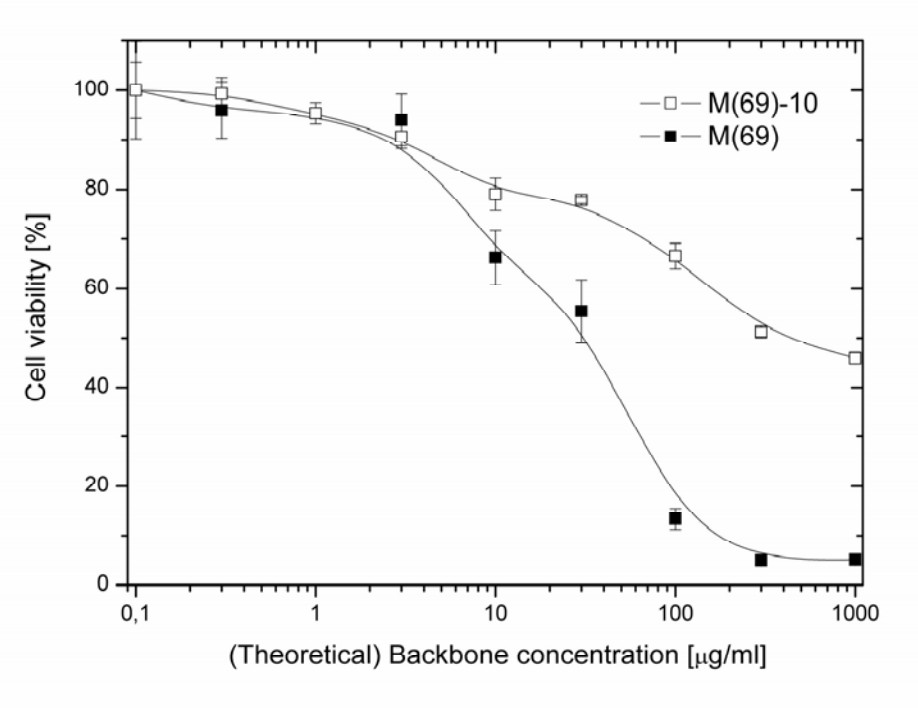

Figure 8: *Shielding effect of PLGA-side chains after treatment of M(69)-10 under stress conditions as compared to ungrafted M(69): Cell viability as a function of theoretical backbone concentration (theoretical amount of DMAPA-PVA backbone contained M(69)-10 has been calculated, an equivalent amount of polymer powder was hydrolyzed for 5 hours with cell culture medium using an autoclave at 121°C. Mouse fibroblasts were cultured with serial dilution of the extract for 24 hours and cell viability was measured by MTT assay (n=8)).*

4.5. Cell attachment, morphology and proliferation of L929 mouse fibroblast cultured on DMAPA-PVA- g-PLGA films

M(7)-10 and M(13)-10 represented polymers with similar side chain length, but differing degrees of amine modification, while M(7)-20 represented a polymer with a longer PLGA side chain. Cells cultured on uncoated glass cover slips were used as a control. L929 mouse fibroblasts were characterized as large, spindle-shaped, adherent cells growing to a confluent monolayer [42] in the control wells.

Depending on the degree of amine substitution and the PLGA side chain length, L929 mouse fibroblasts exhibited morphological changes as compared to controls (Fig. 9). The cell density was higher and morphology was less affected in the M(7)-20 compared to M(7)-10 after 48 h of incubation in accordance with the cytotoxicity results. Hence, an increased side chain

length led to faster cell attachment and proliferation. Comparing fibroblasts growing on M(7)-10 and M(13)-10, cell morphology and proliferation were negatively influenced by the higher degree of amine substitution.

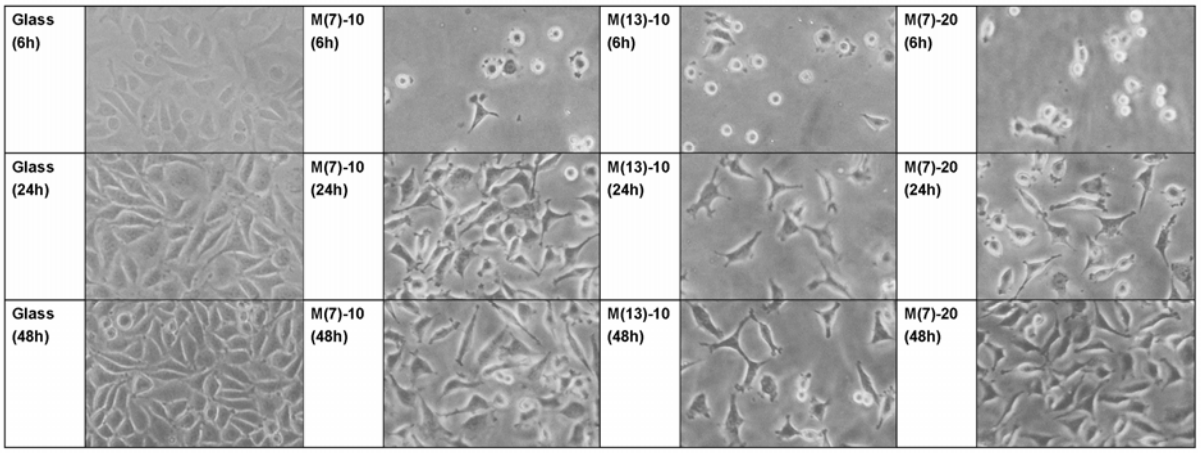

Figure 9: *L929 mouse fibroblasts cultured directly on spin-coated DMAPA-PVA-g-PLGA films for 48 hours; differences in cell number and morphology as compared to cells cultured on uncoated glass cover slips are observable, cell adherence to Amine-PVA-g-PLGA can be controlled by degree of amine-substitution and PLGA-side chain length.*

In accordance with the theory, a reduction of acute toxicity by PLGA-shielding should lead to an increased adherence and proliferation of fibroblasts on polymer films. Depending on the PLGA side chain length and the degree of amine substitution cell morphology and proliferation rate were affected as compared to cells growing on uncoated glass cover slips. In a functional sense the structure of DMAPA-PVA- g-PLGA affected both cell attachment and proliferation and showed the following trends: Longer PLGA side-chains lead to decreased degradation rates and an increased shielding effect, which results in higher cell densities and unchanged fibroblast morphology. Lower degrees of amine substitution leading to an overall lower charge density and a decreased degradation rate, also increased cell attachment and proliferation.

5. Conclusion

Different amine-modified PVA backbone polymers were systematically characterized with respect to the degree and type of amine substitution. Cytotoxicity was investigated using MTT and LDH assays, which quantify cell metabolic activity and membrane integrity, respectively. A molecular weight and dose dependent cytotoxicity was found for amine-modified PVA. The type of amine functionality was of minor importance with, DEAPA being slightly less cytotoxic than DEAEA and DMAPA. The cytotoxic effect is not caused by apoptosis as shown by caspase-3/7 assay but rather by necrotic reaction to the highly charged amine-modified PVA backbone polymers presumably by interactions of polycationic materials with cell membranes. The approach to improve the cytocompatibility of amine-modified PVA polymers using biodegradable PLGA side chains turned out to be successful. Decreased charge densities and shielding positively charged amine moieties by PLGA side chains decreased the cytotoxicity. These results have implications for the design of biodegradable and biocompatible carrier systems containing sensitive molecules such as peptides, proteins, RNA and DNA. Biodegradable nano-complexes based on these polymers are currently under investigation in our labs.

6. Acknowledgements

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Chapter 4

Branched Polyesters based on Poly[vinyl-3-(dialkylamino)alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide): Effects of polymer structure on in vitro degradation behavior

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1. Summary

Branched polyesters of the general structure Poly[vinyl-3-(dialkylamino)alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide) have shown potential for nano- and micro-scale drug delivery systems. Here the *in vitro* degradation behaviour with a special emphasis on elucidating structure-property relationships is reported. Effects of type and degree of amine substitution as well as PLGA side chain length were considered. In a first set of experiment, the weight loss of solvent cast films of defined size was measured as a function of incubation in phosphate buffer (pH 7.4) at 37°C over a time of 21 days. A second study was initiated focusing on three selected polymers in a similar set up, but with additional observation of pH influences (pH 2 and 9) and determination of water uptake (swelling) and molecular weights during degradation. Scanning electron micrographs have been recorded at selected time points to characterize film specimens morphologically after degradation. Our investigations revealed the potential to influence the degradation of this polymer class by the degree of amine substitution, higher degrees leading to faster erosion. The erosion rate could further be influenced by the type of amine functionality, DEAPA-modified polyesters degrading as fast as or slightly faster than DMAPA-modified polyesters and these degrading faster than DEAEA-PVA-*g*-PLGA. As a third option the degradation rate could be modified by the PLGA side chain length, shorter side chains leading to faster erosion. As compared to linear PLGA, remarkably shorter degradation times could be achieved by grafting short PLGA side chains onto amine-modified PVA backbones. Erosion times from less than 5 days to more than 4 weeks could be realized by choosing the type of amine functionality, the degree of amine substitution and the PLGA side chain length at the time of synthesis. In addition, the pathway of hydrolytic degradation can be tuned to be either mainly bulk or surface erosion.

2. Introduction

Drug delivery systems based on biodegradable polymers have received considerable interest as parenteral depots [1, 2] and as non-viral gene delivery systems [3]. Most frequently biodegradable polyesters such as poly(lactic-co-glycolic acid), PLGA, have been used due to their excellent track record of biocompatibility and lack of toxicity [4]. Linear PLGA was shown to degrade by non-enzymatic hydrolysis of ester bonds generating an acidic microenvironment for encapsulated drug substances [5] which is detrimental for sensitive peptides, proteins, RNA and DNA [6, 7]. New biomaterials which allow the design of degradation and release properties would be of considerable interest.

Branched polyesters have been shown to possess physico-chemical properties vastly differing from their linear counter-parts, such as solution behaviour, melt rheology, mechanical properties, and crystallinity [8]. Their potential for drug delivery has not been exploited to a large extend although degradation and release properties are known to be influenced by the polymer architecture. The influence of polymer architecture on drug delivery systems has recently been reviewed [9]. Biodegradable, branched poly(vinyl alcohol), (PVA), grafted with poly(lactic-co-glycolic acid) (PLGA) side chains offer advantageous properties for drug delivery, recently reviewed in [10].

Modification of the branched structure with pendant amine functionalities led to positively charged biodegradable polyesters [11], which have successfully been used for the nano-encapsulation of DNA potentially useful for gene delivery system with surprisingly high transfection efficiency [12, 13]. Polymer-insulin nanocomplexes for transmucosal protein delivery [14-16] and nanoparticles intended for pulmonary delivery of drugs [17-19] are further applications of this polymer class. In addition, amine-modified PVA backbone without grafted PLGA chains was investigated as polycationic, non-viral gene delivery vehicle [20].

A particular advantage of these branched polyesters is their versatility of structural modifications allowing to modify solubility, drug loading and degradation by variation of degree of substitution and type of amine functionalities as well as length of PLGA chains grafted to the PVA backbone. We reported the investigation of structural effects upon cytotoxicity of this polymer class earlier [21]. Preliminary degradation data demonstrated that erosion times could be designed to range from less than 10 days to more than three weeks depending on charge density and degree of branching with PLGA [22]. A systematic evaluation of the structure-function relationships with regard to *in vitro* degradation has not been carried out so far.

Here the degradation behaviour of a series of amine-modified PVA-*g*-PLGA polyesters was investigated under *in vitro* conditions with a special emphasis on elucidating the relationship between polymer structure and degradation properties. In a first set of experiments, 17 amine-modified PVA-*g*-PLGA polyesters covering a broad range of degrees of amine substitution, three different amine functionalities and different PLGA side chain lengths were investigated in a screening model. The weight loss was measured as a function of incubation in phosphate buffer (pH 7.4) at 37°C over a time of 21 days.

In a second set of experiments, we focused on three characteristic polymers selected from the first screening study covering the effects of (a) degree of amine substitution and (b) PLGA side chain length. In addition to the parameters tested in the screening model, the influence of pH on hydrolytic degradation and water uptake (swelling) of this polymer class was investigated.

3. Materials and Methods

3.1. Materials

Diethylaminoethylamine (DEAEA) (purum, >98%), Diethylaminopropylamine (DEAPA) (purum, >98%), Dimethylaminopropylamine (DMAPA) (purum, >98%), carbonyl diimidazole (purum, ~97%), N-methylpyrrolidone (NMP) (absolute) and poly (vinyl alcohol) (PVA) (MW 15 kDa; degree of polymerization 300 (P=300); degree of hydrolysis 86-89%) were purchased from Fluka GmbH (Deisenhofen, Germany) and used as received. D,L-lactide (S-grade) and glycolide (S and A-grade) (Boehringer Ingelheim, Germany) were used as received. Tetrahydrofuran (THF) (BASF, Ludwigshafen, Germany) was dried over sodium and distilled under nitrogen before use. Polyethyleneimine (Polymin water free, 99%, MW 25 kDa) was a gift of BASF (Germany). All other chemicals were used as received without further purification.

3.2. Polymer Synthesis

Synthesis and characterization of the polymers were described in detail elsewhere [11]. Briefly, amine-modified polymer backbones were synthesized by activation of di-amine derivatives using carbonyl di-imidazole (CDI) in THF. Activated CDI components were then added in different ratios to PVA in NMP at 80°C. Amine-modified PVA backbones were purified by ultrafiltration (cut off 1000 g/mol) substituting the solvent by demineralized water. Concentrated solutions were frozen at -20°C and lyophilized. Amine-modified branched polyesters were obtained by grafting D,L-lactide and glycolide (1:1) onto the backbone in stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups: monomer units) by polymerization in bulk at 150°C. The product was dissolved in acetone and precipitated in water: isopropanol (1:1) mixtures.

The source-based IUPAC nomenclature for e.g. DEAPA-modified polymers is Poly(vinyl 3-(diethyl amino) propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly (D,L-lactide-

co-glycolide). As abbreviation the type of amine substitution (P=DEAPA, M=DMAPA, E=DEAEA) was adopted followed in parenthesis by the total average number of amine functions per PVA. For graft copolymers, a suffix indicating the poly (D,L-lactide-co-glycolide) (PLGA) side chain length (y) was used, for example E(12)-10. Data for amine-modified branched polyesters are summarized in tables 1 and 2.

Table 1: *Screened Polyesters: Molar mass, side chain length and amine substitution.*

Polyester	M_n[a] (kg mol^{-1})	Side chain[b]	Amine[c]
P(6)-10	207.6	11.2	2.1
P(6)-20	335.8	18.7	2.1
P(12)-10	201.4	10.8	4.0
P(12)-20	348.2	19.3	4.0
P(33)-10	181.2	9.4	10.9
P(33)-20	315.0	17.2	10.9
P(68)-20	236.2	14.1	22.7
E(6)-10	206.5	11.1	2.0
E(6)-20	341.6	19.1	2.0
E(12)-10	225.6	12.3	3.9
E(12)-20	378.9	21.3	3.9
E(33)-10	203.1	11.1	10.8
E(33)-20	358.0	20.5	10.8
M(7)-10	227.6	12.4	2.3
M(7)-20	374.7	20.9	2.3
M(13)-10	228.0	12.4	4.4
M(13)-20	386.3	21.6	4.4
PVA-10	236.1	12.9	0.0
PVA-20	388.1	21.7	0.0

[a] M_n calculated from ^{1}H-NMR
[b] average side chain length calculated from ^{1}H-NMR
[c] amine substitution of the backbone

Table 2: *DEAPA-PVAL-g-PLGA Degradation study: Molar mass, side chain length, amine substitution.*

Polyester	M_n[a] (kg mol^{-1})	Side chain[b]	Amine[c]
P(12)-10	220.8	11.9	4.0
P(33)-10	203.6	10.8	10.9
P(33)-20	323.7	17.8	10.9

[a] M_n calculated from ^{1}H-NMR
[b] average side chain length calculated from ^{1}H-NMR
[c] amine substitution of the backbone

3.3. Polymer degradation

3.3.1. Polymer Film Preparation

For the screening study, polymer films were cast from a 5% (w/v) solution in dichloromethane using Teflon™ moulds. After 72 h of drying at a temperature of 4°C the samples were recovered and discs with a diameter of 17 mm were punched from the polymer films in a semi-dry state using a cork bore. Residual solvents were then removed in vacuo at room temperature until constant weights were obtained.

Polymer films of selected polymers for the second part of the study were cast from a 5% (w/v) solution in dichloromethane directly into 10-ml snap cap vials with a diameter of 22 mm. After 72 h of drying at a temperature of 4°C the samples were placed in vacuo at room temperature until constant weights were obtained.

3.3.2. Polymer mass loss and swelling

As a screening model, weight loss of water insoluble amine-modified graft polyesters was measured gravimetrically after incubation of polymer films (approx. 30 mg, n=3) in phosphate buffered saline (pH 7.4, 0.15 M, 37°C) over 21 d. The degradation medium was added in great excess (10 ml per glass vial) to guarantee sink conditions for the full testing period of 21 days. As a confirmation, the pH of the degradation media was measured after the study period and did not fall below a pH of 6.8 in any case. To allow even distribution of

potential degradation products, glass vials were gently shaken once a day. After 2, 7, 14 and 21 d, samples were recovered, carefully washed with purified water, blotted dry with Kimwipes™ and wet weight was determined. Wet samples were then frozen at -80 °C and freeze-dried in vacuo for ca. 72 h at room temperature until constant masses were obtained. Polymer mass loss was calculated from the following formula: Mass loss (%) = 100 – (mass (dry) x 100/ original mass).

In the second part of the study, a similar procedure was followed, except that polymer films had a starting weight of approximately 45 mg (n=3) and additional degradation media were used to investigate the effect of pH on degradation. Polymer specimens were incubated in phosphate buffered saline (0.15M, 37°C) at pH 2 and pH 7.4 and in TRIS buffer (0.15M, 37°C) at pH 9. After predefined time points samples were recovered, carefully washed with purified water, blotted dry with Kimwipes™ and the wet weight was determined to calculate the water uptake according to the following formula: Water uptake (%) = (mass (wet) – mass (dry)) x 100/ mass (dry). Mass loss was again determined as described above.

3.3.3. Molecular weight determination

^{1}H-NMR spectroscopic data was collected using a JEOL Eclipse+ 500 and a Joel GX 400 D at a frequency of 500 respective 400 MHz at 50°C in $CDCl_3$ (non-degraded polymers) and *d6*-DMSO (euriso-top, <0.02% HDO+D2O, dried specimen after degradation). Depending on the remaining amount after degradation, 5 to 50 mg sample was used for each measurement. ^{1}H-NMR was performed with 64 scans. The side chain length (SCL) was calculated using the integrals of the lactide and glycolide end groups (I_{end}) and there central groups (I_{cent}) and adding one for the end group using the following formula: $[(I_{cent\_LA\_CH} + (I_{cent\_GA}) / 2) / (I_{end\_LA\_CH} + (I_{end\_GA} / 2)] + 1 = SCL$.

3.3.4. Scanning electron microscopy (SEM)

The morphology of polymer films was characterized by SEM using a Hitachi S-4100 microscope (Hitachi, Germany). A piece of the finally dried polymer film was cut using a razor blade and fixed onto an aluminium pin using double-sided adhesive tape. Prior to microscopic examination the samples were coated with a gold layer for 30 s (Edwards Auto 306, Edwards, Germany).

3.4. Statistical Analysis

All data were obtained at least in triplicate and presented as means ± standard deviation. One-way ANOVA at a significance level of $P \leq 0.05$ with Dunnett's post test was performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, USA). To compare results within one group, Tukey-Kramer post tests were applied.

4. Results and Discussion

In a first screening study, the erosion behaviour of a set of 17 polyesters was investigated, measuring weight loss over time *in vitro* at pH 7.4 and a temperature of 37°C for 21 days. All polymers tested were of the basic structure Amine-PVA-*g*-PLGA with PLGA side chain lengths of 10 and 20 units and three different amine functionalities. As a comparison, two uncharged PVA-*g*-PLGA with PLGA side chain length of 10 and 20 units respectively, were tested (Figure 1d). The weight loss over time of DEAPA-PVA-*g*-PLGA is shown in figure 1a. Similar erosion behaviours could be observed for P(6)-10, P(6)-20 and P(12)-20. However, increasing the degree of amine substitution to 33 or even 68 increased the weight loss enormously to 54.9 ± 6.6 % after 14 days (P(33)-20) and 83.7 ± 8.8 % after 7 days (P(68)-20), respectively. Even shorter erosion times could be observed for polyesters with shorter PLGA side chain lengths with weight losses of up to 97.3 ± 2.4 % after 2 days (P(33)-10). Thus, shorter PLGA chains lead to faster erosion. To investigate the influence of the type of amine

function, also DEAEA- and DMAPA-modified polyesters were investigated as shown in figures 1b and 1c, respectively.

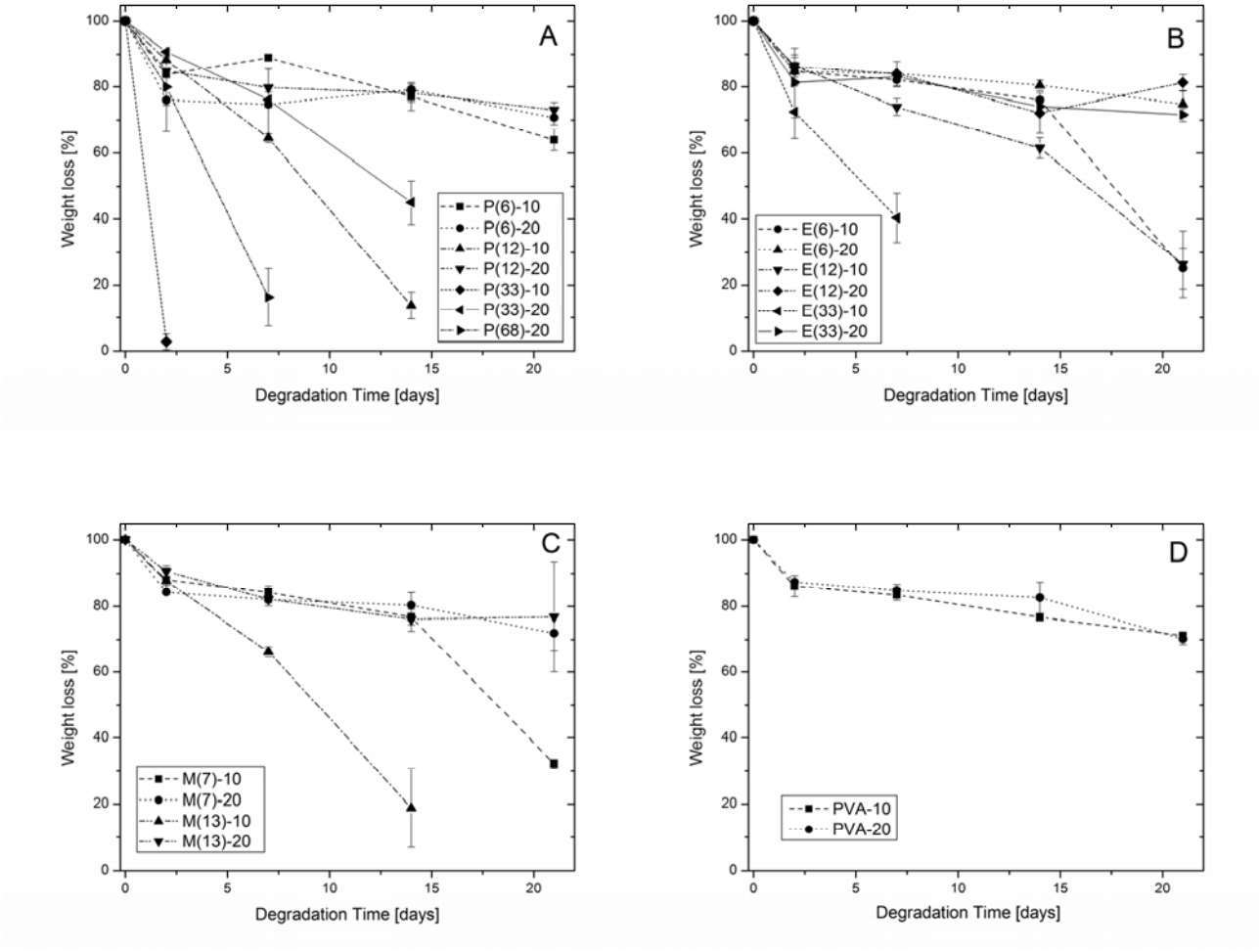

Figure 1: *Screened polyesters: Weight loss of the test specimens as a function of incubation time in phosphate buffer (pH 7.4) at 37°C. (A) DEAPA-PVA-g-PLGA polymers; (B) DEAEA-PVA-g-PLGA polymers; (C) DMAPA-PVA-g-PLGA polymers and (D) unmodified, neutrally charged PVA-g-PLGA polymers (controls).*^

Comparing weight losses of polyesters with different amine functionalities in quantitatively similar degrees of amine substitution, a trend could be observed, DEAPA-modified polyesters degrading as fast as or slightly faster than DMAPA-modified polyesters and these degrading faster than DEAEA-PVA-*g*-PLGA. For example, P(12)-10 lost 86.2 ± 4.1 % weight after 14 days, while M(13)-10 lost 81.2 ± 11.9 % (not significant). In the same time frame, the weight loss of E(12)-10 was significantly lower (38.5 ± 3.1 %). Results obtained from higher degrees

of amine substitution and longer PLGA side chains were in line with that trend. One possible explanation of these differences in erosion rate caused by the different amine modifications could be different pK_a-values leading to unequally protonated polymers at testing conditions of pH 7.4. From the structural point of view, the functionalities directly protecting the amino groups (methyl-/ethyl-) did not significantly influence the polymer erosion. More important, the propyl-spacer of DEAPA and DMAPA considerably increased the erosion rate as compared to DEAEA-modified polymers, which could be explained by a higher conformational flexibility of positive charges, a better steric accessibility and a longer distance to the electron-drawing amide functionality. However, one exemption from that trend could be observed for lower degrees of amine substitution, P(6)-10 degrading significantly slower than E(6)-10 and M(7)-10.

To obtain a more detailed insight into the structure-degradation relationships, a set of three different DEAPA-modified polyesters were resynthesized (Table 2). Two polymers with the same amine-PVA backbone (P(33)), but different PLGA side chain lengths (10 and 20) and one polymer with a lower degree of amine substitution and a short side chain (P(12)-10) were investigated with respect to pH, swelling and incubation time upon degradation. Weight loss and molecular weights were determined at selected time points. Due to the changing physicochemical characteristics of the polyelectrolytes during degradation, molecular weights of degraded samples were not determined by a GPC-MALLS method originally developed for the non-degraded polymers [11] but by ^{1}H-NMR end-group analysis. The actual erosion times obtained in this second part of the study can only be compared in a relative manner to the data observed in the screening study, as the second data set was obtained from (1) a different synthesis and, more important (2) the specimen weights and thickness had to be increased to yield enough material for molecular weight determination after degradation.

PLA and PLGA are known to degrade by bulk-erosion. The polymer samples take up water and hydrolytic degradation is accelerated in the core by the formation of acidic oligomers which cannot leave the sample by pore diffusion. This causes the often described acidic microenvironment inside degrading PLA and PLGA specimens [5]. Another degradation mechanism known as surface erosion is characterized by a decomposition from the outside to the inside with no reaction occurring in the core [23]. Since amine-PVA-*g*-PLGA polymer films are degraded hydrolytically, a bulk-erosion mechanism would be expected. Degradation of amine-PVA-*g*-PLGA polymers was thought to occur by a mixed bulk and surface erosion mechanism [22], but the effect, of polymer composition on degradation profiles remained to be investigated. Figure 2 shows the weight and molecular weight changes of P(12)-10, P(33)-10 and P(33)-20 during *in vitro* degradation at pH 7.4. P(33)-20 showed a degradation profile compatible with bulk hydrolysis. The molecular weight decreased by approx. 100% during 27 days while a mass loss of 27.9 ± 0.9 % was observed. In contrast, P(33)-10 lost 56.1 ± 2.2 % of its original weight during 4 days, while, the molecular weight remained nearly unchanged (- 6%) over this period. This degradation profile is characteristic for surface erosion. The degradation pattern of P(12)-10 lies in-between the two others, the weight loss of 49.9 ± 1.5 % after 19 days was accompanied by a molecular weight decrease of 30 %. Thus, this polymer class can be designed to either degrade by surface erosion using higher degrees of amine substitution combined with short PLGA side chains or to degrade by bulk erosion using lower degrees of amine substitution and/or longer PLGA side chains.

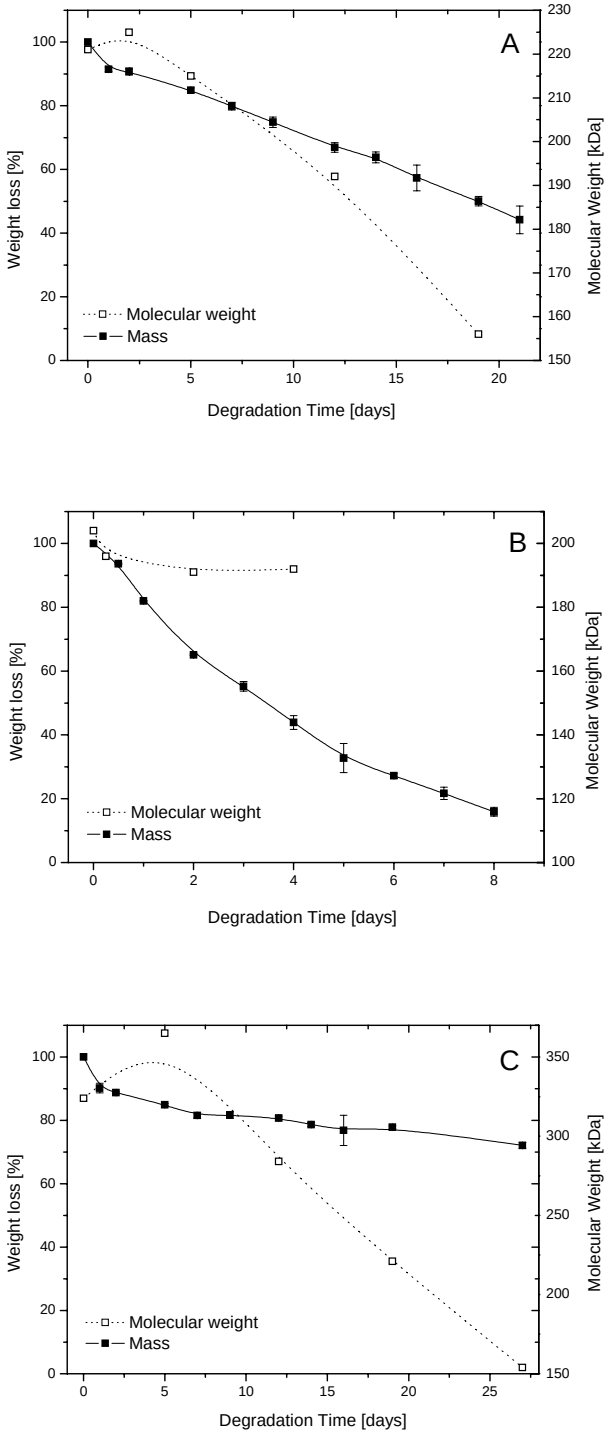

Figure 2: *In vitro degradation profiles of DEAPA-PVA-g-PLGA polymers. Weight loss and molecular weight as calculated from ^{1}H-NMR data as a function of incubation time in phosphate buffer (pH 7.4) at 37°C. (A) P(12)-10 showing a mixed bulk-surface eroding profile; (B) P(33)-10 showing a mainly surface eroding profile and (C) P(33)-20 showing a predominantly bulk eroding profile*

On a molecular level, degradation via selective cleavage of the PLGA side chains at the backbone can be excluded, as in that case the amine-PVA backbone would become soluble and would subsequently leave the polymer bulk. As a result, molecular weights during degradation would suddenly drop, which was not observed. Also, ^{1}H-NMR spectra of degraded specimens (data not shown) revealed presence of the backbone.

Amine substitution had a substantial effect on polymer degradation, presumably due to their positive charges affecting both, swelling and local pH. Hence, the pH of the *in vitro* degradation medium was thought to affect degradation rates. Additional experiments were carried out investigating the degradation of P(12)-10 in buffered media with pH 2 and pH 9, respectively. Degradation of PLGA was shown to be acid catalyzed [24] but polymer erosion (weight loss) was not accelerated unless a pH value of 13 was reached [25]. Similarly, Bittner et al. observed comparable erosion rates for PLGA at pH values of 2, 7.4 or 9 [26]. However, we noticed a base catalysed erosion behaviour for amine-PVA-*g*-PLGA accompanied by acid inhibitory effects as shown in figure 3.

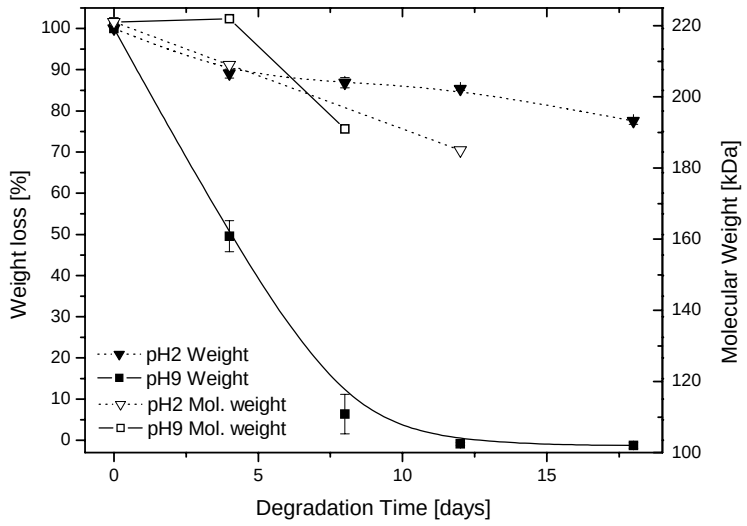

Figure 3: *In vitro degradation profile of P(12)-10. Weight loss and molecular weight as calculated from ^{1}H-NMR data as a function of incubation time in phosphate buffer (at pH 2 and pH 9) at 37°C*

In fact, after 12 days P(12)-10 showed a weight loss of only 14.7 ± 0.4 % at pH 2 as compared to 33.0 ± 1.5 % at pH 7.4, while at pH 9, almost 100 % of the test specimen was degraded. In addition to that, the degradation profile changes from a mainly bulk hydrolysis type at pH 2 over a mixed type at pH 7.4 to a mainly surface erosion type at pH 9. This effect could be explained by a pH dependent hydrolytic cleavage pathway as observed for PLA based polymers earlier [27]. Presumably the PLGA side chains are degraded mainly by random cleavage under acidic conditions, while at basic pH, hydrolytic cleavage happens predominantly via backbiting.

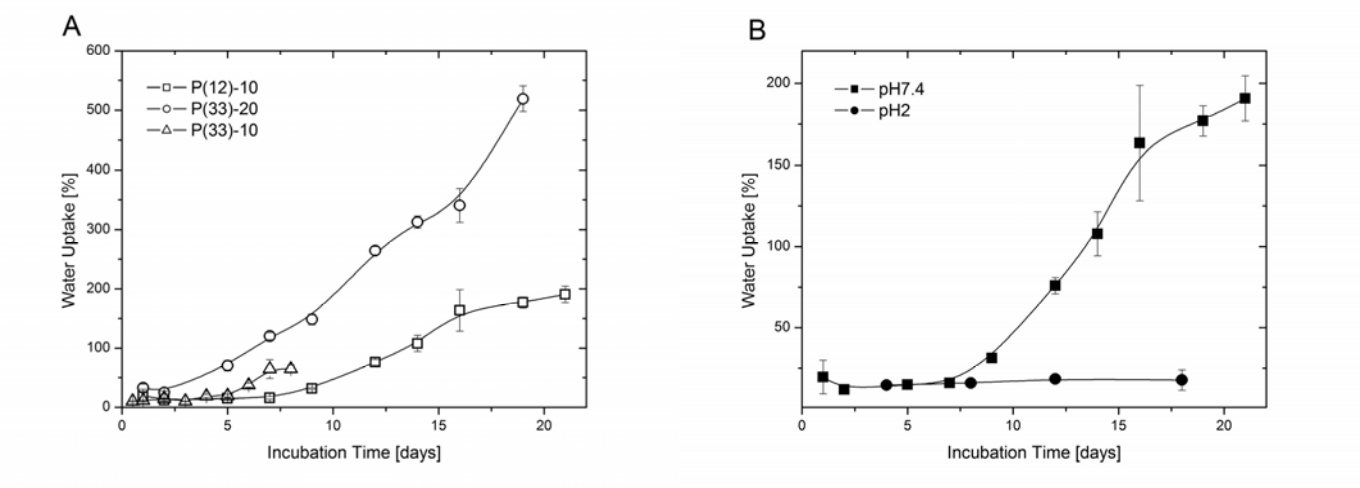

Figure 4: *Water uptake of DEAPA-PVA-g-PLGA polymers during degradation in phosphate buffer at 37°C. (A) Comparison of swelling profiles of P(12)-10, P(33)-10 and P(33)-20 at a pH of 7.4 and (B) Comparison of swelling profiles of P(12)-10 at pH 2 and pH 7.4.*

The swelling characteristics of amine-PVA-*g*-PLGA are in good agreement to the findings for other biodegradable polymers. It has been hypothesized, that bulk erosion is mainly characterized by a diffusion of water into the polymer bulk governed by the formation of breakdown products increasing the osmotic pressure, while surface erosion is mainly characterized by degradation of the polymer being faster than the diffusion [25]. In our studies, the slowest degrading polymer in the second test series, P(33)-20, was able to take up

more than 500 % of water during the testing period, while P(12)-10 took up approx. 200 % of water. The fastest degrading polymer, P(33)-10, showed a swelling of less than 70 % until it was completely degraded. The reason for the different swelling characteristics can be illustrated by scanning electron microscopy taken from specimens during degradation (Figure 5).

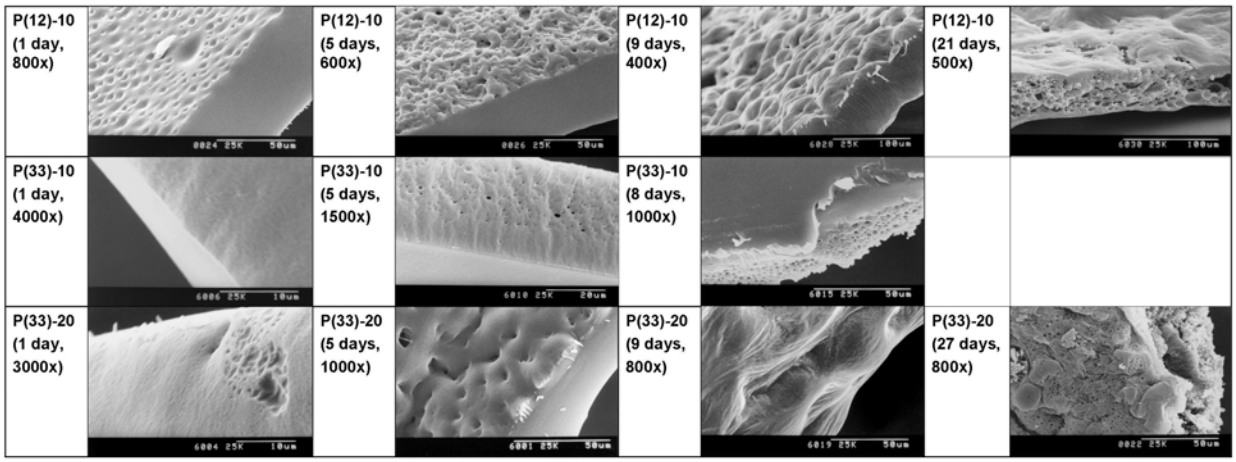

Figure 5: *Scanning electron micrographs of DEAPA-PVA-g-PLGA films after degradation in phosphate buffer (pH 7.4) at 37°C*

It can clearly be seen, that P(33)-10, which was found to be mainly surface eroding, develops only a slightly porous structure, while P(33)-20 showed a heavily porous, sponge-like structure after 27 days of incubation in buffer with a pH of 7.4. Confirming the swelling data during degradation at pH 7.4, P(12)-10 showed a medium pore formation. Interestingly, the observed degradation inhibitory effect of an acidic environment was also accompanied by nearly no swelling at all, as can be seen in Figure 4b. Thus, the degradation inhibitory effect of an acidic environment can not only be attributed to a slower random chain cleavage of PLGA side chains, as the oligomers formed should increase the osmotic pressure inside the specimen attracting even more water. This suggests a considerable role of the unprotonated amine functionalities for facilitating water uptake of degrading amine-PVA-*g*-PLGA polymers. As a hypothesis, at the temperature of 37°C being above the glass transition

temperature, P(12)-10 takes up only very small amounts of water in the acidic environment forming channels inside the specimen with the protonated amine functionalities orientating into these channels leaving a hydrophobic PLGA surface eroding very slowly. However, more detailed studies are required to observe this effect.

In summary, DEAPA-PVA-*g*-PLGA polymers showed a degradation profile, which could not be explained by either of the two known mechanisms, bulk or surface erosion, alone. It may be assumed that, due to the extensive swelling and the sponge-like structure of the polymer matrix formed during degradation of polymers with a higher side chain length, oligomeric acidic degradation products are able to diffuse out of the film leading to a more uniform degradation of the film in both the outer and inner disk regions. Thus, the formation of acidic microenvironments, as observed during the degradation of poly (lactic-co-glycolic acid) (PLGA), can presumably be avoided.

5. Conclusion

The degradation behaviour of a series of amine-modified PVA-*g*-PLGA polyesters was investigated under *in vitro* conditions with a special emphasis on elucidating the relationship between polymer structure and degradation properties. Remarkably shorter degradation times as compared to linear PLGA could be achieved by grafting short PLGA side chains onto amine-modified PVA backbones. By modifying the amine functionality, the degree of amine substitution and the PLGA side chain length the erosion rates of the resulting polymers can be designed to range from < 5 days to > 4 weeks. In addition, the pathway of hydrolytic degradation can be tuned to either mainly follow a bulk eroding profile or to predominantly erode from the surface.

The advantage of this modular concept consists in the ability to select degradation rate, degradation profile, charge density and, as earlier described, cytocompatibility which make

these amine-modified PVA-*g*-PLGA polymers promising materials for the controlled release of bioactive compounds and for gene delivery.

6. Acknowledgements

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Chapter 5

Poly(ethylene carbonate): A thermoelastic and biodegradable biomaterial for drug eluting stent coatings?

Journal of Controlled Release 117 (2007):312-321.

1. Summary

A first feasibility study exploring the utility of poly(ethylene carbonate) (PEC) as coating material for drug eluting stents under in vitro conditions is reported. PEC (Mw 242 kDa, Mw/Mn = 1.90) was found to be an amorphous polymer with thermoelastic properties. Tensile testing revealed a stress to strain failure of more than 600%. These properties are thought to be advantageous for expanding coated stents. *In vitro* cytotoxicity tests showed excellent cytocompatibility of PEC. Based on these findings, a new stenting concept was suggested, pre-coating a bare-metal stent with PPX-N as non-biodegradable basis and applying a secondary PEC coating using an airbrush method. After manual expansion, no delamination or destruction of the coating could be observed using scanning electron microscopy.

The surface degradation-controlled release mechanism of PEC may provide the basis for “on demand” drug eluting stent coatings, releasing an incorporated drug predominantly at an inflamed implantation site upon direct contact with superoxide-releasing macrophages. As a release model, metal plates of a defined size and area were coated under the same conditions as the stents with PEC containing radiolabelled paclitaxel. An alkaline KO_2 - solution served as a superoxide source. Within 12 hours, 100% of the incorporated paclitaxel was released, while only 20% of the drug was released in non-superoxide releasing control buffer within 3 weeks.

2. Introduction

Since the late 1970s percutaneous transluminal coronary angioplasty (PTCA) has revolutionized the treatment of coronary artery disease. Despite significant progress, PTCA frequently causes restenosis of treated arteries ultimately resulting in recurrent myocardial ischemia and anginal symptoms. Over the past decade, coronary stenting (placement of metallic mesh endoprostheses within the coronary artery at the time of PTCA) has been

shown to reduce incidence of angiographic and clinical restenosis compared with conventional balloon angioplasty. As a result, stenting is currently performed in approximately 80% of all PTCAs in the USA [1]. The use of bare metal stents caused a high incidence of restenosis, leading to frequent repetition of revascularization procedures. Polymer-coated stents, some of them releasing antiproliferative drugs, have been investigated extensively as documented by numerous reviews [1-9]. Amongst them, Cypher™ and Taxus™ have become commercially available [10]. Both stents are coated with non-degradable polymers as drug reservoirs. The Cypher™ stent is composed of a polymer matrix loaded with sirolimus and a polymer topcoat serving as diffusion barrier. Polymer blends containing poly(ethylene-co-vinyl acetate) and poly(butyl methacrylate) were reported to release 100% of sirolimus during approximately 1 month [10, 11]. The Taxus™ stent consists of a drug containing coating from poly(styrene-*b*-isobutylene-*b*-styrene) (SIBS), which releases paclitaxel in a biphasic manner [12, 13]. Approximately 10% of the drug incorporated can be released, whereas 90% of the drug remains in the polymer matrix indefinitely [10]. Both drug eluting stents reduced the rate of restenosis significantly as compared to bare-metal stents [14, 15]. Further improvements of existing stent systems are under development. Biological mimicry using a copolymer of phosphorylcholine and methacrylate has been investigated with promising results [16, 17] and recently, the use of crosslinked collagen as a biodegradable stent coating has been reported [18]. Numerous other substances are currently under investigation as potential biomaterials for drug eluting stent coatings and a comprehensive review has recently been published [19].

We hypothesized that a stent system consisting of an inert core coated with a flexible, but strong material with a smooth, biocompatible surface would be beneficial to ensure long-lasting vessel dilatation and controlled endothelialization [20]. This non-degradable core

should then be coated with a flexible, surface-erodable and biocompatible matrix serving as a controlled release drug reservoir.

Here, we present a stenting system consisting of a bare-metal stent coated with PPX-N, which has been described as a non-biodegradable, biocompatible, surface coating earlier [21, 22] and a second coating consisting of poly(ethylene carbonate) (PEC), a rubber-like, biodegradable polymer with good biocompatibility *in vivo* [23], which to our knowledge has never been investigated as a stent coating before. The concept is shown schematically in figure 1.

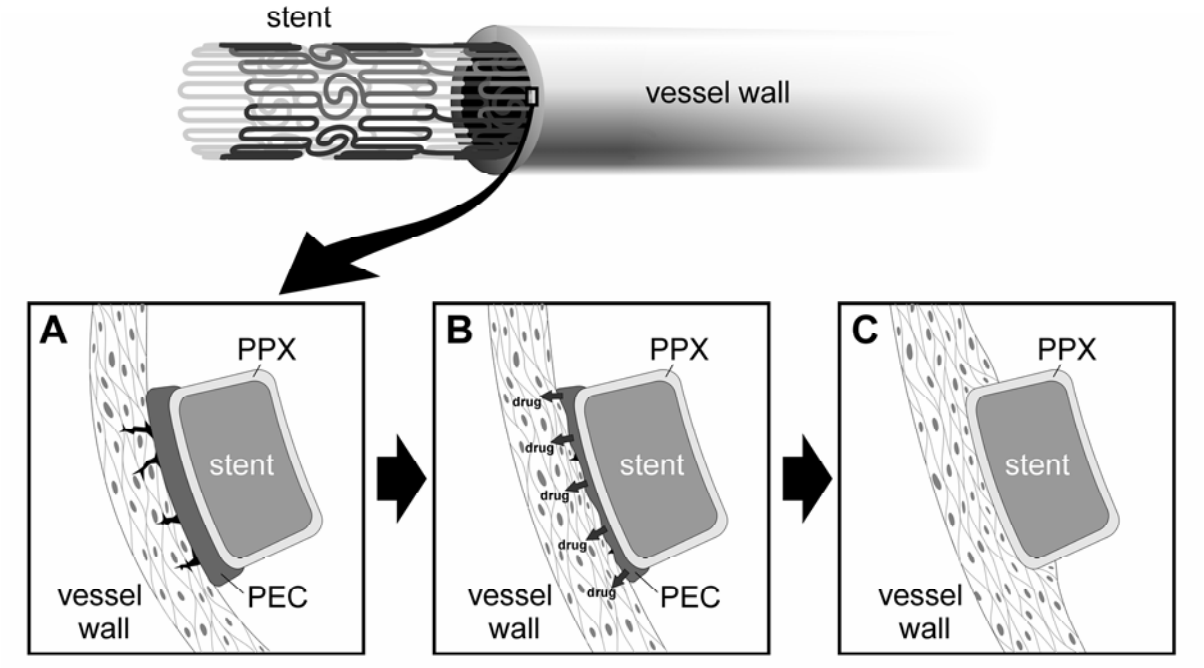

Figure 1: *Schematic illustration of functional and morphological changes of the double coated stent after stent implantation. Primary coating with PPX-N, covering the total stent surface, is generated using chemical vapour deposition (CVD). The outer, lesion site directed, stent surface is coated with a drug containing and biodegradable PEC layer using the airbrush method (A). When positioned into a stenotic vessel, subsequent inflammation attracts polymorphonuclear leucocytes and macrophages degrading the PEC coating; the drug is released towards the inflamed vessel wall preventing excessive over proliferation of smooth muscle cells, whilst the vascular lesion is able to heal (B). After degradation of the drug eluting layer, the smooth, non-degradable PPX coating remains on the stent surface and ensures stent endothelialization and long lasting stent compatibility to the surrounding vascular tissue.*

PEC is a unique polymer known to possess a surface degradation mechanism by superoxide anions produced by adhering polymorphonuclear leucocytes and macrophages [23-25]. Thus, apart from interesting thermoelastic properties, PEC could also serve as a target site specific coating, releasing an incorporated antiproliferative agent primarily at an inflamed implantation site upon direct contact with macrophages. The presence of macrophages and polymorphonuclear leucocytes has been mentioned in several publications dealing with the inflammatory response after PTCA in the restenotic process, which were reviewed recently [26, 27]. Here we report a first feasibility study exploring the utility of PEC as coating material for drug eluting stents under in vitro conditions.

3. Materials and Methods

3.1. Materials

Poly(ethylene carbonate) (PEC) with a weight average molecular weight of 242 kDa and a polydispersity of 1.90 was synthesized by Novartis Pharma AG according to the procedure previously described [28]. DPX of analytical grade was obtained from Speedline Technologies (Indianapolis, USA) and PPX-N was synthesized by CVD as described earlier [29]. Poly(*D,L*-lactic-co-glycolic acid) (RG502H; Resomer 50:50) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Paclitaxel (Genexol™) was kindly provided by Sam Yang Corp. (Seoul, Korea). Radiolabelled paclitaxel (paclitaxel-[2-benzoyl ring-UL-^{14}C]) was obtained from Sigma (Sigma Chemicals, Germany). Liquid scintillation cocktail was obtained from Packard BioScience (Ultima Gold™ LS cocktail, Groningen, Netherlands). All other materials used were of analytical grade.

Table 1: *Physico-chemical properties of PEC, PPX-N and PLGA*

Polymer	M_n (kDa)	M_w (kDa)	PD	T_g (°C)
PEC	127	242	1.90	31.1
PPX-N	n.d.[a]	n.d.[a]	n.d.[a]	80[b]
RG 502H	4.5[c]	7.8[c]	1.73[c]	36.4[c]

[a] n.d. = not determined due to insolubility of PPX – N in most solvents
[b] as published by Gorham [46]
[c] as published by Dani et al. [47]

3.2. Methods

3.2.1. Size exclusion chromatography (SEC)

The molecular weight of PEC was determined by size exclusion chromatography (SEC) using Dichloromethane as an eluent on a PSS-SDV linear pre-analytical column system (Polymer Standard Service, Mainz, Germany) equipped with a multi-angle laser light scattering (Malls) detector (Dawn EOS, Wyatt, Dernbach, Germany) and a refractive index detector (Merck RI-71, Darmstadt, Germany) at ambient temperature. In preliminary experiments, the refractive index increment (dn/dc) was shown to be 0.0565 ± 0.001 for PEC dissolved in dichloromethane (data not shown). Absolute molecular weights were calculated using the Astra software (Wyatt, Dernbach, Germany).

3.2.2. Dynamic water contact angle

The advancing water contact angle was measured using a G-10 Contact Angle Measuring System (Kruess, Hamburg, Germany) combined with a syringe pump set to a pump speed of 3.0 µl/min. and a maximum droplet size of 5.0 µl. As specimen, PEC coated glass cover slips (d = 15mm) obtained by spin-coating a 10 mg/ml PEC solution in dichloromethane at 60 rps (SCI-50, Novocontrol, Hundsangen, Germany) were used. To characterize contact angles after swelling, coated glass cover slips were pre-incubated in phosphate buffered saline (0.15 M, pH 7.4) for 48 hours. Samples were blotted dry using pulp before measurements. Each

measurement was performed in triplicate, and contact angles were calculated using SCA 20 software (DataPhysics Instruments GmbH, Filderstadt, Germany).

3.2.3. Sample preparation for physico-chemical and mechanical investigation

To obtain homogenous test specimen of poly(ethylene carbonate), polymer powder was compression-molded at 90°C and a pressure of 100 bar for 15 minutes using a laboratory press (Schwabenthan Polystat 200T, Servitec, Wustermark, Germany).

3.2.4. Differential scanning calorimetry (DSC)

Water uptake was assessed using a differential scanning calorimeter (DSC7, Perkin Elmer, Rodgau, Germany). Raw polymer powder and compression-molded film samples were incubated in phosphate buffered saline (0.15 M, pH 7.4) for 48 hours. Samples (approx. 5mg) were thoroughly blotted dry using Kimwipes (Kimberly-Clark, Roswell, USA), sealed in aluminum pans and heated in a nitrogen atmosphere. Thermograms covering a range of -50°C to 200°C were recorded at a heating rate of 10°C per minute. Water peaks of first heating runs were used to quantify absorbed water (Pyris Software, Perkin Elmer, Rodgau, Germany). Calibration of the system was performed using gallium and indium standards.

3.2.5. Wide angle X-ray diffraction (WAXD)

X-ray diffraction patterns of compression-molded poly(ethylene carbonate) films mounted on an aluminum cantilever were recorded on a Siemens D-5000 wide-angle goniometer (Siemens, Munich, Germany) equipped with DiffracPlus 3.0 software (Bruker, Rheinstetten, Germany) at room temperature. Nickel filtered Cu-K_α radiation was used as an X-ray source.

3.2.6. Tensile properties

Tensile measurements were performed using an extensometer (Model 4466, Instron, Darmstadt, Germany) equipped with a 500 N load cell and an oven. Polymers strips (30 x 3.55 x 0.7 mm, n = 4) were die-cut from compression-molded films. Results were analyzed using the Series IX software (Instron, Darmstadt, Germany).

3.2.7. Dynamic Mechanical Thermal Analysis (DMTA)

The measurements of the flexural storage (E') and the complex (Young's) modulus (E*) as well as the phase angle tan Delta and the dynamical glass transition temperature were performed using a Gabo Eplexor 100 N (Gabo Qualimeter GmbH, Ahlden, Germany) DMTA (Dynamic mechanical thermal analyzer) in the fixed frequency mode (10 Hz) between -80 and 140°C at a heating rate of 2 K/min. Polymer strips measured 10 x 4.5 x 2 mm and were cut from compression-molded films using a razor blade. Samples were measured in triplicate and results were analyzed using the specialized Gabo instrument software.

Table 2: *Mechanical and swelling properties of Poly(ethylene carbonate)*

Ultimate Tensile strength (MPa)	21°C	>10[d]
	37°C	2.6
Tensile modulus (MPa)[a]	21°C	88.04 ± 19.80
	37°C	20.78 ± 0.27
Flexural modulus (E') (MPa)[b]	21°C	1130
	37°C	11.74
Complex modulus (E*) (MPa)[c]	21°C	1240
	37°C	20.54
Dyn. T_g (°C)		31.1
Dynamic Water Contact Angle (°)	Dry	88
	Swollen	< 15
Water Uptake (%)	Powder	0.6
	Film	0.6

[a] as calculated from the maximum measurable elongation (1100 % at 21°C, 600 % at 37°C)
[b] Flexural storage modulus as measured by DMTA
[c] Complex (Young's) modulus as measured by DMTA
[d] at maximum measurable elongation (no break)

3.2.8. Stent coating

Stents consisting of stainless steel (length 48 mm, diameter of 3.12 mm) (JOSTENT™ peripheral, JOMED Implantate GmbH, Haan, Germany) were coated with PPX-N in a Lab coater (Model PDS 2010, SCS, Indianapolis, USA) using chemical vapor deposition (CVD) as previously described [29]. Basically, [2.2]paracyclophane (DPX) is pyrolyzed in the vapor phase at 600°C in vacuum to form two molecules of p-quinodimethane, which physically condensates and immediately polymerizes on cold surfaces (<30°C) to form a uniform pinholefree coating of PPX-N. In a next step the PPX-N basecoat was covered by a second polymer layer consisting of PEC by using the spray coating method. As a pre-treatment the PPX-N layer was swollen in chloroform for 24 hours to allow good adhesion of the PEC topcoat. Then, a polymer solution of 1% (w/w) in dichloromethane was sprayed onto the stent surface at a distance of 5 cm using an airbrush (Model Aero-pro Classic 10, Hansa, Oststeinbek, Germany) followed by drying under vacuum for 2 days at room temperature.

3.2.9. Scanning electron microscopy (SEM)

The morphology of coated stents was characterized by SEM using a Hitachi S-4100 microscope (Hitachi, Krefeld, Germany). Dried specimens were mounted on aluminum pins using double-sided adhesive tape. Prior to microscopic examination samples were sputter coated with a gold layer under vacuum for 30 seconds (Edwards Auto 306, Edwards, Kirchheim, Germany).

3.2.10. Cell culture

L929 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) and 2 mM glutamine. Cells were cultured as previously described [30].

3.2.10.1. In vitro cytotoxicity testing

In vitro cytotoxicity of polymer extracts was evaluated using the MTT assay as described previously [31]. Briefly, L929 cells were seeded into 96-well microtiter plates (Nunclon™, Nunc, Germany) at a density of 8000 cells/well. After 24 hrs the culture medium was replaced with 100 µl/ well of serial dilutions of polymer stock solutions in antibiotic-free DMEM (n = 4). After an incubation period of 24 hrs MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, Munich, Germany) was dissolved in phosphate buffered saline at 5 mg/ml and 20 µl were added to each well reaching a final concentration of 0.5 mg MTT /ml. After an incubation time of 4 hrs unreacted dye was removed by aspiration and the purple formazan product was dissolved in 200µl/well dimethylsulfoxide and quantitated by a plate reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at wavelengths of 570 and 690 nm. The relative cell viability [%] related to control wells containing cell culture medium was calculated by [A] test / [A] control x 100. Extracts from films of tin stabilized polyvinylchloride (PVC) and polyethylene (PE) were used as positive and negative controls, respectively.

3.2.10.2. Direct contact assay

To allow morphological assessment of L929 mouse fibroblasts directly growing on poly(ethylene carbonate), thin films were prepared by spin-coating polymer solutions in dichloromethane onto glass cover slips. Coated glass cover slips were sanitized with 70% isopropanol, placed in 12-well tissue culture plates and pre-incubated with serum supplemented cell culture medium for 4 hours. L929 mouse fibroblasts were seeded at a density of 35 000 cells /well and observed as described previously [32]. Pictures were taken using a SLR camera (Pentax MZ-7, Asahi Optical Co., Tokyo, Japan). Cell proliferation and morphology were compared qualitatively to uncoated glass cover slips, which were used as negative control.

3.2.11. In vitro release of Paclitaxel

To study the *in vitro* drug release in a model system with defined surface area, PPX-N pre-coated stainless steel plates measuring 2 x 2 cm were coated with PEC films containing paclitaxel (1% thereof ^{14}C-labeled) yielding a final theoretical loading of 1 µg/mm^2 or 40% (w/w). Film thickness was measured using a surface profilometer (Dektak ST, Veeco Instruments Inc., Woodbury, USA) obtaining values of 3.53 ± 0.31 µm (n=3).

Different buffer solutions were used to simulate the *in vivo* situations of paclitaxel-loaded PEC specimen under non-degrading and degrading conditions appropriately. As a physiological, non-degrading release medium, phosphate buffered saline (PBS) (0.05 M, pH 7.4) was chosen. An additional experiment was carried out using PBS (0.05 M, pH 7.4) supplemented with 10% ethanol to generate sink conditions for the lipophilic drug Paclitaxel.

PEC degradation conditions, physiologically caused by adhering polymorphonuclear leucocytes and macrophages, were mimicked by a superoxide containing release medium as previously described [24]. In brief, potassium superoxide (KO_2) (280 mM) was added to PBS (0.05M, pH 12) or PBS (0.05M, pH 12, 10% ethanol). KO_2 - solutions were prepared freshly every 24 hours. PBS (0.05M, pH 12, 10% ethanol) without KO_2 served as a control. Coated plates were incubated in 3 ml buffer solution in sealed tissue culture dishes at 37°C (n=3). At defined time intervals the buffer was withdrawn and replaced by 3 ml of fresh buffer.

3.2.12. Determination of paclitaxel release

To determine the amount of paclitaxel released after a defined time, one milliliter of the 3 ml of supernatant taken from the release protocol was mixed with 5 ml of scintillation cocktail (Ultima Gold™, Packard BioScience, Groningen, Netherlands). The activity of radiolabelled paclitaxel was quantified by liquid scintillation counting (LSC) (Packard Tri-Carb 2900TR, PerkinElmer, Rodgau, Germany) at a counting time of 15 minutes for each sample.

Total paclitaxel release was then calculated by the following equation:

Paclitaxel (*total*) = 3 x 100 x Paclitaxel (^{14}C).

3.2.13. Statistical Analysis

Data were obtained at least in triplicate and presented as means ± standard deviation. One-way ANOVA at a significance level of $P \leq 0.05$ with Tukey-Kramer Multiple Comparisons test was performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, USA).

4. Results and Discussion

4.1. Physico-chemical and mechanical characterization of poly(ethylene carbonate)

According to the investigations of Acemoglu et al. [28], poly(ethylene carbonate)s with molecular weight lower than 100 kDa are virtually non-degradable. Hence, for our studies we chose a PEC with an absolute molecular weight of 242 kDa (M_w) as determined by size exclusion chromatography combined with multi-angle laser light scattering (SEC–Malls) and refractometry. *In vivo* biodegradability of a PEC with comparable molecular weight was shown earlier by Dadsetan et al. [23].

As a measure of wettability, dynamic water contact angles were investigated on dry and pre-incubated PEC surfaces. A contact angle of approximately 88° was measured for dry, spin-coated films. We observed a significantly smaller contact angle of less than 15° for films after pre-incubation in buffer for 48 hours prior to the investigation. Thus, PEC is slightly hydrophilic upon the first contact with water, becoming very hydrophilic upon prolonged water contact suggesting water uptake. This phenomenon was further investigated by differential scanning calorimetry (DSC) of either a raw powder or a heat pressed film incubated in PBS buffer for 48 hours. First heating runs yielded water peaks at approximately 273K suggesting the water to be absorbed by the polymer. From the corresponding peak

areas, the amount of absorbed water was calculated to be 0.6% regardless of the formulation (raw powder or heat pressed film).

It is generally accepted, that hydrophobic surfaces tend to adsorb larger amounts of proteins than hydrophilic ones [33]. Therefore, the observed hydrophilicity of PEC could be beneficial for reducing protein adsorption as compared to more hydrophobic biomaterials such as PLGA. For biomaterials used at the blood-material interface, protein adsorption is known to be a precursor of platelet adhesion followed by activation and subsequent initiation of the coagulation process resulting in the formation of clots [34]. Detailed investigations of protein adsorption to PEC surfaces are currently being performed and will be published elsewhere.

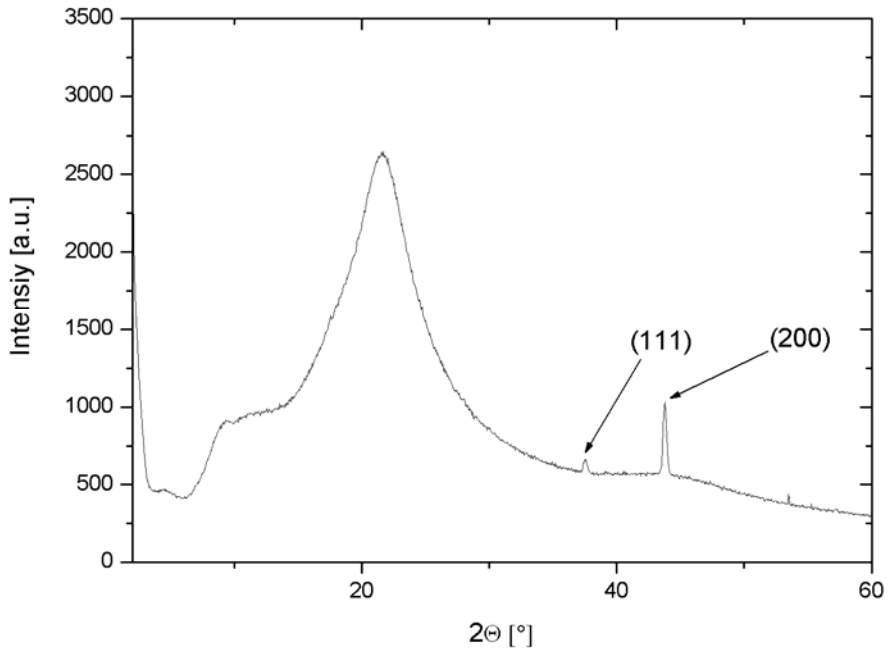

Figure 2: *WAXS diffractogram of Poly(ethylene carbonate). Wide angle X-ray scattering diffractogram of a Poly(ethylene carbonate)-heat compressed film mounted on an aluminium cantilever. The wide halo suggests an amorphous structure, the observed two sharp peaks arise from the underlying cantilever and can be assigned to the (111) and (200) crystalline reflection of the aluminium.*

At the time of PTCA, polymeric stent coatings need to withstand the strain forces during stent expansion. To some extent, the degree of crystallinity has an influence upon the mechanical properties of a polymer. Due to their lower degree of orientation, amorphous polymers are less brittle than crystalline polymers. Therefore, a compression moulded film of PEC was tested using wide angle X-ray scattering. We observed a wide, amorphous halo comprising two sharp peaks which arose the underlying aluminium cantilever and can be assigned to the (111) and (200) crystalline reflection of the aluminium as shown in Figure 2. Thus, poly(ethylene carbonate) can be regarded as an amorphous polymer.

To characterize the mechanical properties of PEC, tensile strength was tested at ambient and human body temperature. Ambient temperature investigations revealed a non-linear stress-strain curve characteristic of a hard-elastic material. PEC can be elongated repeatedly to at least ten times its original length without rupture. The total elongation could not be determined due to the maximum elongation limit of the testing instrument. The tensile Young's modulus of the polymer at ambient temperature is 88.04 ± 19.80 MPa compatible with a relatively soft and flexible material. The ultimate tensile strength is more than 10 MPa, the point where the elongation was about 1000 %, and the instrument limits were reached. Similar results, a tensile strength of 9.4 MPa and an elongation at break of 592%, have been reported for poly(styrene-*b*-isobutylene-*b*-styrene) (SIBS), the polymer applied in the Taxus™ stent [35]. When measured at body temperature of 37°C, the materials' mechanical properties changed considerably. The maximum elongation measurable was again limited by the instruments heating oven at 600%, which was reached without film rupture. The tensile Young's modulus is 20.78 ± 0.27 MPa, four times lower than at ambient temperature, suggesting a softening process during heating. The non-linear stress-strain curve (Fig. 3) shows a hyperbolic character, reaching a maximum tensile stress of 2.56 MPa at about 300% elongation. As a comparison, the tensile Young's modulus of PEC at 37°C is similar to that of

human cartilage (approx. 24 MPa) [36] and the stress to strain failure is superior when compared with that of arteries and veins (up to 260%) [37].

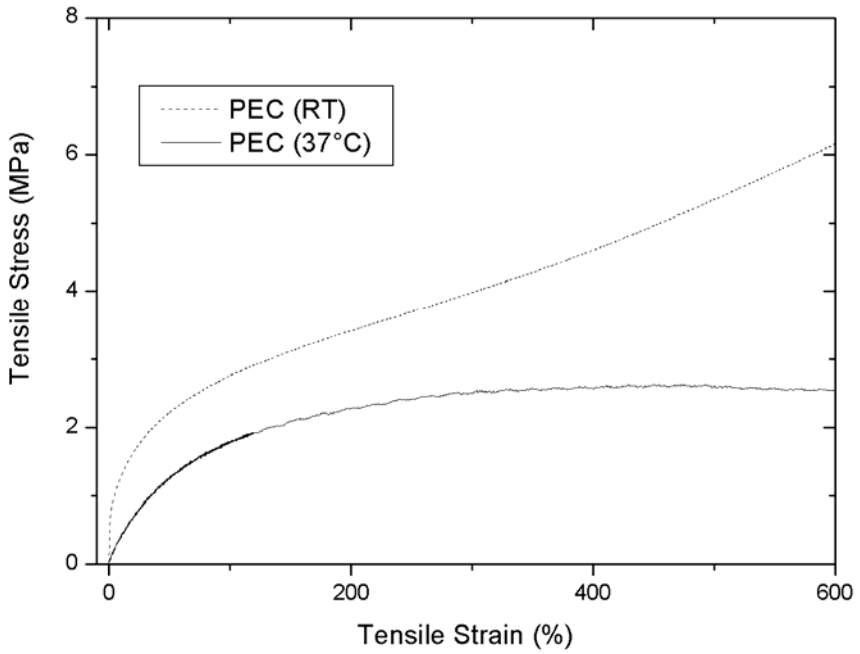

Figure 3: *Stress – strain curves of Poly(ethylene carbonate). At room temperature (RT) PEC shows a low modulus and large elongation ratio indicative for elastomeric materials. At body temperature (37°C), this behavior is even increased.*

Further investigation of PEC by dynamic mechanical thermal analysis (DMTA) yielded valuable data describing the temperature dependency of the materials mechanical properties. The complex (Young's) modulus vs. temperature curve shows a huge drop of material stiffness between 0 and 40°C from more than 2700 MPa to less than 15 MPa. The phase angle tan δ vs. temperature curve shows a maximum of 2.44 at 31.1°C also being the dynamical glass transition temperature. The flexibility of the material at body temperature is expressed by a phase shift of 54° (tan δ = 1.4) as shown in Figure 4. From these results it can be concluded, that PEC retains its form completely, when kept under its dynamic Tg of 31.1°C, e.g. during storage. With reference to the intended use as a stent coating, PEC can be stretched up to at least six times its original length without rupture at human body temperature

of 37°C. Consequently, the PEC film of a spray-coated stent should perform in an in-vitro expansion test without any signs of destruction.

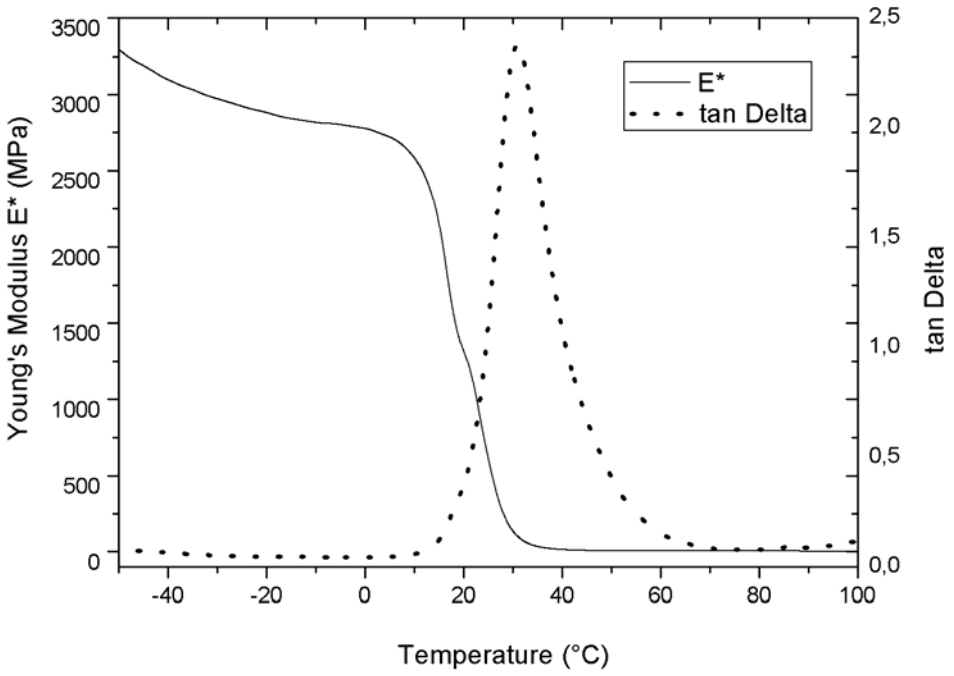

Figure 4: *DMTA Temperature Sweep of Poly(ethylene carbonate). The complex (Young's) modulus E* of Poly(ethylene carbonate) changes from > 3000 MPa at -50° C to less than 30 MPa at 37°C. The phase angle tan delta shows a maximum at 31.3 °C (dynamic glass transition temperature).*

4.2. Stent coating and expansion experiments

To test this hypothesis and to demonstrate the practical applicability of the mechanical properties of PEC in stent-coating, a feasibility trial comparing the surfaces of two differently coated stents after expansion was performed. Both stents were spray-coated, one using a PEC solution and a second one using a solution of a low molecular weight PLGA with thermoplastic mechanical properties (Resomer RG 502H). The thicknesses of the coatings were approximately 3 µm (as measured by SEM). Tempered stents (37°C) were expanded from 3.12 to approximately 5 mm in diameter by crimping onto a stainless steel thorn. Coating conditions after expansion were examined by scanning electron microscopy. As expected, the PEC-coated stent showed a smooth surface without any signs of disintegration

or delamination (Fig. 5 (A, B)), while the PLGA-coated stent showed ruptures and cracks at highly burdened stent parts (Figure 5 (C-F)).

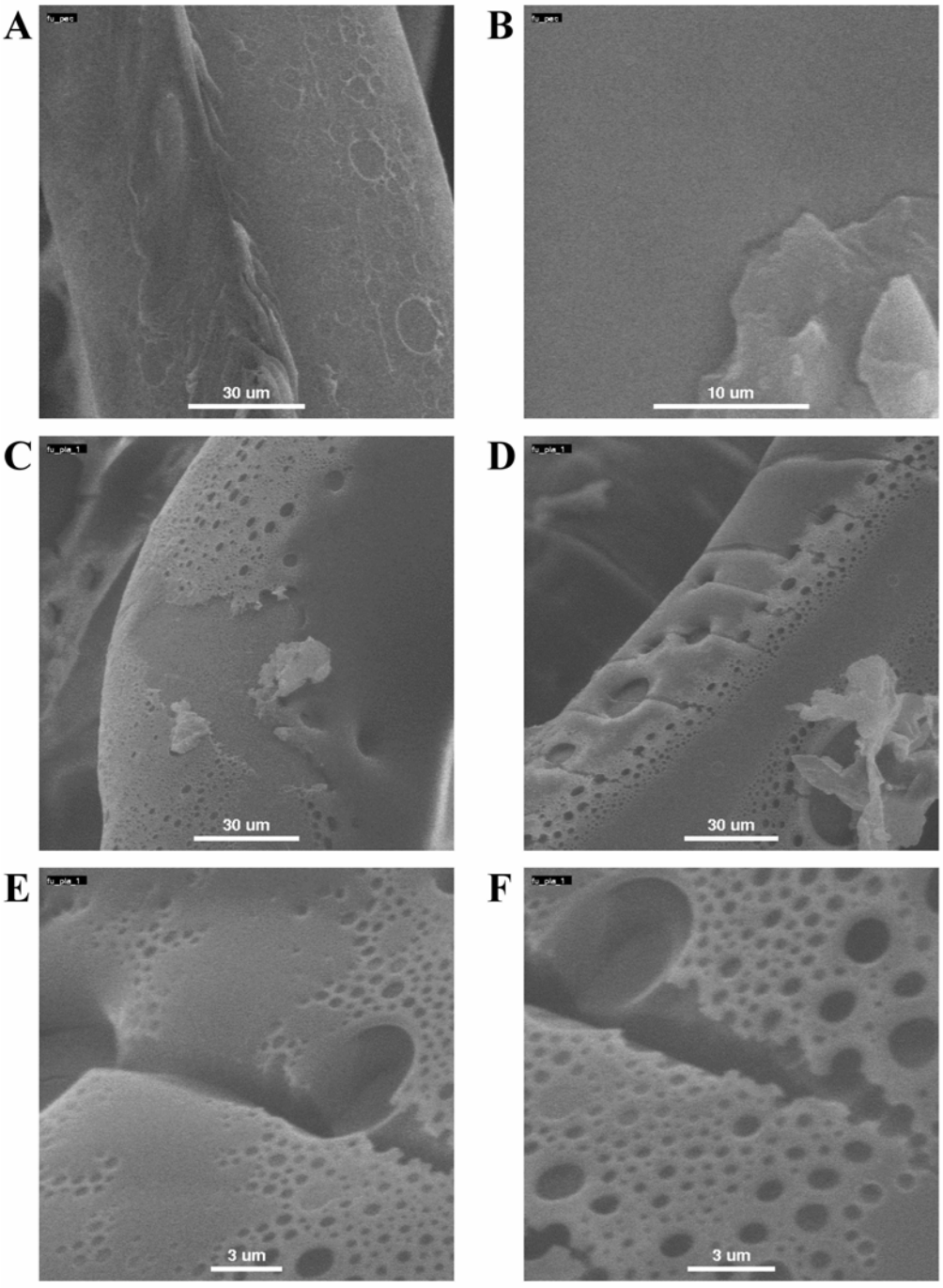

Figure 5: *Scanning electron micrographs of spray-coated stents after dilatation. Smooth PEC surface coating without any signs of disintegration at 1000 x (A) and 4000 x magnification (dust particle as focusing aid) (B). RG 502H surface coating showing ruptures at 900 x magnification (C) and cracks at highly burdened stent parts at 800 x (D), 6000 x (E) and 7500 x magnification (F).*

It is known, that ruptures and cracks could be problematic by inducing fast restenosis, uneven surface structures being a major cause of thrombogenic events with stent implants. In fact, the hope of overcoming the natural roughness of bare metal was one of the first reasons to coat bare metal stents with polymers [38]. In the approach presented in this work, a primary, non-degradable PPX-N layer remains covering all stent parts even after complete degradation of the drug-eluting PEC layer. This is expected to minimize the risk of bare metal parts getting into direct contact to tissue throughout the stents lifetime.

4.3. Cytotoxicity

Following the requirements set by the International standardization organization (ISO) 10993-5 [39], extract and direct contact testing of poly(ethylene carbonate) were carried out accordingly.

Extracts from PEC showed no significant signs of cytotoxicity under *in vitro* conditions as documented by MTT assay. Cell viability of L929-cells after 24 hours of incubation with different extract concentrations was still more than 80% and significantly higher than the positive control (tin-stabilized PVC) at the highest concentration of 6 cm^2/ml as depicted in Figure 6(A). The difference between PEC and the non-toxic, negative control (PE) was not significant at all concentrations tested. Direct contact assay was performed by seeding L929 cells directly onto PEC spin–coated glass cover slips (Fig. 6 (B)) and untreated glass cover slips as control (Fig. 6 (C)). No visible differences could be distinguished between test and control wells in terms of proliferation rate and cell morphology 48 hours after seeding. Cells were able to adhere and proliferate on PEC coated surface. The cells were spindle shaped and did not show any signs of toxic effects. As of our *in vitro* results, PEC is a biomaterial with excellent cytocompatibility. These observations are in close agreement to Stoll et al. [24], who reported good *in vivo* biocompatibility in rats, rabbits and dogs. With regard to hypersensitivity reactions to drug-eluting stents reported by Virmani et al. lately [40], these

findings are already very promising. However, van der Giessen et al. were able to show a major impact of the unique conditions within the blood vessel on the biomaterials biological performance [41] and it must be emphasized, that further *in vivo* studies are required to test the intracoronary biocompatibility of PEC.

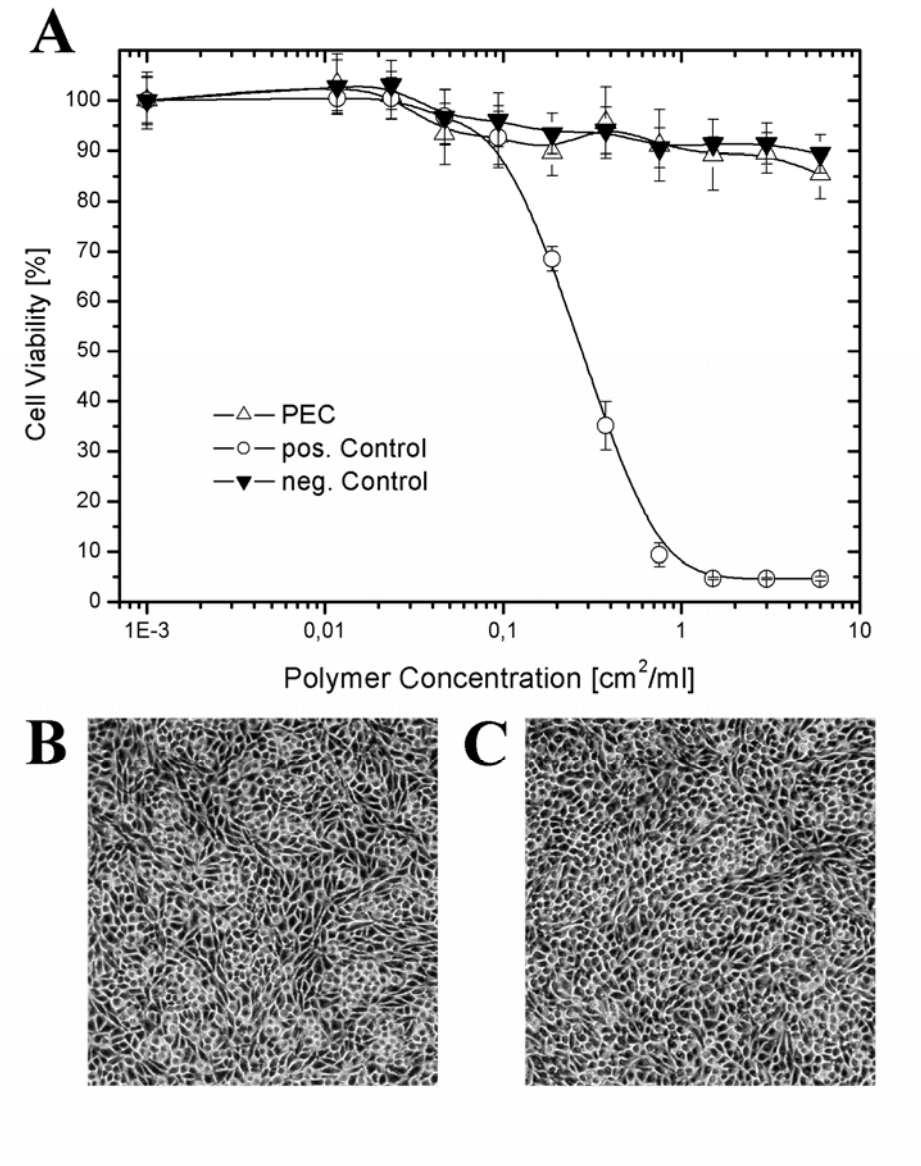

Figure 6: *Cytotoxicity of Poly(ethylene carbonate). Viability of L929 – cells measured by MTT absorption at 570 nm after 24 hours of incubation with different PEC extract concentrations (normalized values) as compared to tin stabilized PVC (positive control) and PE (negative control) (A) and comparison of L929 cell morphology and number on PEC (B) and glass (C) 48 hours after seeding.*

4.4. Drug Release from poly(ethylene carbonate) in an in vitro model

After evaluation of mechanical properties and cytotoxicity we explored drug release from PEC coatings containing Paclitaxel.

PEC is thought to degrade from the surface by superoxide anions produced by adhering polymorphonuclear leucocytes and macrophages [23, 42]. Thus, as a hypothesis, PEC could serve as a target site specific coating, releasing an incorporated antiproliferative agent primarily at an inflamed implantation site upon direct contact with macrophages. After vessel placement, which is usually combined with a balloon dilatation causing local lesions with subsequent inflammation [26], the PEC coating of the stent is supposed to be degraded by attracted polymorphonuclear leucocytes (PMN); the drug would be released towards the vessel wall preventing excessive overproliferation of smooth muscle cells, whilst the vascular lesion would be able to heal.

As we have shown earlier in this work, PEC matrices swell in aqueous solutions and are subject to degradation in the presence of superoxide anions. Therefore, two factors could possibly influence drug release from PEC, diffusion from the swollen matrix and release upon degradation, respectively.

At this stage of development, the hypothesis of a superoxide-triggered, “on demand” drug release from a PEC coating was investigated in a simplified *in vitro* model using paclitaxel as a drug. PPX-precoated stainless steel plates were coated with paclitaxel-loaded PEC films. To simulate the *in vivo* situation, drug release was studied in non-superoxide releasing (diffusion-controlled release) and superoxide releasing (degradation-controlled release) buffer media, respectively.

A physiological situation without inflammation was mimicked using phosphate buffered saline (pH 7.4) as release medium (Fig. 7). A burst release of less than 15 % of the incorporated paclitaxel within the first 24 hours could be shown, reaching a cumulative

release plateau of less than 20 % after 3 weeks of time, while a PLGA coating released nearly 50 % of the paclitaxel incorporated under the same conditions. Additionally, we could observe that during the full time of 3 weeks of immersion in PBS the PEC coating, which turns slightly opaque after swelling, adhered to the PPX precoating on stainless steel plates without any signs of detachment indicating firm adhesion. However, the in vitro model study does not allow full prediction of long-term and stress adhesiveness and further investigations for prolonged time and under in vivo conditions are required.

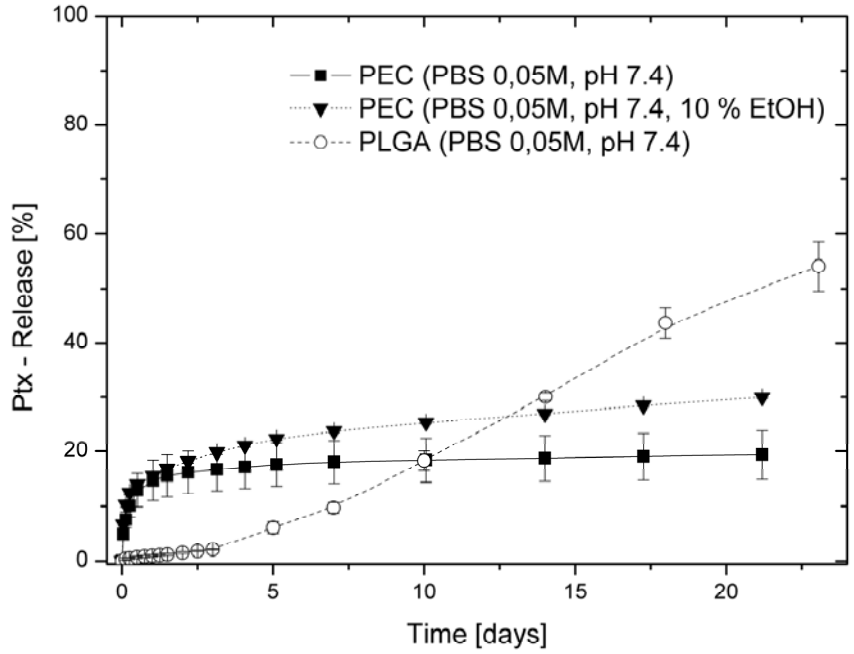

Figure 7: *In vitro-Drug release from PEC in non-superoxide releasing media. ^{14}C-Paclitaxel release from PEC- and PLGA-coated stainless steel plates under physiological conditions as measured by liquid scintillation counting (error bars represent mean ± standard deviation)*

Since paclitaxel is fairly hydrophobic, an additional experiment with a buffer containing 10% ethanol was run in parallel to exclude poor solubility of the drug as a reason for very slow release. As a result, drug release was only slightly increased and still less than 30% of the paclitaxel was released from PEC films after 3 weeks of time. These data confirm the findings of Stoll et al. [24] who stated, that PEC is not degraded hydrolytically under physiological

conditions. Moreover, it can be concluded, that the slight swelling of PEC observed upon contact with aqueous media does not lead to an extensive diffusion-controlled release of incorporated paclitaxel apart from the burst-release of approximately 20%. Nevertheless, further reduction of diffusion-controlled release of the incorporated drug would be beneficial for the *in vivo* situation and could possibly be achieved by adding a second, drug-free, PEC-topcoat as a diffusion barrier.

In a second study, paclitaxel release was investigated under PEC degrading conditions generated by a base stabilized superoxide releasing system containing potassium superoxide (KO_2) (280 mM) in PBS (0.05M, pH 12) or PBS (0.05M, pH 12, 10% ethanol) as shown in figure 8. Within less than 12 hours, the paclitaxel originally incorporated in the PEC layer was completely released in both, superoxide containing media with, and without 10% ethanol, respectively and complete degradation of the PEC topcoat could be visually examined by disappearance of the opacity of the swollen PEC topcoat. In contrast, complete drug release from PEC coated platelets incubated in PBS (pH 12, 10% ethanol) without KO_2 lasted nearly 7 days. Disappearance of opacity and the increased release of 100 % within 7 days of time from the control platelets in this study as compared to only 30 % within 3 weeks in neutral pH PBS could be explained by a base catalyzed degradation at pH 12 supported by the addition of 10 % ethanol. Supporting our hypothesis, a mainly degradation-controlled drug release from a PEC coating depending on the presence or absence of superoxide anions could clearly be shown in the *in vitro* model. However, limitations to the *in vitro* model should also be mentioned here: It is not yet known, how the amount of superoxide anions produced under the *in vitro* model conditions relates to an *in vivo* situation in the stented vessel wall. Drug-release rates measured in the superoxide-releasing degradation model would most likely be too fast for the *in vivo* situation and could in fact cause an excess of drug released from the stent in the event of a strong inflammatory response. An approach to overcome the latter could be the use

of PEC with molecular weights in the range of 100 kDa, since Acemoglu et al. reported reduced degradability for these [28].

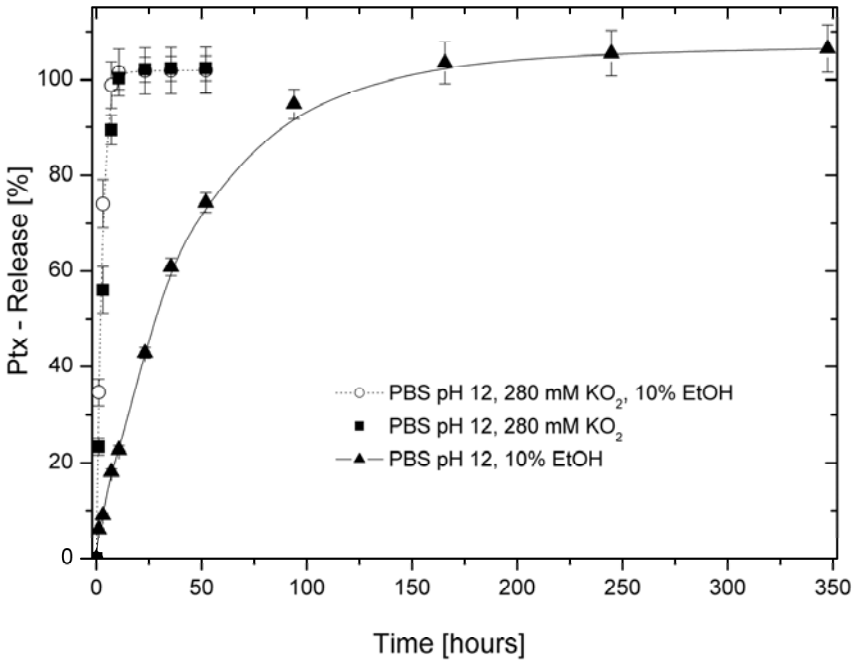

Figure 8: *In vitro-Drug release from PEC in superoxide releasing and non-superoxide releasing media. ^{14}C-Paclitaxel release from PEC-coated stainless steel plates under superoxide releasing conditions as measured by liquid scintillation counting (error bars represent mean ± standard deviation)*

Dadsetan et al. [23] and Stoll et al. [24] reported PEC to be degraded *in vivo* upon attack of superoxide anions generated by adhering polymorphonuclear leucocytes. For the stent situation it is known, that in-stent restenosis happens in four distinct phases: thrombosis, inflammation, proliferation, and matrix deposition/vessel remodeling [43], macrophages and lymphocytes playing a major role in the inflammation phase and proliferating smooth muscle cells forming the neointima in the matrix deposition phase [44]. Welt et al. were able to show a prolonged macrophage accumulation in stented rabbit arteries [45].

Taking into account the *in vitro* drug release results obtained from a superoxide releasing model, this suggests that a PEC-coated stent would release an incorporated anti–proliferative predominantly in the inflammation phase due to degradation by present macrophages. It could

therefore reduce smooth muscle proliferation in the proliferation phase subsequently leading to a reduced neointima formation, the main reason for in-stent restenosis. However, evidence for production of superoxide anions by macrophages in the vascular wall in the event of intimal hyperplasia is still preliminary and future *in vivo* experiments are required to confirm this and also to overcome limitations of the *in vitro* model.

5. Conclusion

The present study demonstrates that poly(ethylene carbonate) is a promising new stent-coating material due to its good biological and superior mechanical and degradation properties. After a short time of slight swelling in aqueous media, a PEC surface becomes very hydrophilic and therefore might substantially reduce protein adsorption and subsequently the formation of blood clots. Excellent i*n vitro* cytocompatibility was shown using standard methods. Based on its amorphous structure, elongation ratios of more than 600% are possible. Due to this flexibility, we were able to prepare a PEC-coated stent that could be expanded without causing any observable damage to the polymeric coating.

Results from *in vitro* paclitaxel release studies clearly show the influence of degradation by superoxide anions. This degradation-controlled release mechanism of PEC may provide the basis for “on demand” drug eluting stent coatings, releasing an incorporated drug predominantly at an inflamed implantation site upon direct contact with macrophages, which have been reported to be present earlier. However, this stent coating strategy merits further investigations under *in vivo* conditions. These experiments are currently under way in our laboratories.

6. Acknowledgements

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Chapter 6

Summary and Perspectives

Zusammenfassung und Ausblick

Summary

In this dissertation, degradation and biocompatibility of biodegradable polymers were investigated with a special emphasis on structure-property relationships.

As an introduction, **Chapter 2** reviews the current scientific knowledge of amphiphilic ABA triblock copolymers containing hydrophobic polyester A-blocks and hydrophilic polyether B-blocks. These block co-polyesters are able to form physically cross-linked hydrogels generated by rapid swelling upon exposure to an aqueous environment providing an attractive alternative to chemically cross-linked systems since they allow incorporation of macromolecular drug substances under mild process conditions. The synthesis and characterization of ABA triblock copolymers as well as their biological and degradation properties were discussed. Their use as biodegradable drug delivery devices in the form of implants, micro- and nano-spheres, especially for proteins, was summarized.

The idea of using co-polymers in drug delivery was enhanced by the generation of comb-like, branched polyesters with a hydrophilic amine-modified PVA backbone and short hydrophobic PLGA side chains. A particular advantage of these branched polyesters is their versatility of structural modifications. A matrix of amine-modified PVA backbone and Amine-PVA-*g*-PLGA polymers was systematically characterized *in vitro* establishing structure-toxicity relationships in **Chapter 3**. Effects of type and degree of amine substitution as well as molecular weight on cytotoxicity were evaluated in cell-based assays. A molecular weight and dose dependent cytotoxicity was found for amine-modified PVA. The type of amine functionality was of minor importance with DEAPA being slightly less cytotoxic than DEAEA and DMAPA. The cytotoxic effect is not caused by apoptosis but rather by necrotic reaction to the highly charged amine-modified PVA backbone polymers presumably by interactions of polycationic materials with cell membranes. The approach to improve the cytocompatibility of amine-modified PVA polymers using biodegradable PLGA side chains

turned out to be successful. Decreased charge densities and shielding positively charged amine moieties by PLGA side chains decreased the cytotoxicity.

A systematic evaluation of the influence of the polymer composition on *in vitro* degradation behaviour is reported in **Chapter 4.** In a first set of experiments, the weight loss of solvent cast films of defined size from 19 polymers was measured as a function of incubation in phosphate buffer (pH 7.4) at 37°C over a time of 21 days. A second study was initiated focusing on three selected polymers in a similar set up, but with additional observation of pH influences (pH 2 and 9) and determination of water uptake (swelling) and molecular weights during degradation. As hypothesized, our investigations revealed the potential to influence the degradation of this polymer class by the degree of amine substitution, higher degrees leading to faster erosion. The erosion rate could further be influenced by the type of amine functionality, DEAPA-modified polyesters degrading as fast as or slightly faster than DMAPA-modified polyesters and these degrading faster than DEAEA-PVA-*g*-PLGA. As a third option the degradation rate could be modified by the PLGA side chain length, shorter side chains leading to faster erosion. As compared to linear PLGA, remarkably shorter degradation times could be achieved by grafting short PLGA side chains onto amine-modified PVA backbones. Erosion times from less than 5 days to more than 4 weeks could be realized by choosing the type of amine functionality, the degree of amine substitution and the PLGA side chain length at the time of synthesis. In addition, the pathway of hydrolytic degradation could be tuned to be either mainly bulk or surface erosion.

The advantage of the modular conception resulting in the ability to predetermine degradation rate, degradation profile, charge density and cytocompatibility makes these amine-modified PVA-*g*-PLGA polymers promising materials for the controlled release of bioactive compounds and for gene delivery.

While the yet investigated PLGA-based polymers were all subject to degradation upon hydrolytic cleavage of ester bonds, "biodegradation" can also occur by other mechanisms such as enzymatic or biocatalytic cleavage. Poly(ethylene carbonate) (PEC) has been shown to exhibit an *in vivo* surface degradation mechanism by superoxide anions produced by adhering polymorphonuclear leucocytes and macrophages.

In **Chapter 5** a first feasibility study exploring the utility of PEC as coating material for drug eluting stents under *in vitro* conditions was reported. PEC was found to be an amorphous polymer with thermoelastic properties. Tensile testing revealed a stress to strain failure of more than 600%. Due to this flexibility, we were able to prepare a PEC-coated stent that could be expanded without causing any observable damage to the polymeric coating. *In vitro* cytotoxicity tests showed excellent cytocompatibility of PEC. Based on these findings, a new stenting concept was suggested, pre-coating a bare-metal stent with PPX-N as non-biodegradable basis and applying a secondary PEC coating using an airbrush method. As an *in vitro* release model, metal plates of a defined size and area were coated under the same conditions as the stents with PEC containing radiolabelled paclitaxel. An alkaline KO_2 - solution served as a superoxide source. Within 12 hours, 100% of the incorporated paclitaxel was released, while only 20% of the drug was released in non-superoxide releasing control buffer within 3 weeks. This degradation-controlled release mechanism of PEC supports our hypothesis of an "on demand" drug eluting stent coating.

Perspectives

In vitro investigations of structure-property relationships have shown that amine-modified PVA-*g*-PLGA polymers are promising materials for the controlled release of bioactive compounds and for gene delivery.

Yet further studies are required to prove the concept of these materials *in vivo.* Establishing *in vitro - in vivo* correlations would then be a major forthcoming for further optimization. In addition, both the cytotoxicity and degradation studies were based on pure polymers investigated in model systems. In the case of application of such polymers in drug delivery systems, further data need to be generated directly from the actual formulations, as the encapsulated drug will presumably alter the characteristics of the sole polymer substantially. With regard to the degradation characteristics, establishing a kinetic model *in vitro* would be an advantage for choosing the correct composition for a given application. This study should include the influence of test specimen design.

Apart from further characterization and optimization studies using the aforementioned components during polymer synthesis, additional parameters could be varied by design: the influence of the lactide to glycolide ratio in the side chains or the molecular weight of the PVA backbone could be tested.

Concerning the use of PEC as an “on demand” drug eluting stent coating, the presented concept should be proven by *in vivo* studies.

As all polymers investigated in this dissertation are intended to be used in parenteral applications, sterilization techniques and their influence on degradation, cytotoxicity and performance should be evaluated.

Zusammenfassung

In dieser Dissertation wurden Abbau und Biokompatibilität bioabbaubarer Polymere untersucht. Ein besonderer Schwerpunkt wurde dabei auf die Aufstellung von Struktur-Funktionsbeziehungen gelegt.

Als Einleitung zum Thema wurde der aktuelle wissenschaftliche Stand der Forschung an amphiphilen ABA Triblockcopolymeren im Rahmen eines Übersichtsartikels in **Kapitel 2** zusammengefasst. Die betrachteten Blockcopolymere bestehen aus hydrophoben Polyester-A Blöcken und hydrophilen Polyether-B Blöcken. Sie zeigen die Eigenschaft, bei Kontakt mit Wasser durch schnelles Quellen quervernetzte Hydrogele auszubilden. Damit stellen sie eine interessante Alternative zu kovalent verknüpften Polymernetzen dar, weil sie sich unter verhältnismäßig milden Prozessbedingungen mit makromolekularen Arzneistoffen beladen lassen. Das Kapitel geht zum einen auf die Synthese und Charakterisierung dieser Polymere ein, zum anderen werden aber auch die biologischen Eigenschaften und der Abbau betrachtet. Des Weiteren wird ein Überblick über die Verwendung von ABA Triblockcopolymeren als bioabbaubare Arzneiform, insbesondere für Proteine, gegeben. Diese Arzneiformen können unter anderem Implantate und Mikro- oder Nanosphären sein.

Eine Weiterentwicklung der Idee, Copolymere in der Arzneistoffverabreichung zu verwenden, stellen kammartig verzweigte Polyester bestehend aus hydrophilen, aminmodifizierten PVA Rückgraten und kurzen, hydrophoben PLGA Seitenketten dar. Ein großer Vorteil dieser kammartigen Polyester besteht in deren vielfältiger, struktureller Modifizierbarkeit. **Kapitel 3** beschreibt die systematische Untersuchung einer Matrix von aminmodifizierten PVA Rückgraten sowie Amin-PVA-*g*-PLGA Polymeren. Im Rahmen dieser *in vitro* Studien konnten Struktur-Funktionsbeziehungen hinsichtlich der Zelltoxizität aufgestellt werden. Die Einflüsse von Amintyp und -substitutionsgrad sowie des Molekulargewichtes auf die Toxizität wurden in zellbasierten Assays untersucht, wobei eine

molekulargewichts- und dosisabhängige Wirkung gezeigt wurde. Als weniger bedeutend für die Toxizität stellte sich die Wahl der Aminfunktion heraus: DEAEA- und DMAPA-modifizierte Polymere waren etwas toxischer, als DEAPA-modifizierte Varianten. Mittels differentialdiagnostischer Tests wurde nachgewiesen, dass die zelltoxische Wirkung zu einem nekrotischen (und nicht apoptotischen) Zelluntergang führt. Diese Beobachtung lässt sich sehr wahrscheinlich durch eine Interaktion der positiv geladenen Polymere mit der negativ geladenen Zellmembran erklären. Der Ansatz, die Zellkompatibilität der aminmodifizierten PVA Rückgrate durch bioabbaubare PLGA Seitenketten zu verbessern, war erfolgreich. Die verminderte Ladungsdichte und der Abschirmeffekt der positiven Ladungen durch die PLGA Seitenketten führte zu einer deutlichen Reduktion der Zelltoxizität.

Eine systematische Untersuchung des Einflusses der Polymerzusammensetzung auf das *in vitro* Abbauverhalten beschreibt **Kapitel 4.** Im ersten Schritt wurden von 19 Polymeren Filme hergestellt. Der Gewichtsverlust durch Abbau bei 37°C in Phosphatpuffer (pH 7,4) für bis zu 21 Tage wurde bestimmt. In einer zweiten Studie wurden dann drei ausgewählte Polymere unter vergleichbaren Bedingungen untersucht. Zusätzlich wurden der Einfluss des pH-Wertes (pH 2 und 9), das Quellungsvermögen und die Molekulargewichtsabnahme während des Abbaus betrachtet. Entsprechend der Arbeitshypothese zeigte sich, dass der Abbau dieser Polymerklasse über den Aminsubstitutionsgrad beeinflussbar ist, wobei höhere Substitutionsgrade den Abbau beschleunigten. Eine weitere Möglichkeit, die Abbaurate zu beeinflussen, war die Wahl der Aminfunktionalität. DEAPA-modifizierte Polyester zeigten einen vergleichbar schnellen oder geringfügig schnelleren Abbau, als DMAPA-modifizierte Polyester. Letztere wiesen im Vergleich zu DEAEA-modifizierten Polyestern eine erhöhte Abbaurate auf. Eine dritte Option, die Abbaugeschwindigkeit zu kontrollieren, war die PLGA Seitenkettenlänge. Kürzere Seitenketten führten zu einer deutlichen Abbaubeschleunigung. Im Vergleich zu linearen PLGA Polymeren konnten durch die strukturellen Variationen

insgesamt deutlich kürzere Abbauzeiten erreicht werden. Zudem war durch die entsprechende Auswahl von Amintyp und -substitutionsgrad, sowie die PLGA Seitenkettenlänge eine Einstellung der Abbauzeit von 5 Tagen bis zu 4 Wochen möglich. Darüber hinaus konnte das Abbauprofil von einem überwiegenden Massenabbau zu einem vorwiegenden Oberflächenabbau verschoben werden.

Durch das Baukastenprinizip der aminmodifizierten PVA-*g*-PLGA Polymere lassen sich Abbaurate, Abbauprofil, Ladungsdichte und Zellkompatibilität schon während der Synthese vorausbestimmen. Aus diesem Grund stellen die untersuchten Kamm-Copolymere eine vielversprechende Basis für die Entwicklung kontrollierte Freigabesysteme für bioaktive Materialien und Gene dar.

Neben den in den ersten Kapiteln untersuchten, PLGA-basierten Polymeren, die durch hydrolytische Esterspaltung abgebaut werden, gibt es auch Polymere, deren „Bioabbau“ durch andere Mechanismen erfolgt. Hierzu zählen enzymatische oder allgemein biokatalytische Abbauwege. Für Poly(ethylen carbonat) (PEC) konnte ein durch Superoxidaninonen hervorgerufener Oberflächenabbaumechanismus gezeigt werden. Diese aktiven Sauerstoffspezies werden *in vivo* von anhaftenden, polymorphonuklearen Leukozyten und Makrophagen freigesetzt. In **Kapitel 5** wurde die Durchführung einer *in vitro* Machbarkeitsstudie zur Verwendung von PEC als Überzugsmaterial für wirkstofffreisetzende Stents berichtet. Es wurde festgestellt, dass PEC ein amorphes Material ist und sich im Zugfestigkeitstest um mehr als 600% elastisch dehnen lässt, ohne zu reißen. Aufgrund dieser Flexibilität war es möglich, einen chirurgischen Metallstent mit PEC zu überziehen, der sich dann manuell aufdehnen ließ, ohne dass Beschädigungen am Überzug sichtbar wurden. Zudem konnte eine sehr gute Zellkompatibilität für das Material *in vitro* gezeigt werden. Aufgrund dieser Erkenntnisse wurde ein neues Stent-Design vorgeschlagen, indem ein Metallstent zunächst mit einer nicht-bioabbaubaren PPX-N Grundschicht überzogen wurde.

In einem zweiten Schritt wurde dann mittels einer Sprühpistole eine PEC Schicht als bioabbaubarer Wirkstoffträger aufgetragen. Die Wirkstofffreisetzung aus solchen Systemen wurde an Metallplättchen definierter Größe und Oberfläche aus Chirurgenstahl *in vitro* untersucht, die entsprechend den entworfenen Stents beschichtet wurden, wobei der PEC Schicht radioaktiv markiertes Paclitaxel als Modellarzneistoff zugesetzt wurde. Als superoxidaninonen-freisetzendes Abbaumedium wurde eine alkalische KO_2-Lösung eingesetzt. Innerhalb von 12 Stunden wurde das gesamte Paclitaxel freigesetzt, während im Kontrollpuffer, der keine Superoxidanionenquelle enthielt, nur 20% des Wirkstoffes innnerhalb von 3 Wochen freigesetzt wurden. Diese abbaugesteuerte, kontrollierte Wirkstofffreigabe unterstützt die Idee eines „nur bei Bedarf“ wirkstofffreisetzenden Stents, also dann, wenn tatsächlich eine entzündliche Gewebsreaktion mit dem Risiko einer Restenose abläuft.

Ausblick

Im Rahmen von *in vitro* Untersuchungen der Struktur-Funktionsbeziehungen konnte gezeigt werden, dass aminmodifizierte PVA-*g*-PLGA Polymere eine vielversprechende Basis für die Entwicklung kontrollierte Freigabesysteme für bioaktive Materialien und Gene darstellen. Folgestudien sollten nun den Beweis antreten, dass das zugrundeliegende Konzept auch *in vivo* seine Gültigkeit hat. Die Aufstellung von *in vitro* - *in vivo* Korrelationen wäre dann eine große Hilfe für die weitere Optimierung dieser Materialien. Weiterhin wurden die hier berichteten Zelltoxizitäts- und Abbaustudien an reinen Polymeren durchgeführt. Sobald eine Anwendung für ein bestimmtes Arzneistoffverabreichungssystem geplant ist, sollten zusätzliche Studien mit den jeweiligen Formulierungen erfolgen, da der eingeschlossene Arzneistoff die Eigenschaften des Polymers beeinflussen kann.

Bezüglich der Abbaueigenschaften dieser Polymere sollte ein kinetisches *in vitro* Modell aufgestellt werden, um anhand der Daten für eine entsprechende Applikation das richtige Polymer auswählen zu können. In diesem Rahmen könnte auch der Einfluss des Probendesigns (z.B. Film, Mikropartikel, Nanopartikel, Implantat) untersucht werden. Eine zusätzliche Möglichkeit, Materialien dieser Polymerklasse weiter zu optimieren, stellen auch konzeptionelle Änderungen dar. So wurde bislang nicht geprüft, welchen Einfluss das Verhältnis von Lactid zu Glycolid in den Seitenketten hat. Auch ein PVA Rückgrat mit einem anderen Molekulargewicht könnte getestet werden.

Das vorgestellte Stent-Design mit PEC als wirkstofffreisetzender Schicht sollte, insbesondere hinsichtlich des Konzepts der bedarfsgesteuerten Freisetzung, *in vivo* untersucht werden.

Da alle in dieser Dissertation betrachteten Polymere als Arzneistoffträger für die parenterale Verabreichung angewendet werden sollen ist es wichtig, geeignete Sterilisationstechniken und deren potentiellen Einfluss auf Zelltoxizität und Abbau zu durchleuchten.

Appendices

ABBREVIATIONS

LA	D,L-lactide
LLA	L-lactide
GA	Glycolide
CL	ε-caprolactone
PEO	Poly(ethylene oxide) or poly(ethylene glycol)
PLA	Poly(D,L-lactide)
PLLA	Poly(L-lactide)
PGA	Poly(glycolide)
PCL	Poly(ε-caprolactone)
PLGA	Random copolymer of D,L-lactide and glycolide
PLC	Random copolymer of D,L-lactide and ε-caprolactone
PLLC	Random copolymer of L-lactide and ε-caprolactone
PLA-PEO-PLA	Triblock copolymer of poly(D,L-lactide-block-ethylene oxide-block-D,L-lactide)
PLLA-PEO-PLLA	Triblock copolymer of poly(L-lactide-block-ethylene oxide-block-L-lactide)
PLGA-PEO-PLGA	Triblock copolymer of poly[(D,L-lactide-co-glycolide)-block-ethylene oxide-block-(D,L-lactide-co-glycolide)]
PLLGA-PEO-PLLGA	Triblock copolymer of poly[(L-lactide-co-glycolide)-block-ethylene oxide-block-(L-lactide-co-glycolide)]
PCL-PEO-PCL	Triblock copolymer of poly(ε-caprolactone-block-ethylene oxide-block-ε-caprolactone
PLC-PEO-PLC	Triblock copolymer of poly(D,L-lactide-co-ε-caprolactone-block-ethylene oxide-block-D,L-lactide-co-ε-caprolactone)
PLLC-PEO-PLLC	Triblock copolymer of poly(L-lactide-co-ε-caprolactone-block-ethylene oxide-block-L-lactide-co-ε-caprolactone)
AB	Diblock copolymer of polyester-polyether
ABA	Triblock copolymer of polyester-polyether-polyester
BAB	Triblock copolymer of polyether-polyester-polyether
DP	Degree of Polymerization
DMAPA	3-dimethylaminopropylamine = M
DEPAPA	3-diethylaminopropylamine = P
DEAEA	2-diethylaminoethylamine = E
PVA	Poly(vinyl alcohol)
A(x)-y	A indicates the type of amine substitution [P= DEAPA, M= DMAPA, E= DEAEA], x is the number of monomers in the backbone carrying amine substitutions, y is the PLGA side chain length calculated from feed
PEI	Polyethylenimine
NMR	Nuclear magnetic resonance
GPC	Gel permeation chromatography
TGA	Thermo-gravimetric analysis
DSC	Differential scanning calorimetry
MALLS	Multi angle laser light scattering
WAXD	Wide angle x-ray diffratometry
SEM	Scanning electron microscopy
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
LDH	lactate dehydrogenase

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- F. Unger, M. Wittmar, T. Kissel: Branched Polyesters of the type PVAL-g-PLGA allow design of rapidly degrading parenteral delivery systems; 30th Annual Meeting and Exposition of the Controlled Release Society (July 2003, Glasgow, UK) - Student Poster Highlight Award Presentation

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