Chapter 6

Cell behavior on polymeric amines derived from alternating polyketones

Abstract

Polymeric amines (polyamines) derived from chemical modifications of alternating polyketones were used as thin films to study the response of vascular smooth muscle cells (VSMC) and bovine arterial endothelial cells (BAEC). The physical and chemical properties of the polyamines were characterized at different cross-linking levels by FTIR spectroscopy, elemental analysis, gel content (cross-linking degree), water contact angle, and atomic force microscope (AFM). In this study, we find that polyamines without cross-linking or at low cross-linking levels may induce cell death by apoptosis, this being confirmed by activation of Caspase-3/7 assay and direct visual observation at the microscope. However, polyamines at high cross-linking levels display good biocompatibility with both VSMC and BAEC (i.e. supporting proper attachment and proliferation).

Key word: Polyamines; Polyketones; Apoptosis; Biomaterials

6.1 Introduction

In the past two decades, there has been a growing interest in the use of polymers as biomaterials for application as tissue scaffolds, polymer carriers for drug delivery or medical devices.\textsuperscript{1-3} Currently, many studies in tissue engineering are driven by a few classes of polymers such as polylactic acid, polyglycolic acid (PGA), chitosan, polyurethane, and polycaprolactone. These polymers are generally required to be non-toxic, biocompatible with living cells, and biodegradable. In more recent years, a promising class of novel biocompatible materials, alternating aliphatic polyketones prepared by using homogeneous palladium catalyst systems, have been reported for potential applications in pharmacological and biological fields.\textsuperscript{4,5} The remarkable advantage of this class of polymers is that their average molecular weight (and therefore chain length), polarity, crystallinity, mechanical and surface properties can be easily tuned to meet the specific requirements of many diverse biomedical applications. Nontoxic behavior of the polyketones was demonstrated by \textit{in vitro} tests over a period of 60 days.\textsuperscript{4} Polyketones have been even utilized as biocompatible scaffolds for primary urothelial cells \textit{in vitro} and \textit{vivo} studies.\textsuperscript{6} In addition, bioactive moieties such as monosaccharide fragments and protected tyrosine groups could be linked to the backbone of the polyketones by palladium catalyzed insertion polymerization.\textsuperscript{7,8}

Polymers containing amino functionality (e.g. polyethylenimine, chitosan, and polylysine) have been used and studied for many biomedical applications, such as drug- and DNA-carriers for gene delivery.\textsuperscript{9,10} Polyketones can potentially act as excellent precursors for the preparation of polymeric amines (polyamines) by chemical modifications via the Paal-Knorr reaction due to the presence of the highly reactive 1,4-di-carbonyl groups along the backbone.\textsuperscript{11} Different kinds of polyamines, containing different amino functionality (which can be either primary, secondary, tertiary or aromatic) as side chains, can be prepared via this kind of modifications. The easiness of the chemical modification makes the synthesis of different polyamines (different cross-linking degrees, amount of cationic species, and chemical structure of the backbone) a straightforward and fast task. In the present work, we demonstrate that the polyamines derived from alternating aliphatic polyketones have the ability to induce and moulate apoptosis of cells (i.e. a programmed cell death\textsuperscript{12-14} that is essential for tissue and organ development, physiologic adaptation, and disease).

In this study, we have investigated the behavior of rat vascular smooth muscle cells (VSMC) and bovine arterial endothelial cells (BAEC) on exposure to the polyamines \textit{in vitro}. Apoptosis assays were used to analyze the ability of the polyamines to induce apoptosis. A detailed structure-effect relationship was investigated in order to fully
understand which characteristics of the polyamines are actually responsible for apoptosis. Since cell adhesion and consequent states are dependent not only on the cell type but also on the physical and chemical properties of the polymers, a detailed characterization of the physical and chemical properties of the polyamine (e.g. wettability, surface roughness, and cross-linking level) was also carried out.

6.2 Experimental

Materials. The alternating polyketones (Mw 3970), ter-polymers of carbon monoxide, ethylene, and propylene were synthesized according to a reported procedure\textsuperscript{15}. 1,2-diaminopropane (Acros, \(\geq 99\%\)) and tetrahydrofuran (THF, Acros, \(\geq 99\%\)) were purchased and used as received.

Preparation of polymeric amines. Polymeric amines (polyamines) were synthesized in bulk by reacting two components (polyketones and 1,2-diaminopropane) in a single one-pot synthesis.\textsuperscript{11} After reaction, the resulting mixtures were washed several times with deionized Milli-Q water. After filtering and freeze-drying, light brown polymers were obtained as the final products. The polyamines with low (2.7 mmol/g) and high (4.5 mmol/g) degree of amino functionality were prepared here for study, corresponding to 40\% and 70\% conversion value of carbonyl groups of the polyketones, respectively.

Preparation of polyamine films. Polyamine films were prepared by a solvent-casting method on glass petri dishes (Diameter 40 mm). Polyamines were first dissolved in THF at a concentration of 50 mg/ml. After passing through a 200 nm syringe filter twice, 0.5 ml of polyamine solution was added into the petri dishes, which was then covered with a lid and placed in a fume hood at room temperature overnight for slow evaporation. Cross-linking of the casted films was carried out at 140 °C in vacuum oven at the different time intervals.

Characterization of polyamine films. The FTIR spectroscopy was performed using a Perkin-Elmer Spectrum 2000. Thermogravimetric analysis (TGA) was conducted in a nitrogen environment on a Perkin-Elmer TGA 7 instrument from 20 °C to 600 °C at a heating rate of 10 °C/min. Elemental analysis of C, H, N were performed with an Euro EA elemental analyzer. The gel content of the cross-linked polyamines was determined by solvent extraction with tetrahydrofuran (THF). Water contact-angle measurements were carried out at room temperature (20 °C) by the sessile drop method, using a custom-built microscope–goniometer system. A 1.5 \(\mu\)l drop of ultrapure water was placed on a freshly prepared sample using a Hamilton micro-syringe and the contact angle was measured after 30 s. At least five different places on the film surface were measured and all quoted angles are subject to an error of ± 2°. The atomic force microscope (AFM) measurements of morphology of polyamine films were performed in tapping mode using a NanoScope IV
multimode scanning probe microscope from Digital Instruments. The leaching of polyamines was studied on a HP 8453 Spectrophotometer. A phosphate buffer solution of pH 7.4 was added to the coated petri dish with polyamines. The coated petri dish was placed in an oven at 37 °C. After one day, 2 ml solution was withdrawn from the petri dish for UV-VIS spectrophotometer analysis.

Cell culture Studies. Rat vascular smooth muscle cells (VSMC) and bovine arterial endothelial cells (BAEC) were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Life Technologies) supplemented with 10% Fetal calf serum, 2% Penstrep and incubated under 5% CO₂ at 37 °C in a humidified incubator in all of the experiments described herein. Cell morphology was evaluated using a phase contrast, confocal laser microscope at 633 nm wavelength (Zeiss Microsystems LSM, Axiovert 135M) at different time intervals.

Cell behavior on polyamine films. Before being seeded with BAEC and VSMC, the polyamine films were first washed with 70% ethanol and three times with culture medium to remove contaminants. The cells were seeded at a density of 800 cells/cm² (i.e. 10000 per dish) for BAEC cells and 1600 cells/cm² for VSMC cells (i.e. 20000 cells per dish). To assess time-dependent cell viability and cell adhesion, the cells were cultured for various durations as indicated in the result section.

Cell response to polyamine solutions. The stock solution of polyamines (50 mg/ml) after protonation with acetic acid in de-ionized milli-Q water was diluted with culture medium to obtain different concentrations of polyamines, as indicated in the result section. BAEC were seeded into 96-well polystyrene plates (10000 cells per well) and incubated for 48 h at 37 °C with a 5 % CO₂ atmosphere. The medium was replaced after 48 h with the medium containing the various concentrations of polyamines. After 24 h, the apoptosis was determined by Caspase-3/7 assay (Promega). The positive control for apoptosis was performed by incubation with menadione (45 µM for 1 h). The medium was removed and a 50/50 solution of Caspase 3/7 assay and DMEM was added to the cells. The cells were incubated in the dark for 1 h and the fluorescence was recorded on a Wallac Victor 2 1420 multilabel counter luminometer.

6.3 Results and discussion
6.3.1 Characterization of polyamine films

Polymeric amines (polyamines), which have N-substituted 2,5-pyrrolediy1 groups incorporated in the backbone bearing an amino functional group pendant from the main chain, were synthesized by reacting a class of low molecular weight polyketones with 1,2-diaminopropane. The route of the chemical modifications of the polyketones described
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here just consists of a two component/one-pot reaction without the need of any catalysts, organic solvent or any additives. Furthermore, the reaction can be easily carried out by using mild conditions during the whole process. The TGA analysis performed in nitrogen shows a degradation temperature of the polyamines at around 200 °C. The degree of amino functionality was finely adjusted by varying the initial molar ratio between the diamine and the 1,4-di-carbonyl functions of the polyketones. A maximum of around 70% of carbonyl groups on the polyketone backbone can be actually converted into pyrrole units. This is due to statistical factors (two adjacent carbonyls must react in order to obtain ring formation) and steric hindrance. Here polyamines with low (2.7 mmol/g) and high (4.5 mmol/g) degree of amino functionality were used for study: one with 40% carbonyl conversion (denoted as PA40) and the other with 70% carbonyl conversion (denoted as PA70). Upon heating at high temperature (140 °C), the polyamines can be cross-linked by formation of either instable imine bonds or stable bis-pyrrole units (Figure 6.1). The exact pathway is truly dependent on the degree of amino functionality and in turn on the availability of the 1,4-di-carbonyl functions at the main chain of the polyamines.

**Figure 6.1** Cross-link reactions of the polyamines at high and low degree of amino functionality.

FTIR spectra for PA70 and PA40 are presented in Figure 6.2 before and after cross-linking. The peaks at 1707 cm⁻¹, corresponding to carbonyl group stretching vibration, as well as the range of 1500-1680 cm⁻¹ due to the skeletal stretching of the pyrrole ring, represent characteristic absorptions for the given system in all the spectra. At high degree of amino functionality (Figure 6.2a), the intensity of the absorption peak at 1707 cm⁻¹ (carbonyl groups) decreases significantly after cross-linking. This, together with the invariance of the pyrrole rings absorption, constitute an indirect evidence of imine
formation. With respect to low degree of amino functionality (Figure 6.2b), the increased intensity of the absorption peak at 1657 cm\(^{-1}\) (stretching vibration of the pyrrole rings) clearly indicates the bis-pyrrole formation upon cross-linking.

**Figure 6.2** FTIR spectra of polyamines at (a) high (PA70) and (b) low (PA40) degree of amino functionality before and after cross-linking.

The cross-linking level can be easily tuned as a function of the cross-linking time. Such dependence makes it possible from PA70 to prepare low-cross-linked samples after 2 h (PA70-IX-l), medium-cross-linked ones after 4 h (PA70-IX-m), and highly-cross-linked ones after 8 h (PA70-IX-h). The same holds for PA40 despite the differences in chemical structures of the cross-linked points. For the latter medium-cross-linked samples (PA40-PX-m) and highly-cross-linked ones (PA40-PX-h) were prepared after 4 h and 8 h cross-linking time. The increased cross-linking level of the polyamines with the cross-linking time was further verified by the oxygen content values deduced from the elemental
analysis (Figure 6.3). An increase in the cross-linking time leads to a gradual decrease of the oxygen content because of the water formation and release during the cross-linking process. The same trend is confirmed by the gel content measurements (Table 6.1). The gel content of the crosslinked PA70 slightly increases with the cross-linking time and no weight loss is found for the crosslinked PA40 for 4 h and 8 h cross-linking time, indicating a fully-cross-linked structure and stable bis-pyrrole formation.

![Figure 6.3 Oxygen content of the polyamines at high (PA70) and low (PA40) degree of amino functionality as a function of cross-linking time.](image)

**Figure 6.3** Oxygen content of the polyamines at high (PA70) and low (PA40) degree of amino functionality as a function of cross-linking time.

<table>
<thead>
<tr>
<th>Cross-linked polyamines</th>
<th>Cross-linking Time (h)</th>
<th>Gel content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA70-IX-l</td>
<td>2</td>
<td>81</td>
</tr>
<tr>
<td>PA70-IX-m</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>PA70-IX-h</td>
<td>8</td>
<td>96</td>
</tr>
<tr>
<td>PA40-PX-m</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>PA40-PX-h</td>
<td>8</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 6.1** Gel content of the polyamines at high (PA70) and low (PA40) degree of amino functionality as a function of cross-linking time.

Water contact angle measurements have been commonly used to characterize the relative hydrophilicity or hydrophobicity of polymer surfaces. The surface of the unmodified polyketones is relatively hydrophilic with an average contact angle of 56°. After chemical modifications, the contact angle of the polyamine film remains virtually unchanged: 52° for PA70 and 54° for PA40 before cross-linking. However, it is interesting to observe that the surface of the polyamines becomes more hydrophobic after cross-
linking (Figure 6.4). The water contact angles of the polyamine surface gradually increase with cross-linking time from 52° to 100° for PA70 and from 54° to 90° for PA40. Such dramatic change can be explained by the fact that the cross-linking reaction between carbonyl groups and amino groups leads to a reduction in the number of the polar groups (amino and carbonyl) at the polymer surface. This also confirms the results of the elemental analysis and gel content with respect to the number of polar groups as a function of cross-linking time. All these observations indicated that the cross-linking process could effectively modify the surface wettability of the polyamines.

**Figure 6.4** Contact angle of the polyamine films at high (PA70) and low (PA40) degree of amino functionality as a function of cross-linking time.

Surface morphology of the polyamines before and after cross-linking was examined with atomic force microscope (AFM) (Figure 6.5). The AFM images indicate that the morphology of the surface of PA70 is rather smooth and flat. Roughness analysis was performed over the entire image and the surface roughness is expressed as the root mean
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The surface roughness of the PA70 thin films before cross-linking is around 0.44 nm, which remains almost unchanged with respect to the cross-linking time: 2 h (0.44 nm), 4 h (0.42 nm), and 8 h (0.52 nm). A very similar surface morphology is also observed for the surface of the PA40 thin films before and after cross-linking. Thus it may conclude that the cross-linking process has no effect on the surface roughness of the polyamine films.

Figure 6.5 AFM images of the polyamine films (PA70) as a function of cross-linking time: (a) 0 h; (b) 2 h; (c) 4 h; (d) 8 h.

6.3.2 Cell culture studies

It has been reported that the use or application of a given biomaterial is closely related to cell behavior upon contact with them and particularly to cell adhesion to the material solid surface.22 Cell adhesion and proliferation on the polyamine films of PA70 with different cross-linking levels have been qualitatively examined by using phase contrast microscopy here. Since physical and chemical properties of the polyamines vary with the cross-linking level, the latter is also found to play an important role in determining the cell behavior. The cell behavior and response to solid films of PA70 at different cross-linking level are summarized in Table 6.2.
Table 6.2 Behavior of BAEC and VSMC on the films of PA70-IX-l, PA70-IX-m, and PA70-IX-h at the culture time of 1 h, 1 day, and 4 days. Qualitative scoring from ++ (= high / very well) to – (= absent / poor) is applied.

In general, BAEC and VSMC should attach and stretch out as to maintain viability. Poor attachment and rounding up of these cells are indicative for improper cell-material interaction, which may be followed by cell death due to apoptosis.\textsuperscript{23,24} In 1 h, both BAEC and VSMC started to attach at the surface of PA70 at different cross-linking levels. On day 1, it was observed that BAEC died upon contact with the poorly cross-linked PA70-IX-l. On the medium cross-linked PA70-IX-m, they attached, but did not stretch out over the surface. On the highly cross-linked PA70-IX-h, attachment and subsequent morphological change, i.e. stretching out over the surface and developing the typical shape of BAEC, was good (Figure 6.6). With respect to VSMC, better tolerance to apoptosis effect of the polyamines can be observed than for BAEC: some cells were still attached at the film surface and assumed normal VSMC morphology whilst stretching over the surface on day 1. On day 4, no survived BAEC and VSMC were detected at the surface of PA70-IX-l and PA70-IX-m. However, PA70-IX-h supports both BAEC and VSMC after 4 days. Both BAEC and VSMC were well-attached and stretched out over the surface of PA70-IX-h to obtain proper morphology (Figure 6.7). Colonies of VSMC cells were even observed at the surface of PA70-IX-h (Figure 6.7b) on day 4. As a general observation, we must make note here that VSMC attached better and proliferated faster than BAEC. The observed behavior might in any case be explained by assuming that the released polyamines (from the surface of the films) is responsible for the apoptotic effect due to the non-stable imine crosslinking bond. By using UV-Vis spectroscopy, the absorbance at the wavelength of 350 nm, ascribed to pyrrole rings of the polyamines was detected in phosphate buffer at pH of 7.4 at 37 °C, supporting the probability of the release of the polyamines into the cell culture.
medium at a function of culture time. As a consequence, we might conclude that cross-linking density (a structural factor) of the films is essential in determining cell survival and a high cross-linking level can lead to the disappearance of the apoptotic effect.

Figure 6.6 On day 1, morphology of BAEC on the films of (a1) PA70-IX-M, (a2) PA70-IX-h and VSMC on the films of (b1) PA70-IX-1, (b2) PA70-IX-m, and (b3) PA70-IX-h at the magnification (10×).

Figure 6.7 On day 4, morphology of (a) BAEC at the magnification (10×) and (b) VSMC at the magnification (10×) on the films of PA70-IX-h.

To further confirm that polyamines can induce cell death by apoptosis, the effect of a wide concentration range of the PA70 solutions on BAEC was studied to determine the cellular response by microscopical examination. Depending on the dose of PA70, substantial changes in cell morphology were detected upon exposure to polyamines (Figure 6.8). Upon treatment with increasing doses of polyamines, BAEC turned into bubbled spheres and partially detached, which is suggestive for an apoptotic death.23,24 At the
highest dose of 250 μg/ml of PA70, cells perished. Cell death was either observed as a loss of cell morphology followed by detachment, or an appearance of cytoplasm-free cell remainders at the bottom of the wells, presumably representing bare cytoskeletons. Thus, it seems that at this very high dose necrosis (uncontrolled cell death) becomes the dominant death mechanism.

**Figure 6.8** Morphology of BAEC after treatment with PA70 after 4 h at different concentration: (a) 2.5 μg/ml; (b) 7.5 μg/ml; (c) 25 μg/ml; (d) 75 μg/ml; (e) 250 μg/ml.
The activation of the Caspase-3/7 at different doses of PA70 was measured (Figure 6.9) in order to further pinpoint the concentration at which apoptosis occurs. The results show that the cells died from apoptosis already at a concentration of 2.5 \( \mu \text{g/ml} \). At 25 \( \mu \text{g/ml} \) of PA70, activation of Caspase-3/7 was substantially less than at lower doses (2.5 to 12.5 \( \mu \text{g/ml} \)), reaching the level of the negative control experiment. However, microscopical examination of these cultures showed that cell death occurred rapidly at 25 \( \mu \text{g/ml} \) of PA70. Thus, it can be concluded that at or below doses of 12.5 \( \mu \text{g/ml} \) of PA70, cell death involves apoptosis, while at 25 \( \mu \text{g/ml} \) of PA70 causes necrosis.

![Figure 6.9 Caspase activation of BAEC at a effect of dose concentration of PA70.](image)

Normally, apoptosis can be induced in various ways, such as a loss of intracellular water, chromatin condensation, internucleosomal DNA fragmentation, mitochondrial swelling, interaction with cell membrane receptors.\(^{25,26}\) The cause of apoptosis in this study may be correlated with the primary amino groups and in turn by the presence of positive charges on the polyamines. It has been well established that natural low-molecular aliphatic amines (e.g. putrescine, spermidine, and spermine) and many of their structural analogues are involved in the apoptosis process of cells and thus utilized as tools for apoptosis-based cancer therapies.\(^{27-29}\) However, the exact mechanism of cytotoxicity here must be further investigated to check whether cytotoxic effects are mainly mediated by interactions of the polycations with cell membranes or by cellular uptake and subsequent activation of the intracellular signal transduction pathway here.\(^{30}\) The observation for apoptosis effects of the polyamines may can find its application in the design of drugs or medical implants that require cytostatic properties.\(^{31}\) A most illustrative example is the drug-eluting stent (an implant designed to revascularize obstructed arteries), which is currently covered with cytostatic drugs to prevent hyperplastic renarrowing of the vessel lumen.\(^{32,33}\)
On the other hand, the study of cell behavior on the films of PA40 after cross-linking demonstrated good biocompatibility with both BAEC and VSMC. The apoptotic effect completely disappeared for both PA40-PX-m and PA40-PX-h due to a non-reversible and stable cross-linking bond (Bispyrrole). Similar cell behavior and morphology were observed for PA40-PX-m and PA40-PX-h. The morphology of BEAC and VSMC on the surface of PA40-PX-h (Figure 6.10) with respect to different culture time was shown as

**Figure 6.10** Morphology of BAEC and VSMC on the films of PA40-PX-h on day 1, day 3, and day 9 at the magnification (10×).
example here. Both types of cells were well-attached at the surface of PA40-PX-h. The increasing number of both type of cells over time demonstrated the occurrence of proper proliferation at the surface of polymer films. After 9 days of culture time, both types of cells had grown into confluent layers with a considerably higher cell population density than that of samples on day 3. The morphology of cells grown onto the surface of PA40-PX-h was found to be comparable with that of cells on polystyrene (cells on polyamine films and on polystyrene platforms both reached confluency at day 9). The results for good biocompatibility of PA40 after cross-linking may be utilized to enhance cell adhesion and tissue integration for tissue scaffold application.

### 6.4 Conclusions

In the present study, a new type of polyamines, synthesized from the chemical modifications of alternating aliphatic polyketones, was used as biomaterial to study the behavior of BAEC and VSMC. Depending on the degree of the attached amino functionality, the polyamines can be cross-linked either by imine or bis-pyrrole formation. The water contact angle gradually increases with the cross-linking level, thus indicating that the material becomes more hydrophobic during the cross-linking process. In addition, a flat, smooth nature of the polyamine films can be obtained upon solvent casting before and after cross-linking. Based on Caspase-3/7 assay and the study of cell morphology at the surface of the polyamine films, it was found that cell behavior (apoptosis, growth, and proliferation) can be finely tuned by adjusting the cross-linking degree/time of the polyamines. The observed cell behavior with respect to the polyamines (i.e. low cross-linking level induce cell apoptosis and high crosslinking level support cell growth and proliferation) may open many special biomedical applications, e.g. using them as platforms to local delivery of drugs (such as drug eluting stent) or medical implants that require good biocompability. The present study warrants extensive studies with respect to biocompatibility of the polyamines that are specifically modified to serve these respective goals.

### 6.5 References

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