 DOI: 10.1002/masy.201300107

Development and Noninvasive Characterization of Hormone Releasing In Situ Forming Implants

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Summary: In the human body aldosterone plays a major role in the regulation of the salt water balance and the blood pressure. To investigate the pathophysiological effects of aldosterone in a mouse model appropriate drug delivery systems, that release the drug in a constant and continuous manner, are needed. Therefore novel in situ forming implants were developed as alternative aldosterone releasing depots. The non-invasive analytical method electron spin resonance (ESR) spectroscopy was applied to characterize in situ the implant formation by direct and continuous quantification of polymer precipitation and solvent exchange rates. Therewith influences of several key formulation parameters, such as type of the solvent and the polymer have been investigated. In this context AB di- and triblock copolymers of poly(ethylene glycol) (PEG) and poly(lactide-co-glycolide) (PLGA) were firstly explored as polymeric matrices for in situ forming implants. The phase separation kinetics and therewith the aldosterone releases were highly dependent on the hydrophilic character and the molecular weight of the used polymers.

Keywords: aldosterone; block copolymer; EPR; ESR; in situ forming implant; PEG-PLGA; PLGA; solvent removal

Introduction

Diseases of the cardiovascular system rank as the number one cause of mortality and morbidity worldwide.[1] One of the recognized risk factors for vascular diseases is the excessive activation of the renin-angiotensin-aldosterone system. Aldosterone is one of the endogenous effectors.[2] By activation of the mineralocorticoid receptor it increases the reabsorption of water and ions in the kidneys and expands the blood volume resulting in increased blood pressure.[3] Further aldosterone can lead to inflammation, proliferation and remodeling of connective tissue in the blood vessels, the heart and the kidneys. However, the mechanism, by which aldosterone exerts its harmful effects on the cardiovascular system, is largely unknown. To investigate the pathophysiological effects of aldosterone in a mouse model appropriate drug delivery systems, that release the drug in a constant and continuous manner over a period of several days to months, are needed. Classical drug delivery systems in preclinical research, like osmotic pumps or implants, need surgery in its administration, which is related to high physical stress for the research animal. After anesthesia it is often seen that mice remove the suture used for wound closure. There are devices to inhibit this, but they are accompanied with major limitations in mobility for the animals. Furthermore, recent studies demonstrate that the desired constant release of mini pumps with poor water soluble drugs, to which belongs aldosterone, is often not reached.[4] Our own studies also proved that commercially available aldosterone releasing implants, contrary to the manufacturer's

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instructions for their "hormone-containing pellets", possessed no continuous release (data not shown).

So the controlled release of hormones is not only desired in human drug therapy but also in preclinical animal disease models. Therefore novel in situ forming implants (ISFI) were developed as alternative aldosterone releasing depots. ISFI are an attractive formulation principle, because they are (compared to microparticles) easy to manufacture and (compared to implants) avoid the use of large needles or surgery. Especially systems based on solvent exchange induced phase separation have attracted research interest in the recent years.[5] They consist of water insoluble polymer dissolved in the water miscible, physiological compatible organic solvent. After injection the polymer precipitates during solvent/non-solvent (body fluids) exchange.[6] Initial drug release occurs simultaneously with the formation of the implant. In this connection the phase inversion dynamics of the polymer solutions directly influence the drug release behavior and the implant morphology.[5] Over the past decades, polymers composed of poly-(lactide-co-glycolide) (PLGA) have been investigated extensively for a wide variety of ISFI based on solvent exchange induced phase separation.[7,5] The advantage of PLGAs includes their biodegradability by simple hydrolysis of the ester backbone in aqueous environment. The degradation products can be further metabolized to carbon dioxide and water. However the strong hydrophobic character of these polymers has caused some limitations, especially hydrophobic interactions of drugs with the matrix, resulting in a lag phase in drug release prior to the onset of erosion and often incomplete release.[8] Furthermore PLGA degradation is characterized by bulk hydrolysis and autocatalysis.[9] The carboxylic end groups of degraded products are able to accelerate the degradation and decrease the local pH inside matrices to values as low as pH 2.[10,11] The acidic microclimate is responsible for loss of integrity of acid-labile drugs.[12,11] One strategy to minimize hydrophobic interactions and to prevent the internal pH decrease is the incorporation of the hydrophilic poly(ethylene glycol) (PEG) into biodegradable polyesters.[5] PEG is a non-toxic water soluble polymer. Low molecular PEGs can be easily excreted by humans. Various kinds of block copolymers with different block structure and composition have been developed. According to their block structure they can be classified as AB diblock, ABA, or BAB triblock, multi-block, and star-shaped block copolymers, in which A is a hydrophobic block made up of biodegradable polyesters and B is a hydrophilic PEG block.[13,14] Recently di- and triblock copolymers of PEG and PLGA have become commercial available, what foster their use for nano- and microparticles.[15–17] But the use of these polymers for in situ forming depots based on phase inversion has not been investigated yet. Another drawback of PLGA based drug delivery systems is the typical irregular tri-phasic release. The initial drug release occurs simultaneously with the formation of the implant by non-solvent induced phase separation. In this connection, the phase inversion dynamics of the polymer solutions have a direct effect on the drug release behavior and the implant morphology.[5,18,19] Typical release profiles consist of distinct regions of initial drug burst, commonly 20–80% of the total drug load. The initial release of large amounts of particularly good solvent or water soluble drugs provoke increased local and systemic toxicity.[20] The high burst release rates are directly related to the lag between the injection of the polymeric solution and the formation of the solid implant, and the solubility of the drug in the organic solvent. In addition the phase inversion dynamics of PLGA solutions are complex processes, which are directly affected by the solvent properties,[21] solvent/non-solvent affinity[22] as well as the molecular weight,[23,24] end-group functionality[25] and concentration of the polymer.[26] Usually the initial burst is followed by a lag time of little or no

release. After this lag phase one observes an increase in diffusion and erosion controlled release and a final period of drug depletion.

Despite the intensive research in this field, the knowledge about the detailed mechanism of implant formation and disintegration is still very limited. In particular quantitative characterization of the in situ implant formation is quite challenging because of the complex nature of these systems. In previous studies on PLGA based ISFI, the non-invasive method Electron Spin Resonance (ESR) spectroscopy was applied to characterize for the first time the implant formation by direct and continuous quantification of both the polymer precipitation and the solvent exchange as well as in vitro and in vivo.[6,27] The current study describes the development and characterization of aldosterone loaded ISFI for preclinical animal models of vascular diseases. The influence of several formulation parameters, such as type of the solvent and the polymer has been investigated. In this context AB di- and triblock copolymers of PEG and PLGA were firstly explored as polymeric matrices for ISFI.

Experimental Part

The polymers PLGA (Resomer® RG503H, Mw ~ 34.000), PEG-PLGA (Resomer® RGP d 50155, Mw ~ 39.000) und PLGA-PEG-PLGA (Resomer® RGP t 50106, Mw ~ 73.000) were obtained from Boehringer Ingelheim, Germany. The solvents N-methyl-2-pyrrolidone (NMP) and Dimethyl sulfoxide (DMSO) were purchased form Sigma Aldrich, Germany. The preparation of the ISFI was as followed: briefly, either the spin probe tempolbenzoate (TB) or the drug aldosterone was dissolved together with the corresponding polymer in the solvent by stirring (Table 1). The polymeric solutions were prepared one day in advance for each injection.

ESR Studies Phase Separation and Solvent Exchange

The ESR studies on the phase separation and the solvent exchange followed the recently published protocols.[6,27] Briefly 200 μl of the TB-containing polymer solution were injected through a 21 gauge needle into 50 ml of 0.1 M phosphate buffer (pH 7.4; 37 °C) set in an incubation shaker

Table 1.
Composition of the investigated ISFI formulations.

Formulation	Polymer type	Polymer content (%)	Solvent	Drug content (μg/ml)	
				TB	Aldosterone
1	PLGA	30	NMP	415.5	
2		40	NMP	415.5	
3		50	NMP	415.5	
4		30	DMSO	415.5	
5		40	DMSO	415.5	
6		50	DMSO	415.5	
7	PEG-PLGA	30	NMP	415.5	
8		40	NMP	415.5	
9		50	NMP	415.5	
10		30	DMSO	415.5	
11		40	DMSO	415.5	
12		50	DMSO	415.5	
13	PLGA-PEG-PLGA	30	NMP	415.5	
14		40	NMP	415.5	
15		50	NMP	415.5	
16		50	NMP		400
17		30	DMSO	415.5	
18		40	DMSO	415.5	
19		50	DMSO	415.5	
20		50	DMSO		400

(30 rpm). The polymer solutions were injected into perforated plastic cylinders placed in the buffer solutions, where the implants were formed immediately. The incubation medium was exchanged periodically to prevent the accumulation of degradation products. At determined time points the implants were taken out of the buffer, the plastic cylinders were dried with wipes and covered with plastic foil to prevent drying and subsequently transferred to the EPR spectrometer. For the calibration measurement 0.5 mmol/l of TB was dissolved in different NMP or DMSO buffer mixtures. The values distance between the first and the third peak of the integrated ESR spectra ($2a_N$) were determined from the spectra and were plotted against the concentrations of the organic solvent in different organic solvent buffer mixtures (Figure 1). The EPR measurements were performed using an L-band spectrometer (MagnetTech, Germany) with a re-entrant resonator, operating at a microwave frequency of about 1.1–1.3 GHz. Measurement parameters were set to: modulation amplitude 0.14 mT, scan width 10 mT, scan time 100 s, centre field 49.0 mT, number of accumulations 3. All measurements were performed in triplicate; data are reported as mean ± standard deviation (SD). The "Cu3 v.6.1" program (MagnetTech, Germany) was used for data recording. The program "Analysis" (MagnetTech, Germany) served for calculation of polarity shifts inside the implants by determining $2a_N$ after integration of the recorded and simulated spectra. The proportion of immobilized TB inside the implants was calculated from EPR spectra fitted with "Nitroxide Spectra Simulation Freeware Version. 4.99-2005" (Jozef Stefan Institute, Department of solid state physics, Ljubljana, Slovenia). In case of baseline drifts or low signal to noise ratios the recorded EPR spectra were simulated and the simulated spectra were used for double integration, to improve the accuracy of the double integration and to eliminate noise and baseline distortion.

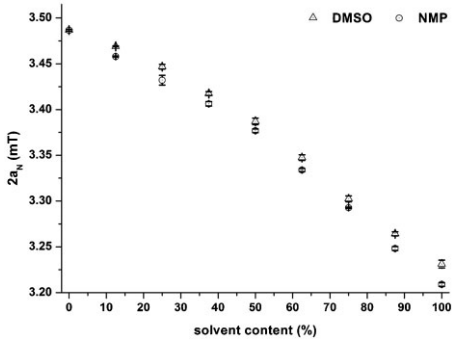

Figure 1.
Dependence of the isotropic hyperfine splitting $2a_N$ in different NMP-buffer and DMSO-buffer mixtures. The hyperfine splitting parameter correspond to the NMP and DMSO concentrations and can therefore be used to determine phase inversion induced changes in micropolarity inside the implants.

In Vitro Release Studies

200 μl polymeric solution, containing 800 μg aldosterone, were injected through a 21 gauge needle into 5 ml of 0.1 M phosphate buffer (pH 7.4; 37 °C) placed in an incubation shaker (30 rpm). After predetermined time points 200 μl of the release media were taken and replaced by fresh buffer solution. The samples were filtered through a 0.45 μm membrane filter (Millipore, Gemany) and the amount of released aldosterone was analysed by HPLC-UV using a Merck Hitachi HPLC system consisting of a model AS 4000A autosampler, L 6200A programmable pump and a L 4250 UV–Vis detector (Merck, Darmstadt, Germany), using a RP-8-LiChro®Cart column. The mobile phase, composed of methanol/water (50:50), was pumped at a flow rate of 1 ml/min. 20 μl was injected and the column effluent was monitored at a wavelength of 240 nm.

Results and Discussion

In the development of ISFI, the control the initial burst release is one of the greatest challenges, closely followed by the securing of the afterwards continuous and consistent release. Different parameters

were investigated to obtain an ISFI formulation that possesses a low initial burst and subsequent nearly zero order aldosterone release. Therefore the influence of polymer hydrophilicity and molecular weight, as well as the polymer concentration, on the phase inversion dynamics was examined by comparing PLGA with PEG-PLGA and PLGA-PEG-PLGA in two different solvents, namely DMSO and NMP. PLGA and PEG-PLGA possessed nearly equal molecular weight (Mw), but showed different hdyrophilic characteristics. In contrast, the triblock copolymer PLGA-PEG-PLGA had about twice of the molecular weight. As aldosterone is readily soluble in both solvents, and the solvent exchange rates directly correlate to the initial drug release,[5,18,19] the solvent content inside the implants during implant formation was investigated by ESR spectroscopy; as a marker for the burst release.[6]

As Figure 2 shows, the higher the PLGA loading in the solvent, the slower the solvent exchange within the first 24 hours. This effect, already reported in literature, can be explained by the increase of the viscosity of the system, slowing the solvent removal from the implants.[28,8] Differences in polymer concentration had a higher impact in systems with DMSO as a solvent, which can be explained by a higher water affinity resulting in a faster phase inversion.[20,29] Solutions of PLGA in NMP remain longer in the non-precipitated state after contact with water, as NMP is a better solvent for PLGA than DMSO.[29] Similar effects have been reported by Kranz and Bodmeier.[29] They observed that the slightly slower polymer precipitation resulted in a less porous implant surface, thus decreasing the initial drug and solvent release.

The same concentration dependent initial solvent exchange could be also detected for PLGA-PEG-PLGA (Figure 2, bottom). The higher Mw of the triblock copolymer additionally decreased the

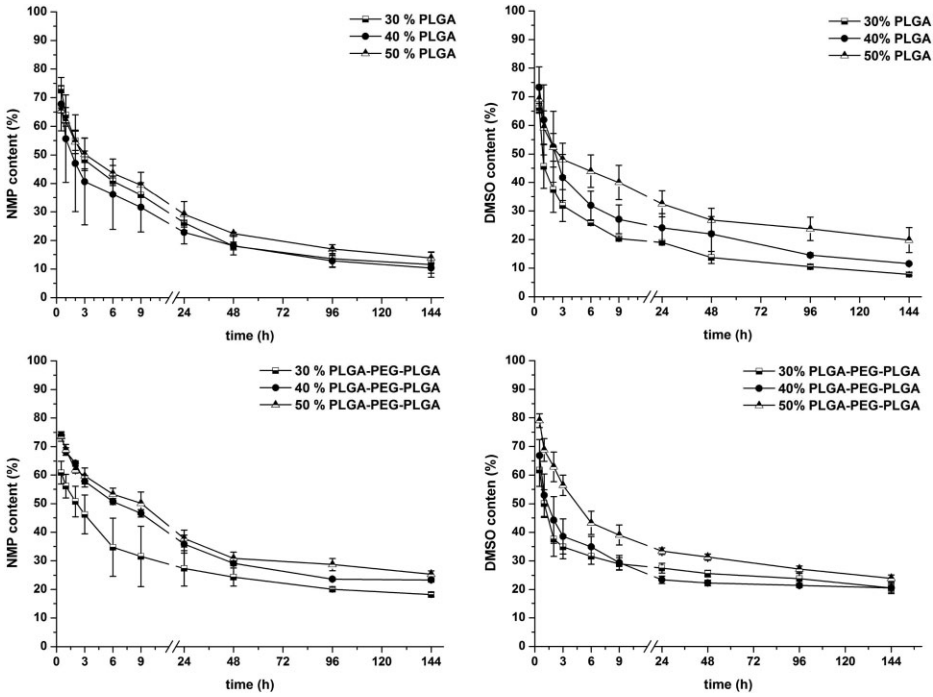

Figure 2.
Effect of polymer concentration (PLGA top, PLGA-PEG-PLGA bottom) on the solvent (NMP left, DMSO right) removal from in situ formed implants (determined by ESR spectroscopy).

solvent exchange rate. It is usually assumed that in solutions of higher molecular weight polymers, the longer polymer chain lengths get more entangled and therefore the system diffusivities decreased and therewith the solvent exchange. Eliaz et al. investigated the effect of PLGA molecular weight on the release of tumor necrosis factor receptor and found, that the higher molecular weight caused a faster solidification of the slow phase inversion systems and thereby decreased the diffusion and release rates of proteins.[26]

The introduction of PEG-end chains increased the hydrophilicity of the PLGA polymer and enforced both the polymer precipitation and the solvent exchange (Figure 3). PEG-PLGA possesses nearly the same molecular weight as PLGA. But due to its more hydrophilic character, a significant faster solvent exchange was observed after three hours ($p < 0.05$). As expected the incorporation of PEG blocks favored the water influx into the polymer and therefore accelerated both, the solvent exchange (Figure 3, top) and polymer precipitation rate (Figure 3, bottom). Similar effects have been observed by Witt et al..[14] They found that the water uptake and swelling degree of pre-shaped implants of PEG-PLGA-PEG triblock copolymers was directly related to the PEG content of the polymers.

Whereas distinct differences in the phase inversion kinetics of the different polymers could be detected in the NMP solute systems, this effect was less pronounced in the systems containing DMSO (Figure 3, left). Due to the higher water affinity of DMSO, the implant solidified faster within one hour, independent of the polymer type. In both solvents PLGA-PEG-PLGA showed the lowest rate of solvent exchange, which can be attributed to its higher Mw and therefore higher intrinsic viscosity of the polymeric solutions.

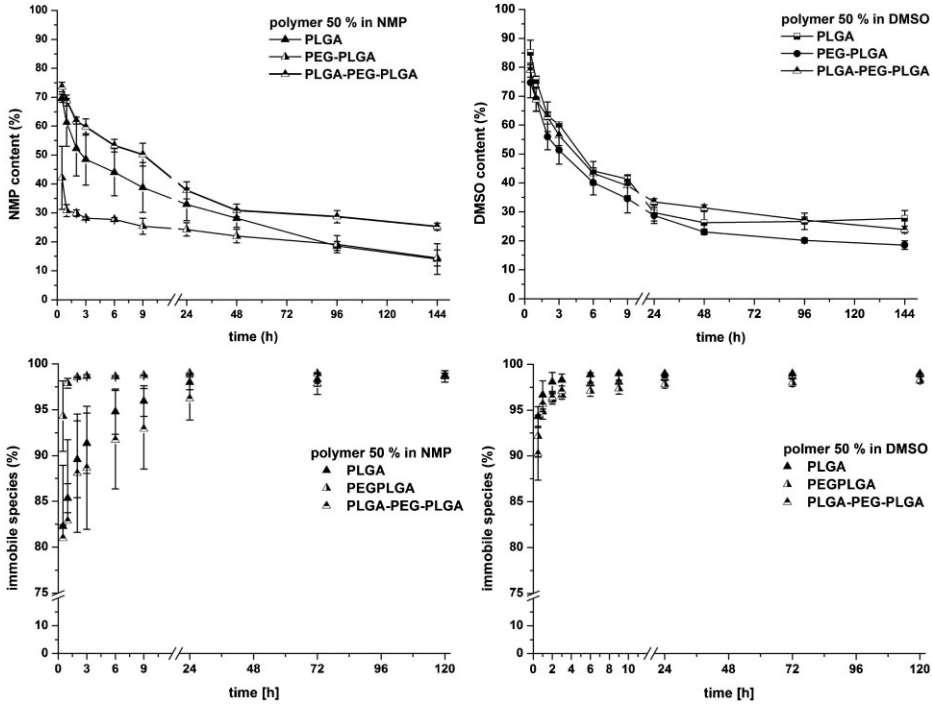

Figure 3.
Influence of the hydrophilic character of the polymer on solvent exhange rate (top, left: NMP as solvent, right: DMSO as solvent) and polymer precipitation rate (bottom, left: NMP as solvent, right: DMSO as solvent).

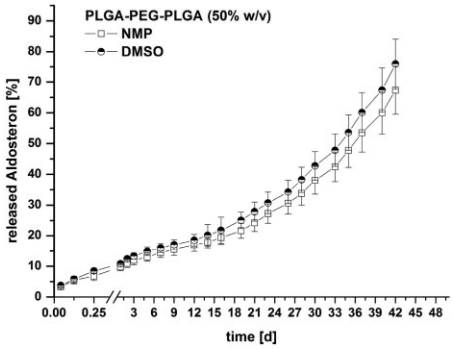

Figure 4.
Influence of the solvent on aldosterone release rate of PLGA-PEG-PLGA-based in situ forming implants.

As these systems, using PLGA-PEG-PLGA with a polymeric concentration of 50% (w/v), showed the slightest initial solvent release rates, they were further analyzed in their in vitro aldosterone release (Figure 4). A remarkable low initial drug burst below 10% could be realized for both solvents, NMP and DMSO. Nearly constant release rates followed the next two weeks. As degradation and erosion start immediately in PLGA-PEG-PLGA implants after incubation,[14] no phase of no drug release was observed. With progressing degradation of the polymer, the release rate increased after 21 days.

Similar release profiles have been reported for triblock copolymer microparticles,[17] where bovine serum albumin (BSA) was released in a slow and continuous way without initial burst and lag time. This release behavior was correlated to the rapid hydration of the copolymer and associated formation of a gel-like structure. The BSA was released by diffusion through the swollen matrix and polymer degradation.

Conclusion

For the first time di- and triblock copolymers of PEG and PLGA have been investigated as matrices for ISFI. This study demonstrated that the phase inversion kinetics were highly dependent on the hydrophilic character, the polymer concentration and the molecular weight of the used polymers. All of the investigated systems can be classified as fast inverting systems. A slightly faster phase inversion was observed for the PEG-PLGA based systems, and polymeric solutions with DMSO as the solvent. A higher molecular weight of the polymer, as well as a higher polymer concentration, delayed the solvent exchange. A 50% solution of PLGA-PEG-PLGA showed the minimal initial solvent exchanged, which is displayed in a very low initial aldosterone release (below 10%), followed by nearly constant release rates over 1.5 months. However further in vivo release studies are necessary to proof whether the systems show the same release profile than in vitro, and can be used as appropriate drug delivery systems to study the pathophysiological effects of aldosterone in a mouse model.

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