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Biocompatibility of poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) microspheres after subcutaneous and subcapsular renal injection

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ABSTRACT

Poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) is a biodegradable copolymer with potential as a novel carrier in polymeric drug delivery systems. In this study, the biocompatibility of PLHMGA microspheres (PLHMGA-ms) was investigated both *in vitro* in three different cell types (PK-84, HK-2 and PTECs) and *in vivo* at two implantation sites (by subcutaneous and subcapsular renal injection) in rats. Both monodisperse (narrow size distribution) and polydisperse PLHMGA-ms were prepared with volume weight mean diameter of 34 and 17 μm, respectively. Mono and polydisperse PLHMGA-ms showed good cytocompatibility properties upon 72 h incubation with the cells (100 μg microspheres/600 μL/cell line). A mild foreign body reaction was seen shortly after subcutaneous injection (20 mg per pocket) of both mono and polydisperse PLHMGA-ms with the presence of mainly macrophages, few foreign body giant cells and myofibroblasts. This transient inflammatory reaction diminished within 28 days after injection, the time-point at which the microspheres were degraded. The degradation profile is comparable to the *in vitro* degradation time of the microspheres (*i.e.*, within 35 days) when incubated at 37 °C in phosphate buffered saline. Subcapsular renal injection of monodisperse PLHMGA-ms (10 mg) in rats was characterized with similar inflammatory patterns compared to the subcutaneous injection. No cortical damage was observed in the injected kidneys. In conclusion, this study demonstrates that PLHMGA-ms are well tolerated after *in vivo* injection in rats. This makes them a good candidate for controlled delivery systems of low-molecular weight drugs as well as protein biopharmaceuticals.

1. Introduction

Poly(D,L-lactic-*co*-glycolic acid) (PLGA) is a biodegradable aliphatic polyester that has been investigated for controlled delivery of low molecular weight drugs (Kim et al., 2011), peptides (Shmueli et al., 2013; Xuan et al., 2013), proteins (Menon et al., 2014; Reguera-Nuñez et al., 2014; Wink et al., 2014) and vaccine antigens (Huang et al., 2014; Joshi et al., 2013). PLGA is degraded by hydrolytic cleavage of ester bonds that connect the monomer units, and the final degradation products are lactic and glycolic acid, both endogenous compounds (Spenlehauer et al., 1989; Vert et al., 1994). An important drawback of PLGA matrices, however, is the formation of acidic degradation products which are detrimental for the stability and integrity of entrapped (therapeutic) proteins (Estey et al., 2006; Park et al., 1995). Denaturation of the formulated protein or structural modifications due to acid-catalyzed reactions will affect both therapeutic efficacy and can cause potential immunological responses to the formulated protein (Hermeling et al., 2004; Patten and Schellekens, 2003).

A novel copolymer, poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) (Leemhuis et al., 2006) has a similar molecular

Abbreviations: α-SMA, alfa-smooth muscle actin; BMMG, 3*S*-(benzyloxymethyl)-6*S*-methyl-1,4-dioxane-2,5-dione; DCM, dichloromethane; ED-1, mouse anti rat CD68 monoclonal antibody; FBGCs, foreign body giant cells; HK-2, human proximal tubular cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffered saline; PK-84, human skin fibroblasts; PLGA, poly(D,L-lactic-*co*-glycolic acid); PLBMGA, poly(D,L-lactic-*ran*-benzyloxymethyl glycolic acid); PLHMGA, poly(D,L-lactic-*co*-hydroxymethyl glycolic acid); PLHMGA-ms, poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) microspheres; PTECs, human primary tubular epithelial cells; PVA, polyvinyl alcohol; SEM, scanning electron microscope; T_g, glass transition temperature.

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structure as PLGA with additional pendant hydroxyl groups on the polymer backbone (Fig. 1). The degradation of this co-polymer and the release of entrapped proteins can be tailored by its copolymer composition (Ghassemi et al., 2010; Leemhuis et al., 2007; Samadi et al., 2013b). Furthermore, PLHMGA based microspheres are peptide and protein friendly (Ghassemi et al., 2010, 2012; Samadi et al., 2013b). Owing to the more hydrophilic nature of PLHMGA compared to PLGA, it has been demonstrated that the water-soluble acidic degradation products of PLHMGA are rapidly released from degrading microspheres into the external medium (Liu et al., 2012). As PLHMGA is intended for use of delivering drugs *in vivo*, characterization of the *in vivo* biodegradation as well as biocompatibility properties of these copolymeric microspheres is required.

The aim of this study is to evaluate the *in vitro* cytotoxicity and *in vivo* biocompatibility of PLHMGA microspheres (PLHMGA-ms). These tests are mandatory according to the International Organization for Standardization (ISO) guidelines for biological evaluation of implantable medical devices (ISO Guidelines April 23, 2013). PLHMGA-ms were prepared with two different methods, a conventional single emulsion solvent evaporation method for preparation of polydisperse microspheres and by membrane emulsification method for generating uniform size microspheres. Previously, we have shown that microspheres prepared by this method of emulsification have high batch-to-batch reproducibility in terms of particle characteristics and release kinetics (Kazazi-Hyseni et al., 2014). Moreover, due to the uniform size, monodisperse microspheres also have better injectability and hence allows the use of smaller needles for the administration of microsphere suspensions. This is of special attention in the present study, in which we investigated the feasibility of injecting PLHMGA microspheres under the renal capsule. Subcapsular renal injection is a relatively new method for local delivery of therapeutics to the kidneys which was earlier tested for the injection of hydrogels (Dankers et al., 2012). We created a small pocket between the capsule and the soft cortex tissue with a small blunt needle and used the same needle to inject a concentrated dispersion of the microspheres, to study their biocompatibility at this injection site. In addition, we studied the biocompatibility of PLHMGA microspheres after subcutaneous injection.

The *in vitro* cytocompatibility was assessed in three different cell types, namely dermal fibroblasts (PK-84), proximal tubular epithelial cells (HK-2) and primary tubular epithelial cells (PTECs). For the *in vivo* biocompatibility assessment, both monodisperse and polydisperse PLHMGA-ms were injected subcutaneously in rats. The inflammatory response was studied along with the influence of particle size and polydispersity on the foreign body reaction. Furthermore, the degradation profile of PLHMGA-ms was studied *in vitro* and correlated to the *in vivo* degradation as observed in histopathology tissue samples.

2. Materials and methods

2.1. Materials

O-benzyl-L-serine was purchased from Senn Chemicals AG (Dielsdorf, Switzerland). Tin(II) 2-ethylhexanoate ($SnOct_2$), poly (vinyl alcohol) (PVA; M_w = 13,000–23,000 g/mol), palladium 10 wt % (dry basis) on activated carbon, hematoxylin solution and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (Germany). 1,4-Butanediol, 99+% was obtained from Acros Organics (Belgium). Carboxymethylcellulose (CMC, with viscosity of 2000 mPa s of a 1% solution in water) was obtained from Bufa B. V. (255611, The Netherlands). Sodium phosphate dibasic (Na_2HPO_4) and sodium azide (NaN_3) were purchased from Fluka (The Netherlands). Dichloromethane (DCM) and tetrahydrofurane were purchased from Biosolve BV (The Netherlands). Sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH) and sodium chloride (NaCl) were supplied from Merck (Germany). Mouse anti rat CD68 monoclonal antibody (clone ED-1) was obtained from AbD Serotec (MCA341R, Germany). Monoclonal mouse anti-human Actin (α-SMA) was obtained from Dako (Clone 1A4, Denmark).

2.2. Polymer synthesis and characterization

Poly(D,L-lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) was synthesized as previously described (Leemhuis et al., 2006), using butanediol as an initiator, to obtain a hydroxyl terminated co-polymer. In brief, BMMG (3*S*-(benzyloxymethyl)-6*S*-methyl-

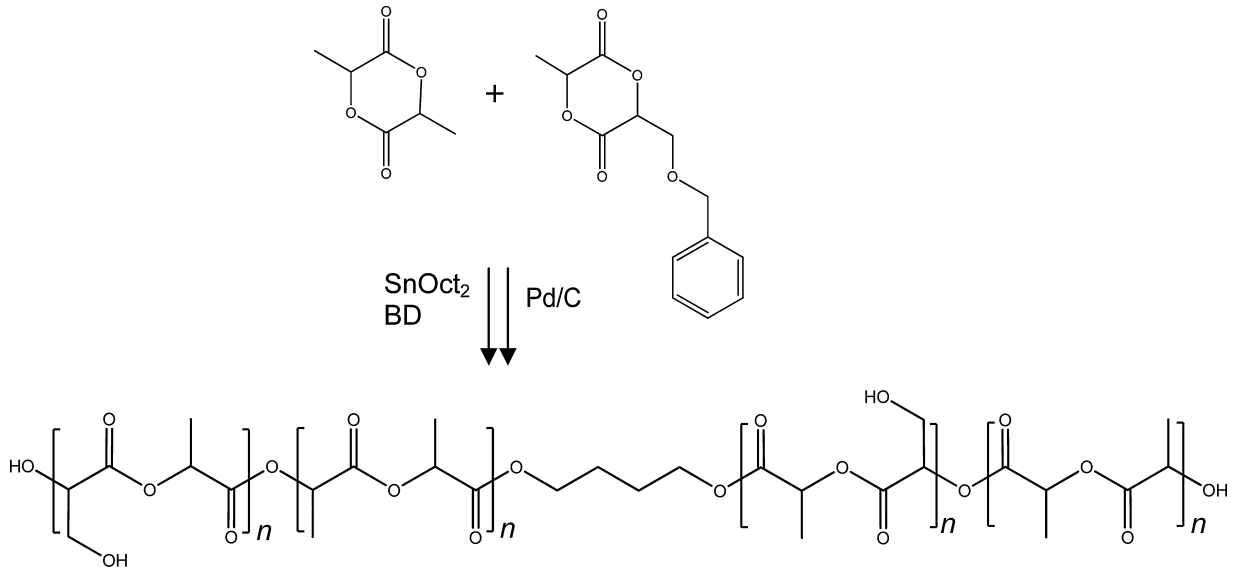

Fig. 1. Synthesis of poly(lactic-*co*-hydroxymethyl glycolic acid) (PLHMGA) from D,L-lactide and 3*S*-(benzyloxymethyl)-6*S*-methyl-1,4-dioxane-2,5-dione (BMMG) by melt copolymerization with $SnOct_2$ as catalyst and 1,4-butanediol (BD) as initiator. The protective benzyl groups were removed by hydrogenation using palladium on activated carbon (Pd/C) as a catalyst.

1,4-dioxane-2,5-dione) was synthesized from *O*-benzyl-L-serine (Leemhuis et al., 2006). In the second step BMMG (35 mol%) and D, L-lactide (65 mol%) were copolymerized in the melt at 130 °C using butanediol and tin(II) 2-ethylhexanoate as initiator and catalyst, respectively, to yield poly(D,L-lactic-*ran*-benzyloxymethyl glycolic acid) (PLBMGA). Next, the resulting PLBMGA was dissolved in chloroform and subsequently precipitated in cold methanol, and dried *in vacuo*. In the third step, the protective benzyl groups of PLBMGA were removed by hydrogenation of the polymer dissolved in tetrahydrofuran, using 10% w/w palladium on activated carbon (Pd/C) as a catalyst, for 16 h at room temperature. The catalyst was removed by filtration through 0.2 μm nylon filters (Alltech Associates) and the formed copolymer, PLHMGA, was dried *in vacuo* (Fig. 1).

The molecular weight of the polymer was determined by GPC (Waters Alliance System) with a Waters 2695 separating module and a Waters 2414 refractive index detector, using tetrahydrofuran as solvent at a flow rate of 1 mL/min; polystyrene standards (PS-2, M_w = 580–377,400 Da, EasiCal Varian) were used for calibration. Two PL-gel 5 μm Mixed-D columns fitted with a guard column (Polymer Labs, M_w range 0.2–400 kDa) were used. The composition of the copolymer was determined by NMR (Gemini-300 MHz) in chloroform-*d*, 99.8 atom% (Sigma–Aldrich) as a solvent (Leemhuis et al., 2006).

PLHMGA- ^{1}H NMR ($CDCl_3$): δ = 1.4–1.6 (m, 3H, $-CH_3$), 3.8–4.1 (m, 2H, $-CH_2-OH$), 5.0–5.3 (m, 2H, $-CH-CH_2-OH$ and $-CH-CH_3$)

The thermal properties of the copolymer were measured with differential scanning calorimetry (DSC – Q2000, TA Instruments). Approximately 5 mg of copolymer was transferred into an aluminum pan (T zero pan/lid set, TA Instruments) and the sample was scanned with a modulated heating method in three cycles (Ghassemi et al., 2010). The sample was heated until 120 °C (5 °C/min) and then cooled down to −50 °C, followed by a heating until 120 °C (5 °C/min). The temperature modulation was ±1 °C/min. The glass transition temperature (T_g) was determined from the second heating scan. Residual palladium in PLHMGA, used as a catalyst during the de-protection step, was measured with instrumental neutron activation analysis (Technical University of Delft). Around 100 mg of PLHMGA was packed in high purity polyethylene capsules and was irradiated at a neutron flux of $4.5 \times 10^{16}\ m^{-2}\ s^{-1}$. The γ ray spectra were acquired using various independently calibrated detectors. The spectra obtained were interpreted using the nuclear data set (Blaauw, 1995). The detection limit of palladium with this method is 2.4 ppm.

2.3. Preparation of polydisperse and monodisperse PLHMGA-ms

Monodisperse PLHMGA-ms were prepared using a membrane emulsification method with a single emulsion (O/W) as described in detail elsewhere (Kazazi-Hyseni et al., 2014; Nakashima et al., 2000). The particles were prepared aseptically in a flow cabinet using autoclaved equipment and sterile water. The oil phase (O) contained 3 g of polymer dissolved in 20.3 mL DCM (10%; w/w). This solution was then pushed through the microsieve™ membrane (Iris-20, Nanomi B.V., The Netherlands) at a rate of 12 mL/h by using a syringe pump (Nexus 6000, Chemyx, USA) into the continuous phase (W) containing 400 mL of 4% PVA (the ratio of the oil phase and the continuous phase was 1:20). Polydisperse PLHMGA-ms were prepared with conventional single emulsion (O/W) method. Two grams of polymer were dissolved in 13.5 mL DCM (10%; w/w) and 67.5 mL of 0.5% PVA solution was added. The mixture was homogenized with Ultra-Turrax T8 (Ika Works, USA) with dispersing element S10N-10G, at a speed of 20,000 rpm for 30 s, and then added dropwise to 270 mL of 4% PVA solution. For both methods, the collected droplets were stirred for three hours at room temperature to evaporate DCM. The hardened microspheres were washed three times with water by centrifuging at 3,000 rpm for 2 min (Rotina 380, Hettich, Germany) and subsequently collected after freeze-drying (Alpha 1–2, Martin Christ, Germany). Single batches were used for *in vitro* cytocompatibility and *in vivo* biocompatibility testing.

2.4. Characterization of PLHMGA-ms

The size of the particles was measured with an optical particle sizer (Accusizer 780, California, USA). At least 5000 microspheres were analyzed and the volume–weight mean particle diameter is reported as the mean particle size. The morphology of the microspheres was analyzed with scanning electron microscope (SEM, Phenom, FEI Company, The Netherlands). Lyophilized microspheres were transferred onto 12-mm diameter aluminum specimen stubs (Agar Scientific Ltd., England) using double-sided adhesive tape. Prior to analysis, the microspheres were coated with platinum using an ion coater under vacuum. The residual amount of DCM in the microspheres was measured with NMR (Varian Gemini-300) with DMSO-d_6 as a solvent (Avdovich et al., 1991; Jones et al., 2005). Samples of 50 mg were dissolved in 1 mL of DMSO for one hour and spiked with 5 mg of 1,4-dinitrobenzene (Oekanar®, Fluka) as the internal standard. The amount of the DCM was calculated from the NMR spectra according to the following equation, as adapted from Jones et al. (2005):

$$DCM_{ppm} = \frac{integral_{(DCM)} \times No.H_{(standard)} \times weight_{standard} \times M_{w(DCM)}}{integral_{(standard)} \times No.H_{(DCM)} \times M_{w(standard)}} \times 1.10^6$$

where M_w is the molecular weight and No.H is the number of protons of the peak (4H for 1,4-dinitrobenzene and 2H for DCM). The residual amount of DCM in the microspheres should be below the maximum concentration allowed by FDA, *i.e.*, concentration limit of 600 ppm or the permitted daily exposure of 6 mg/day) (FDA Guidance Documents December, 1997; Grodowska and Parczewski 2010).

Potential bacterial contamination of the microspheres was determined by inoculation of 5 mg of dry PLHMGA-ms (dispersed in sterile water) on blood agar plates. The plates were incubated at 37 °C for 4 days and were checked daily for the presence of bacterial colonies. The endotoxin levels were determined using the Limulus assay (Toxicon Europe, Leuven, Belgium).

2.5. In vitro *degradation studies*

PLHMGA-ms (10 mg) were suspended in 1.5 mL PBS buffer, pH 7.4 (0.056 M NaCl, 0.033 M Na_2HPO_4, 0.066 M NaH_2PO_4 and 0.05% (w/w) NaN_3) and incubated at 37 °C while mildly shaking. A total of six vials was used. At predetermined time-points one vial was removed, centrifuged (4000 rpm, 5 min) and the pellet was washed three times with water and freeze-dried overnight. The microspheres were measured for dry weight and the molecular weight of the polymers was analyzed using GPC as described in Section 2.2.

2.6. In vitro *cytotoxicity study*

2.6.1. Cell culture

Monodisperse and polydisperse PLHMGA-ms were incubated with three different cell types (human skin fibroblasts (PK-84), human proximal tubular cells (HK-2) and human primary tubular epithelial cells (PTECs)). The PK-84 were cultured in RPMI

1640 medium (Lonza, Breda, The Netherlands), supplemented with 10% v/v fetal calf serum (Perbio Science, Etten-Leur, The Netherlands) and with standard additives. The HK-2 and PTECs were cultured in 1:1 v/v Ham's F12 (L-glutamine) and in Dulbecco's modified Eagle's medium supplemented with 1% v/v glutamine, 1% v/v penicillin, 0.01 mg/L epidermal growth factor, 10 mg/L insulin, 5.5 mg/L transferrin, 6.7 μg/L sodium selenite, 36 μg/L hydrocortisone and 2 mM glutamax. The medium of HK-2 was supplemented with 10% v/v fetal calf serum, whereas the medium of PTECs was supplemented with 1% v/v human pooled serum. All cell cultures were incubated at 37 °C with 5% CO_2.

2.6.2. Extraction test

For the preparation of the extracts of microspheres, 5 mg PLHMGA-ms was incubated for 24 h at 37 °C in 25 mL of complete culture medium. This method allows the extraction of both polar and nonpolar leachables from the microspheres (ISO Guidelines April 23, 2013). After 24h-incubation, the samples were centrifuged at 300 g. In a similar way we prepared extracts of latex rubber (thickness 3–4 mm; Hilversum Rubber Factory, Hilversum, The Netherlands) and of polyurethane film (thickness about 1 mm; made from 2363–55D-pellethane® resin; Dow Chemical, Midland, MI, USA) that were used as a positive cytotoxic control (Latex) and as a negative non-cytotoxic control (polyurethane), respectively. PK-84, HK-2 and PTECs were seeded in 24-well plates (cell density of 15,000 cells/cm^2) and after 24 h the medium of the cells was replaced with 500 μL of the extracts of PLHMGA-ms (corresponding to 100 μg microspheres), latex and polyurethane. Cells were incubated for 48 h followed by measurements with CyQuant cell proliferation assay (for quantification of nucleic acid content) and MTS assay (for mitochondrial activity measurements) as described in Sections 2.6.4 and 2.6.5.

2.6.3. Direct contact assay

For direct contact assay, the microspheres were dispersed in complete medium (100 μg in 600 μL) and added to the cell cultures (cell density of 15,000 cells/cm^2). Small pieces of polyurethane film and latex rubber were used as a negative and positive control to show the behavior of the cells in the presence of a biocompatible and cytotoxic material, respectively (De Groot et al., 2001). Cells were cultured for 72 h and the cell morphology was examined every day. The cell viability was analyzed with CyQuant cell proliferation assay and MTS assay as described in Sections 2.6.4 and 2.6.5.

2.6.4. Cell proliferation assay

The CyQuant® cell proliferation assay (Invitrogen, The Netherlands) was performed according to the manufacturer's instructions. In brief, after removing the culture medium (including floating and dead cells) the cells were stored at −80 °C for 48 h. Subsequently, culture plates were defrosted at room temperature and the CyQuant® green dye/cell-lysis buffer was added to each

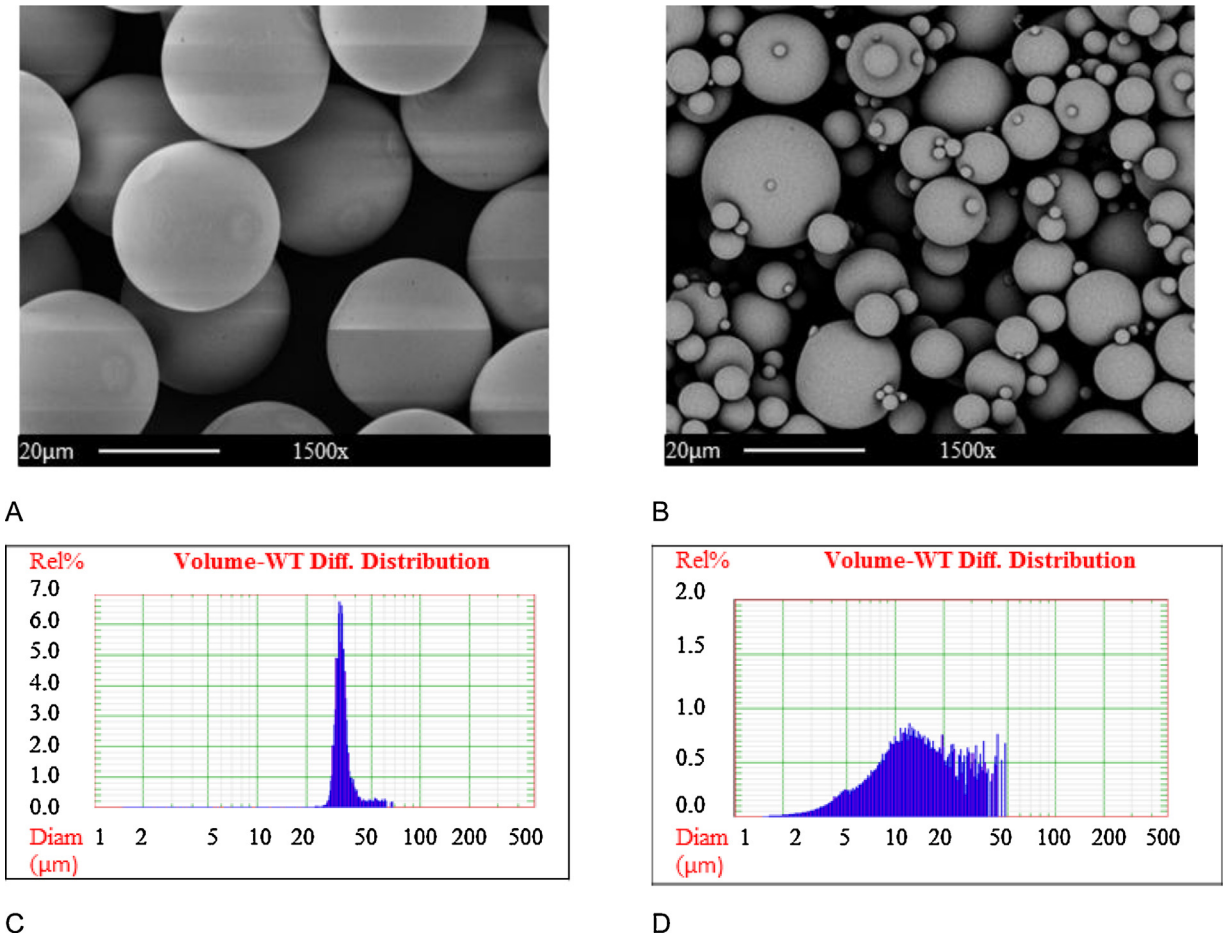

Fig. 2. Representative SEM photographs of PLHMGA-ms (A and B; magnification 1500×) and the results of the volume weight particle diameter as measured with AccuSizer (C and D). A and C: monodisperse PLHMGA-ms prepared with membrane emulsification method. B and D: polydisperse PLHMGA-ms prepared with a conventional solvent evaporation method.

well. The green dye exhibits strong fluorescence enhancement when bound to cellular nucleic acids. After 5 min incubation at room temperature, the fluorescence was quantified using a fluorescence microplate reader (Varioscan, Thermo Fisher Scientific Inc.) with a 480/520-nm filter set.

2.6.5. Mitochondrial activity assay

The mitochondrial activity – MTS assay (CellTiter 96® AQueous One Solution, Promega Benelux Bv, The Netherlands) was performed according to manufacturer's instructions. Briefly, 100 μL of the culture medium containing the samples was mixed with 20 μL of the MTS reagent. The MTS reagent is reduced by metabolically active cells into a colored product. After 2 h incubation at 37 °C and 5% CO_2 atmosphere the absorbance was recorded at 490 nm with a fluorescence microplate reader (Varioscan, Thermo Fisher Scientific Inc.).

2.7. In vivo *experiments*

2.7.1. Animals

Animal experiments were carried out in 10–12 week old male Fischer 344/NCrHsd rats (Harlan Nederland, The Netherlands; $n = 3$/time-point). Animals were fed laboratory chow and acidified water *ad libitum*, and were housed according to institutional rules with 12:12 h dark/light cycles. The protocol was approved by the Animal Ethical Committee of the University of Groningen. During the injections, rats were anesthetized under general isoflurane/O_2 inhalation and palliative treatment was used consisting of buprenorphine. At specific time-points rats were sacrificed by cervical neck dislocation.

2.7.2. Subcutaneous injection

Mono and polydisperse PLHMGA-ms suspensions were prepared by mixing 20 mg of the microspheres with 150 μL of an autoclaved viscous carrier (0.4% carboxymethylcellulose-CMC, 0.02% Tween-20 and 5% mannitol in water). Microparticle suspensions were injected subcutaneously on the back of the rats. Injection sites were explanted at day 7, 14 and 28. Implants were fixed in zinc fixative solution (0.1 M Tris-buffer, 3.2 mM calcium acetate, 23 mM zinc acetate, 37 mM zinc chloride, pH 6.5–7; Merck, Darmstadt, Germany) overnight, prior to paraffin embedding. Implants were cut into 4 μm thick sections.

2.7.3. Subcapsular renal injection

For injection under the renal capsule, monodisperse PLHMGA-ms were used. A midline incision was made under the left kidney capsule of a rat and 50 μL of microsphere suspension (10 mg of microspheres in 50 μL of 0.4% CMC, 0.02% Tween-20 and 5% mannitol in water) was injected with a 26G blunt Hamilton needle (Chrom8 International, The Netherlands). The kidneys were explanted at day 3, 7 and 14. Kidneys were flushed *in vivo* with saline solution, excised and paraffin-embedded. Implants were cut into 4 μm thick sections.

2.8. (Immuno)histochemistry

Tissue sections were stained for infiltration of macrophages (ED-1 macrophage marker) and for myofibroblasts (α-SMA staining). Four μm thick sections were deparaffinized and antigen retrieval was performed overnight in a 0.1 M Tris–HCl buffer, pH 9.0, at 80 °C (Koopal et al., 1998). The non-specific binding was blocked with 2% bovine serum albumin for 30 min, while the endogenous peroxidase activity was suppressed by incubating the samples in 0.1% H_2O_2 for 10 min. In ED-1 staining, sections were then incubated with mouse-anti-rat ED-1 monoclonal antibody (10 μg/mL) for 1 h followed by horseradish peroxidase-conjugated rabbit-anti-mouse polyclonal antibody (13 μg/mL; DAKO, Denmark) for 30 min. For α-SMA staining, after antigen retrieval and blocking of the non-specific binding, tissue sections were incubated in mouse α-SMA monoclonal antibody (0.44 μg/mL) for 1 h, followed by incubation in horseradish-conjugated rabbit-anti-mouse polyclonal antibody (13 μg/mL; DAKO, Denmark) for 30 min. After the incubation with the secondary antibody all sections were washed three times with PBS and the enzyme activity was developed with 3-amino-9-ethylcarbazole (AEC; Sigma–Aldrich, The Netherlands). All tissue sections were counter-stained with hematoxylin for 5 min at 37 °C.

3. Results and discussion

3.1. Characteristics of the PLHMGA copolymer

The synthesized PLHMGA (Fig. 1) had an average molecular weight of 22 kDa (relative to the polystyrene standards) with a PDI of 1.7 as measured by GPC. The copolymer composition was 34/66 mol/mol (BMMG/D,L-lactide before hydrogenation) as measured with NMR (feed ratio 35/65). The glass transition temperature of PLHMGA (T_g) was 35.6 °C. Due to the use of palladium-based catalyst during the de-protection step, the obtained copolymer might contain residual amounts of this metal. Instrumental neutron activation analysis showed that the palladium content in PLHMGA was 174 ppm, which corresponds to 1.74 μg of palladium in 10 mg of PLHMGA-ms. According to European Medicines Agency, the parenteral permitted daily exposure to palladium is 10 μg/day (for a 50 kg person) while LD50 values for palladium salts range from 3 to 4,900 mg/kg depending on the type of palladium salt and route of administration (EMEA Guideline: Doc. Ref. EMEA/CHMP/SWP/4446/2000). Based on these criteria,

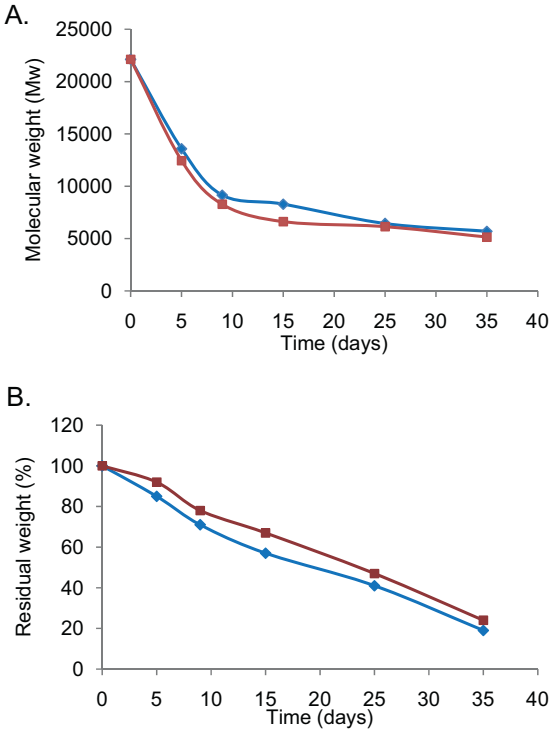

Fig. 3. *In vitro* degradation of monodisperse (in blue diamonds) and polydisperse (in red squares) PLHMGA-ms. (A) Weight average molecular weight (M_w) and (B) residual weight (%) over time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

we do not expect adverse events in the animal studies due to the residual amounts of palladium catalyst.

3.2. Characteristics of the PLHMGA-ms

Monodisperse PLHMGA-ms were prepared with a membrane emulsification method. The obtained microspheres had a volume weight mean diameter of 34 μm and were quite monodisperse (distribution: 30–38 μm) (Fig. 2A and C). Polydisperse PLHMGA-ms were prepared with a conventional single emulsion method and had a mean particle size of 17 μm (distribution: 5–46 μm) (Fig. 2B and D). Scanning electron microscopy (SEM) showed that the microspheres had smooth surface and no visible pores (Fig. 2A and B). The residual DCM content measured with NMR was <400 ppm for both microsphere batches which is below the maximum recommended amount by Food and Drug Administration (600 ppm or 6 mg/day) (FDA Guidance Documents, December, 1997; Grodowska and Parczewski, 2010). No bacterial contamination was detected in the prepared microsphere batches. The endotoxin level of the microsphere dispersions was within the approved FDA norm (0.5 EU/mL).

When incubated in PBS buffer at 37 °C, both mono and polydisperse PLHMGA-ms showed 80% weight loss within 35 days, with gradual decrease in the molecular weight (Fig. 3). This is in agreement with previously published data of PLHMGA with similar copolymer composition and molecular weight (Ghassemi et al., 2009). No apparent differences were seen in the degradation profile between mono and polydisperse microspheres, most probably due to the small differences of the average size of the microspheres (34 and 17 μm). According to another study, PLGA microspheres with an average diameter of 3 and 20 μm had similar degradation patterns, whereas nanoparticles of 300 nm in size degraded slower (Samadi et al., 2013a).

PLHMGA-ms are known to degrade by hydrolysis into lactic acid and hydroxymethyl glycolic acid (Leemhuis et al., 2007; Samadi et al., 2013b), both endogenous small molecular weight acidic compounds. The latter compound is a derivative of serine, which is converted into glyceric acid and further metabolized *via* the glycolytic pathway (Rabson et al., 1962).

3.3. In vitro *cytocompatibility of PLHMGA-ms: extraction test and direct contact assay*

The *in vitro* cytocompatibility of PLHMGA-ms was tested using three different cultured cell types, *i.e.*, PK-84 (human skin fibroblasts), HK-2 (human proximal tubular cells) and PTECs (primary human proximal tubular epithelial cells). These cell types also reflect the tissues in which the microspheres were evaluated for *in vivo* biocompatibility (PK-84 for the subcutaneous injection and HK-2 and PTECs for the subcapsular renal injection). Fig. 4 shows the results from the cytocompatibility study of PLHMGA-ms incubated with PK-84 cells. PLHMGA-ms did not influence the confluency of the cultured cell layer in both direct contact assay and upon incubation with the 24 h-extracts of the microspheres.

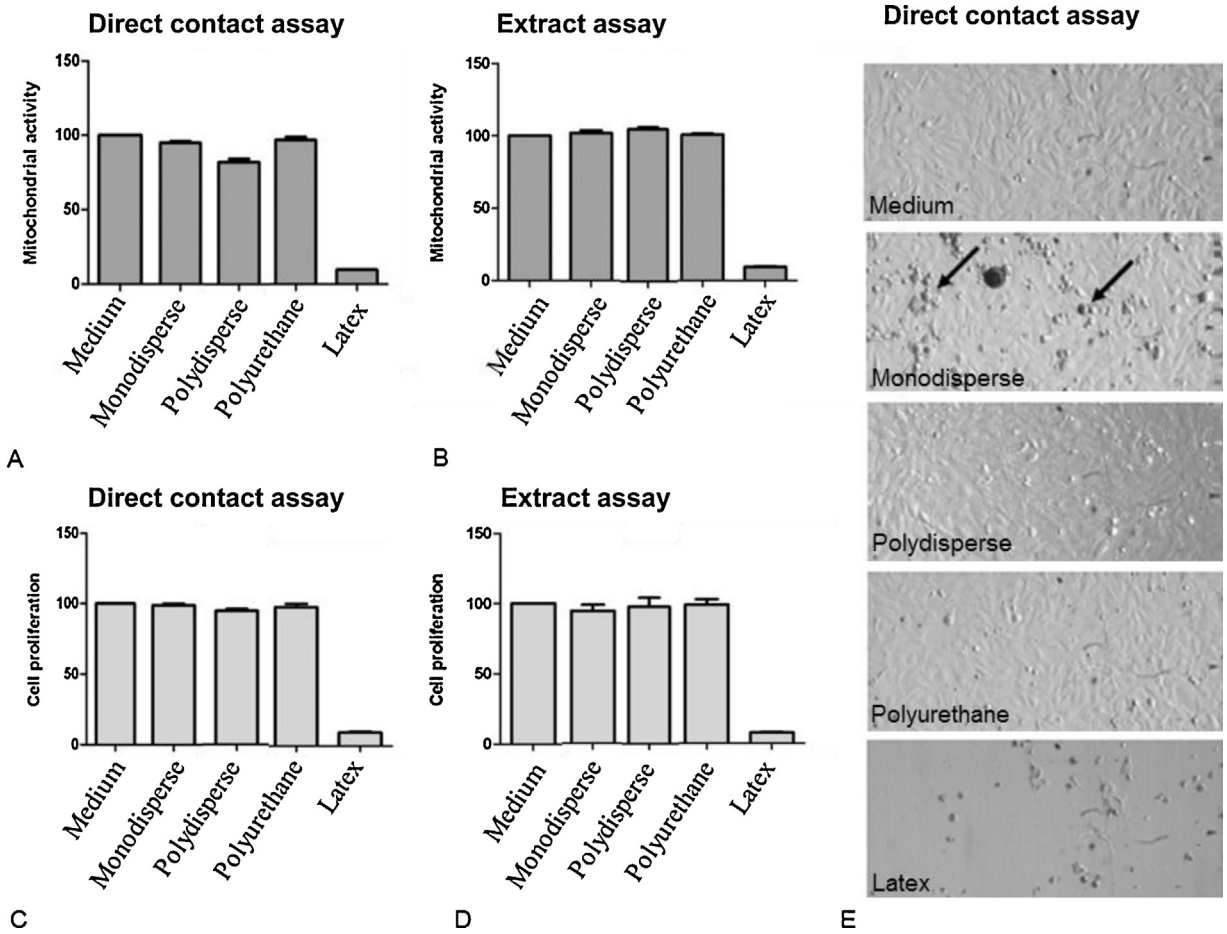

Fig. 4. *In vitro* cytocompatibility of PLHMGA-ms (5 mg/600 μL) upon incubation with human skin fibroblasts (PK-84). PK-84 were exposed to monodisperse and polydisperse PLHMGA-ms in the direct contact assay for 72 h (A and C) and to their 24-h extracts for 48 h (B and D). E: cell morphology in direct contact assay (magnifications 100×); arrows indicate the monodisperse PLHMGA-ms. Cell viability was assessed with MTS and cell proliferation assay. Polyurethane and latex were used as a negative and a positive control, respectively.

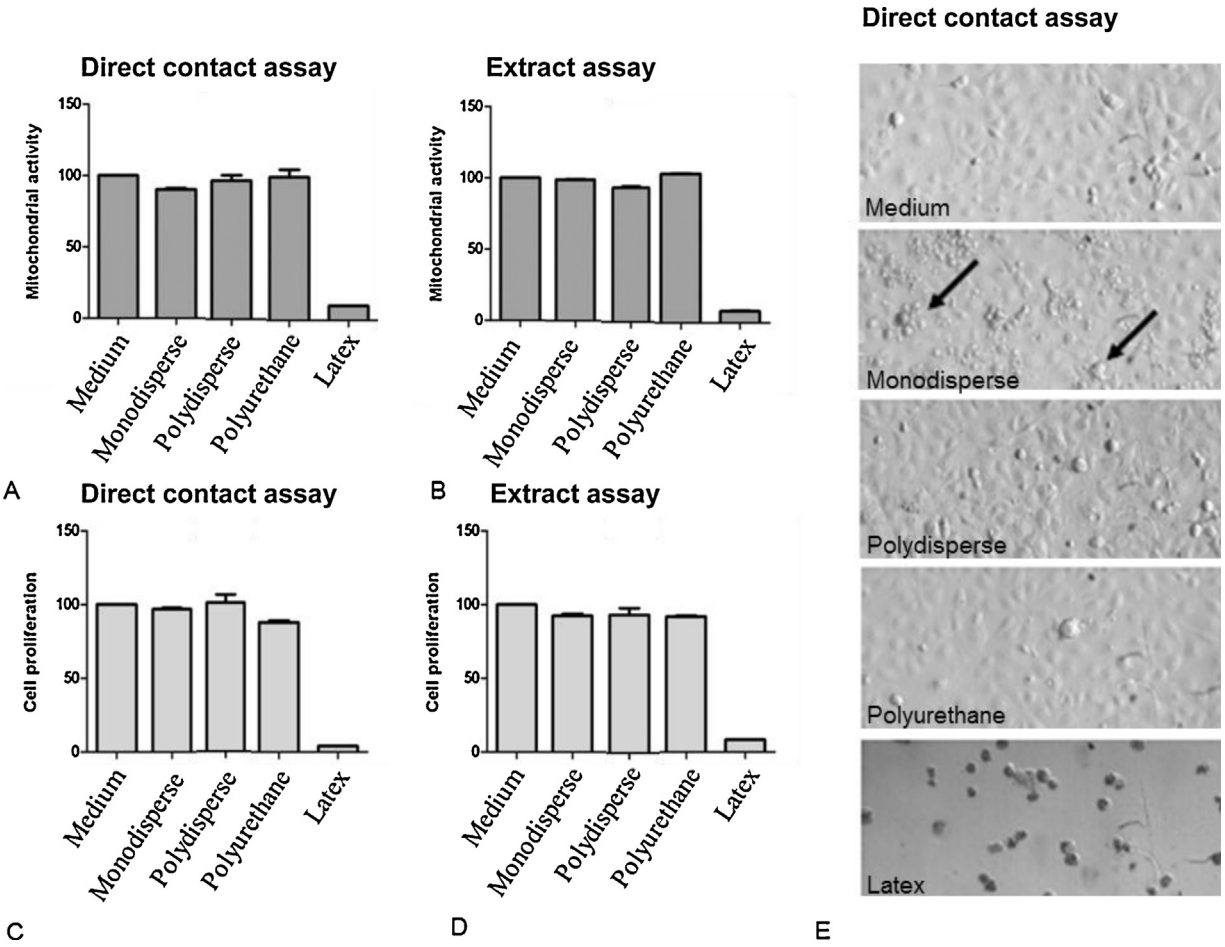

Fig. 5. *In vitro* cytocompatibility of PLHMGA-ms (5 mg/600 μL) upon incubation with human proximal tubular cells (HK-2). HK-2 cells were exposed to monodisperse and polydisperse PLHMGA-ms in the direct contact assay for 72 h (A and C) and to their 24-h extracts for 48 h (B and D). E: cell morphology in direct contact assay (magnifications 100×); arrows indicate the monodisperse PLHMGA-ms. Cell viability was assessed with MTS and cell proliferation assay. Polyurethane and latex were used as a negative and a positive control, respectively.

No significant differences were seen between polydisperse and monodisperse PLHMGA-ms in the cell viability assays. Proliferation of the cells was comparable to the control cultures and to polyurethane exposed cells, which served as a control material with good biocompatibility. As a positive (*i.e.*, cytotoxic) control in our assays, we exposed the cells to latex rubber and latex rubber extracts. Extracts of small pieces of latex or direct contact with this material resulted in detachment of exposed cells from the culture plate and extensive cellular lysis was observed within the first 24 h (Fig. 4E; last panel). Similar cytocompatibility data were also obtained using HK-2 (Fig. 5) and PTECs (Fig. 6). Thus, PLHMGA-ms showed excellent cytocompatibility with the studied cells. These data encouraged further *in vivo* biocompatibility studies with this copolymer (described in the next sections).

3.4. In vivo *biocompatibility after subcutaneous injection of PLHMGA-ms*

The *in vivo* biocompatibility of mono and polydisperse PLHMGA-ms was assessed after subcutaneous injection of 20 mg microspheres in rats. The tissue samples were explanted at day 7, 14 and 28 and tissue sections were stained with ED-1 and α-SMA (Fig. 7). PLHMGA-ms were visible in the tissue sections as unstained white round spheres. In tissues injected with monodisperse PLHMGA-ms, a mild inflammatory reaction was observed at day 7 after injection with the recruitment of few inflammatory cells, from which the majority were ED-1 expressing macrophages (Fig. 7A) capable of phagocytosis (Dijkstra et al., 1985). The presence of few foreign body giant cells (FBGCs) was also observed, which are formed by the fusion of macrophages in response to the foreign material (Anderson, 2001). Few myofibroblasts in samples explanted at day 7 were detected with α-SMA staining (Fig. 7D). Myofibroblasts are cells with features of smooth muscle cells and are responsible for the wound contraction (Desmoulière et al., 2003) and are also responsible for synthesizing collagen. Collagen forms the basis of the fibrous capsule, which plays a crucial role in the tissue repair and is considered a normal reaction feature towards the implanted foreign material (Anderson and Shive, 1997; Anderson, 2001). Staining for α-SMA also allows detection of blood vessels, since vascular smooth muscle cells express this marker (Skalli et al., 1986). Scattered capillaries and arterioles were observed in sample tissues injected with monodisperse PLHMGA-ms and explanted at day 7. The presence of erythrocytes in the vessel lumina (Fig. 7D) suggests functional blood vessels. The inflammatory reaction (macrophages, FBGCs) and myofibroblasts were also seen at day 14, when the microspheres fragmented into smaller residues <10 μm (Fig. 7B and E). At day 28, no particle residues were detected and few infiltrating macrophages were still present (Fig. 7C). Myofibroblasts were virtually absent (Fig. 7F), marking the end of the fibrotic response towards monodisperse PLHMGA-ms. No fibrous capsule was detected, which indicates a relatively mild foreign body reaction (Shishatskaya et al., 2008).

In a recent study, PLGA monodisperse microspheres with a similar size of 30 μm were investigated for their biocompatibility

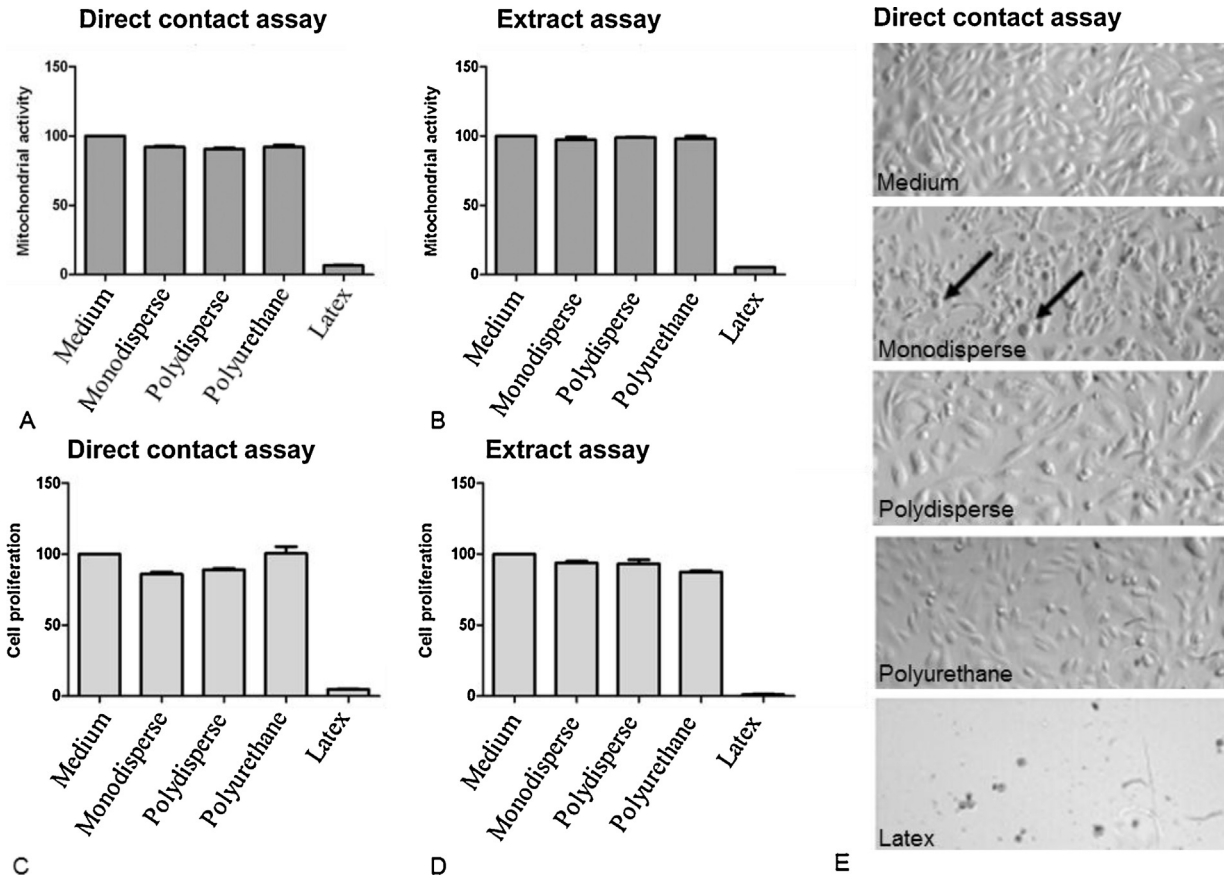

Fig. 6. *In vitro* cytocompatibility of PLHMGA-ms (5 mg/600 μL) upon incubation with human primary tubular epithelial cells (PTECs). PTECs were exposed to monodisperse and polydisperse PLHMGA-ms in the direct contact assay for 72 h (A and C) and to their 24-h extracts for 48 h (B and D). E: cell morphology in direct contact assay (magnifications 100×); arrows indicate the monodisperse PLHMGA-ms. Cell viability was assessed with MTS and cell proliferation assay. Polyurethane and latex were used as a negative and a positive control, respectively.

after subcutaneous injection in rats up to 4 weeks after their administration (Zandstra et al., 2014). As expected from the type of PLGA used in this study, these microspheres hardly showed degradation during the time course of the study and only low numbers of infiltrated inflammatory cells were observed, in agreement with the mild foreign body reaction to PLGA. Polydisperse PLGA microspheres as well as other types of PLGA matrices have been studied extensively for their foreign body reaction and biocompatibility (Anderson and Shive, 1997; Athanasiou et al., 1996; Cadée et al., 2001; Kohane et al., 2002). Visscher et al., 1985, 1987, 1988 reported studies of the biocompatibility of 30 μm diameter PLGA (50/50) microspheres after intramuscular injection in rats. The authors observed a mild inflammatory reaction for a period of nine weeks with the presence of lymphocytes, macrophages and FBGCs. Phagocytosis of particles was observed around day 42 after injection, the time-point when particles became smaller than 10 μm in size (Cadée et al., 2001). Increased infiltration of macrophages was reported at day 56 (Visscher et al., 1985). The end of the inflammatory response in tissues injected with PLGA microspheres was observed around day 60 after administration (Visscher et al., 1985). Prolonged inflammatory reaction of PLGA microspheres as compared to the 28 days observed for PLHMGA-ms is caused by the longer (two-month) degradation time of PLGA (Anderson and Shive, 1997; Visscher et al., 1985, 1988).

The intensity of the inflammatory reaction towards injected polymeric microspheres is also dependent on particle size distribution. In this study, the effect of particle size distribution on the biocompatibility was tested by subcutaneous injection of polydisperse PLHMGA-ms in rats, prepared by conventional single emulsion method, with size distribution between 5 and 46 μm in diameter (mean: 17 μm). Tissues were explanted at day 7, 14 and 28 and stained with ED-1 and α-SMA (Fig. 8). As was the case for monodisperse PLHMGA-ms, prepared by membrane emulsification, substantial numbers of macrophages were observed on 7, 14 and 28 days, as well as FBGCs on day 7 and 14 (Fig. 8A–C). In comparison with monodisperse microspheres, increased vascularization was observed in tissue samples at day 14 (Fig. 8E). Only a very few myofibroblasts were observed at day 7 and 14 (Fig. 8D and E). Interestingly, some large particles were still present at day 28 after injection (Fig. 8C and F). Although increased inflammatory responses towards smaller particles have been reported in previous studies (Cadée et al., 2001; Champion et al., 2008; Tabata and Ikada, 1988; Thomasin et al., 1996; Visscher et al., 1988), in the current study no significant differences were observed in the inflammatory reaction between the tissues injected with monodisperse and polydisperse PLHMGA-ms.

3.5. In vivo *biocompatibility after subcapsular renal administration of monodisperse PLHMGM-ms*

The biocompatibility of monodisperse PLHMGA-ms was also tested after subcapsular renal injection, which is a novel strategy to for local drug delivery in the kidney. The injected amount of microspheres (10 mg in 50 μL vehicle) was optimized as the highest concentration of the microspheres that could be delivered under the kidney capsule in view of the high viscosity of such dispersion and the injection *via* a small size needle of only 26G.

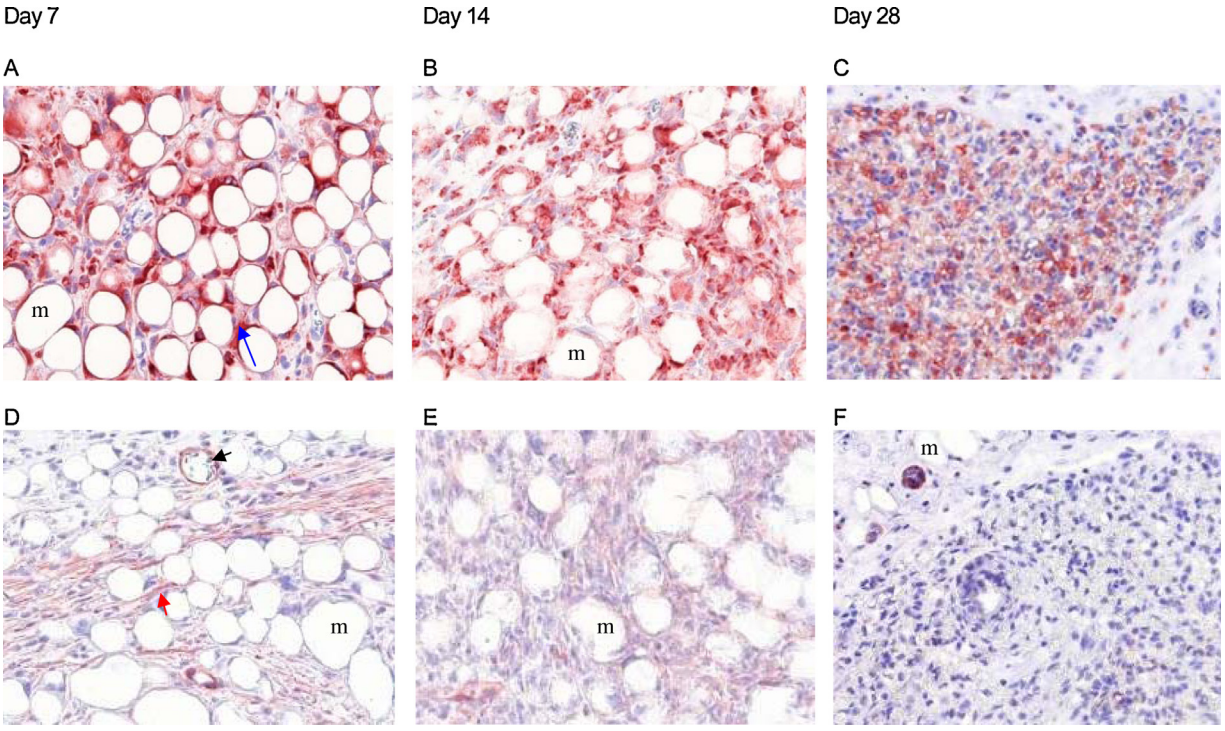

Fig. 7. Histological pictures of subcutaneous tissues in which monodisperse PLHMGA-ms were injected. A–C: ED-1 staining (macrophages are stained in brown, blue arrow); D–F: α-SMA staining (myofibroblasts are stained in pink; red arrow), blood vessels are stained in red (black arrow). Microspheres (m) remain unstained in both stainings and are visible as white spheres at all time-points; (magnification 40×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

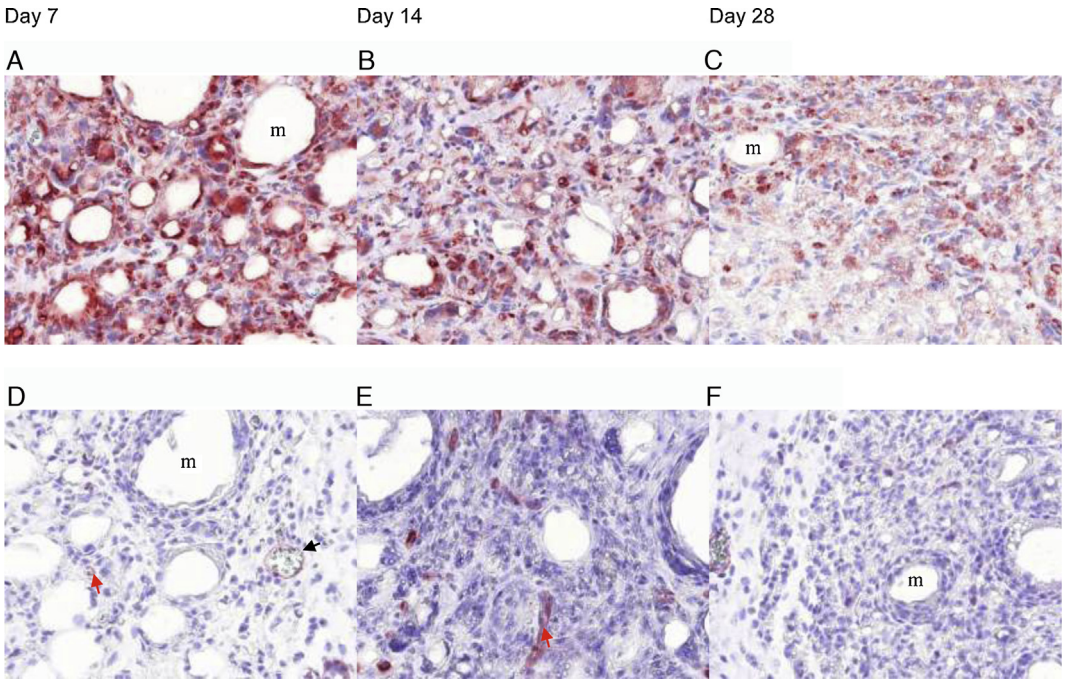

Fig. 8. Histological pictures of subcutaneous tissues in which polydisperse PLHMGA-ms were injected. A–C: ED-1 staining (macrophages are stained in brown); D–F: α-SMA staining (myofibroblasts are stained in pink (red arrow), blood vessels are stained in red (black arrow). Microspheres (m) remain unstained in both stainings and are visible as white spheres; (magnification 40×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

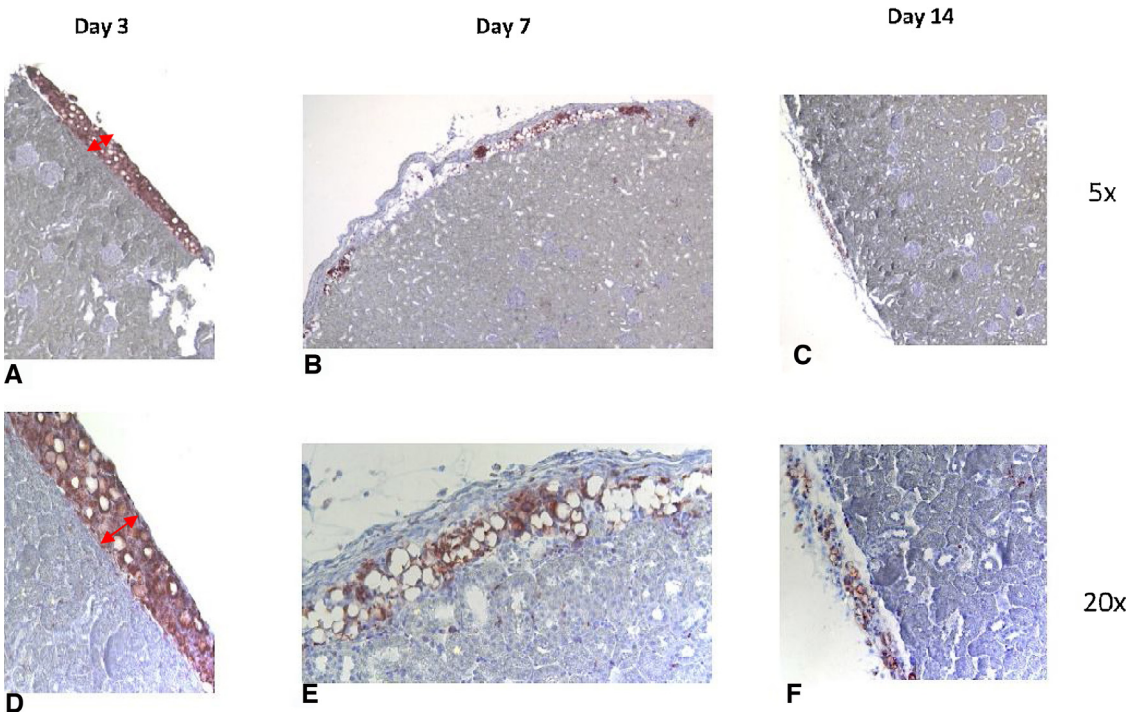

Fig. 9. Foreign body reaction elicited by monodisperse PLHMGA-ms injected under the kidney capsule, stained with ED-1 and counterstained with hematoxylin. The area marked with red arrows represents the subcapsular space where the microspheres where injected. This area was analyzed for histological examinations and for possible inflammatory responses. Macrophages are stained with brown, nuclei in blue while microspheres remain unstained and are visible as white spheres. Magnification: 5× (A–C) and 20× (D–F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

After the injection, the kidneys were explanted at day 3, 7, and 14. The results of the tissue sections stained with ED-1 and hematoxylin are given in Fig. 9. Similar to the subcutaneous injection, microspheres injected under the renal capsule were visible until day 14 as small particulates. Macrophages again appeared as the most abundant inflammatory cells in the injected tissues. They localized only at the implantation site between the cortex and the renal capsule. Macrophages were mainly visible in the tissue samples explanted at day 3 and 7, with significant reduction at day 14 after injection. The injected microspheres and the inflammation reaction were localized between the cortex and renal capsule with no penetration into the peritubular space (Fig. 9). This result shows that polymeric microspheres can be injected under the renal capsule without cortical damage or damage to the capsule due to the injection method. The biocompatibility of a subcapsular depot was previously studied for supramolecular hydrogels in rats (Dankers et al., 2012). Similar to our results, they showed that subcapsular injected biomaterials primarily resulted in a thickening of the renal capsule with only minimal responses in the renal cortex. From our data we conclude that monodisperse PLHMGA-ms injected under the kidney capsule have a good biocompatibility and can therefore be used for local delivery of therapeutic molecules in the kidney.

3.6. In vitro–in vivo *degradation of PLHMGA-ms*

In vitro studies in a PBS buffer showed that PLHMGA-ms undergo 80% mass loss during 35 days as described in paragraph 3.2 and at day 28, around 30% of the original mass was present. After subcutaneous and subcapsular renal injection of monodisperse PLHMGA-ms, particles were virtually absent in the tissue sections explanted at day 28 and 14, respectively. This indicates a slightly faster *in vivo* degradation compared to *in vitro*. Similar findings have been reported for PLGA microspheres (Jiang et al., 2005). It has been demonstrated that PLGA particles inside macrophages degrade faster than particles in buffer likely due to the relatively low pH and/or the presence of esterases in the phagosomes (Van Apeldoorn et al., 2004; Walter et al., 2001), which may also have contributed to faster *in vivo* degradation of PLHMGA microspheres in the present study.

4. Conclusion

Monodisperse and polydisperse PLHMGA-ms showed good cytocompatibility after incubation with PK-84, HK-2 and PTECs cells and are biocompatible *in vivo* after subcutaneous administration. Therefore both monodisperse and polydisperse PLHMGA-ms are promising drug delivery systems for subcutaneous injection. In addition, monodisperse PLHMGA-ms injected under the kidney capsule induced only a very localized inflammatory reaction at the site of the depot, showing the feasibility of this type of microspheres for local drug delivery to the kidney.

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