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Emulsion-based synthesis of PLGA-microspheres for the *in vitro* expansion of porcine chondrocytes

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Abstract

The *in vitro* cell expansion of autologous chondrocytes is of high interest in regenerative medicine since these cells can be used to treat joint cartilage defects. In order to preserve chondrocyte phenotype, while optimizing adhesion on microspheres, several processing parameters for the microsphere synthesis were varied. In this study three different polylactide-*co*-glycolides were used with differing lactide–glycolide ratios (85:15 and 50:50) and differing inherent viscosities. An emulsion route was established, where the polymer was dissolved in chloroform and then injected into a stirred polyvinyl alcohol–water solution at different polymer concentrations and different stirring velocities to produce microspheres with varying diameters. The sphere size distribution and morphology was analyzed using image processing software on SEM pictures. Based on previous experiments with commercial microspheres, three optimum samples were selected for further investigations. The degradation of the microspheres was determined in a long-term experiment in culture medium for 3 months. Adherent cells were characterized after 3 and 5 days by FDA + EB vital staining and in SEM.

Keywords: Cartilage tissue engineering; PLGA-microspheres; *In vitro* expansion; Degradation; Microcarrier culture

1. Introduction

For tissue engineering of joint cartilage *in vitro*, cell expansion is necessary because the amount of cells which can be harvested from cartilage biopsies is normally insufficient to fill a given defect size. Conventional cell expansion uses two-dimensional cell culture flasks obtaining a confluent monolayer, often resulting in cellular dedifferentiation and in the loss of the native phenotype. Consequently, the cultivated cartilage cells (chondrocytes) loose their ability to synthesise extra cellular matrix substances like collagen type II and instead synthesise collagen type I, while the phenotype resembles fibroblasts (Von der Mark et al., 1977). It is postulated that dedifferentiation is mediated by actin-stress filaments resulting from cellular adhesion to a flat surface (Benya et al., 1978). Therefore, a main goal is to achieve a regenerated tissue in a three-dimensional conformation with a normal cell phenotype and differentiation status (Bonassar and Vacanti, 1998). One example for a three-dimensional cultivation system is the application of microspheres enabling cells to adhere to the sphere surface. This allows a three-dimensional cell conformation *in vitro* and inhibits the production of actin filaments (Benya et al., 1988) but preserves the ability to generate a typical extra cellular matrix (Freed and Vunjak-Novakovic, 1995).

Microsphere cultivation systems (also known as microcarrier cultivation) are well established for polysaccharide (Cytodex) and aliphatic polyesters of the poly(α-hydroxy)acids, especially poly-L-lactides (PLA) and polyglycolides (PGA) or their copolymers poly-L-lactide-*co*-glycolides (PLGA). This material system can be easily tailored in its physical and chemical properties by varying the lactide–glycolide ratio (Temenoff and Mikos, 2000). Particularly the degradation rate can be adjusted to allow degradation between several months (pure PLA) and a few weeks (pure PGA) (Minuth et al., 2003). Further, PLGA contrary to collagen-based materials has been shown to promote the

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production of proteoglycans, cellular differentiation and cell maturation (Grande et al., 1997).

Most microspheres currently researched have diameters between 100 and 250 μm and feature a hydrophilic surface (Bardouille et al., 2001) resulting in a significantly larger surface area related to the cell suspension volume than is possible in conventional 2D-cell expansion. It has been approximated that the surface to volume ratio of microspheres is approximately 20 cm^2/ml opposed to 4 cm^2/ml in 2D-cultivation (Clark and Hirtenstein, 1981).

A major benefit using biodegradable microspheres is the possibility to abandon trypzinisation which is of high interest in injectable applications when the used polymer microspheres completely degrade *in vivo* or *in vitro* (Hong et al., 2005).

Mechanical shear stresses are known to induce beneficial cellular responses *in vivo* (Sah et al., 1989) which can be translated into the *in vitro* system through moderate stirring of the microsphere-based culture system (Smith et al., 2000). These mechanical forces are also reported to result in redifferentiation of chondrocytes having already established a fibroblast-like phenotype (Malda et al., 2003). To further stimulate the adhering cells, the microspheres can be modified by collagen coatings (Chun et al., 2004) or can be modified with active agents being released over the degradation period (Hong et al., 2005).

In this study we used an o/w-emulsion based processing route to synthesise PLGA-microspheres from three different polymers in different sizes by varying stirring velocity and dissolved polymer content in the oil-phase. The microspheres have been tested *in vitro* using porcine chondrocytes.

2. Experimental

2.1. Synthesis of microspheres

Microspheres were produced by dissolving three different PLGA–polymers (50:50 PLGA-type "a" with a residual monomer content of 0.90% and an inherent viscosity (i.V.) of 0.84 dl/g, 50:50 PLGA-type "b" with a residual monomer content of 0.83 (i.V.: 0.69 dl/g) and 85:15 PLGA with a residual monomer content of 1.20% (i.V.: 3.27 dl/g), all Resomer RG type, Boehringer Ingelheim) in $CHCl_3$ (Merck), and casting this polymer-solution into stirring deionized and sterile water with the addition of 0.5% polyvinyl alcohol (Fluka). Three different stirring speeds have been used (200, 300 and 400 rpm) with the three different polymers, which were further used in three different polymer concentrations (0.05, 0.10 and 0.15 g/ml). The stirring speed has been adjusted using a Hall-sensor in combination with a multi position magnetic stirrer (RO 15 power Ikamag, IKA) and has been performed overnight to allow for complete removal of the solvent via evaporation (solvent evaporation technique). Microspheres were washed shortly in 70% 2-propanol for disinfection purposes and were washed afterwards thrice in deionized and sterile water. To further ensure sterility, samples were subjected to UV-irradiation for 20 min in cooled condition on freeze packs. To limit the parameter set and to obtain optimum microsphere sizes being related to commercial type microspheres (Cytodex, Amersham Biosciences) only concentrations of 0.10 g polymer/ml $CHCl_3$ and a stirring velocity of 300 rpm were chosen.

2.2. Evaluation of microsphere size

Microspheres were homogenously distributed on aluminium SEM-stubs (diameter 1.2 mm) with self-adhesive graphite-pads and imaged at a fixed magnification (100×) using a Philips XL 20 scanning electron microscope. Images were processed using image processing software (NI Vision 7.1, National Instruments) using an automated batch script to obtain the size distribution of the microcarriers.

2.3. Microsphere degradation

Microspheres were subjected to hydrolytic degradation in RPMI 1640 culture medium with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (all Biochrom). Therefore, 100 μg microcarriers were incubated in falcon tubes (12 ml culture medium) with partly opened lid in humidified (95%) atmosphere and 10% CO_2 for 80 days. Degradational behavior has been investigated after 20, 40, 60 and 80 days in respect of morphology using SEM imaging, pH-shift using a pH-electrode (Schott) and overall mass decrease using a balance (CP324 S, Sartorius). Mass loss has been analyzed by measuring the dry weight, using a freeze dryer (Modulyo, Edwards) to completely remove water residues from the microspheres before weighing.

2.4. Cell culture

Primary porcine chondrocytes were harvested from adult pigs from a slaughterhouse by mechanically separating healthy joint cartilage from the knee condyles followed by enzymatic digestion for 18 h using 1.5 U/ml collagenase P (Boehringer Ingelheim), 500 U/ml collagenase II (Biochrom) and 50 U/ml hyaluronidase (Sigma) in RPMI 1640 medium with 10% FCS supplemented with 100 U/ml penicillin (Biochrom) and 100 μg/ml streptomycin (Biochrom). Afterwards the cell suspension has been washed thrice with Hanks-buffer-solution (Biochrom). This suspension was centrifuged to obtain the porcine chondrocytes which were resuspended in culture medium to obtain passage 0 (P0) chondrocytes or were seeded in cell culture flasks to obtain passage 1 (P1) chondrocytes after further cultivation for several weeks. Overall cell vitality has been estimated using trypan blue staining in a Neubauer chamber. 2.0×10^6 cells (P0 and P1 passages) were cultivated in 12-well plates with 200 mg microcarriers per well and 2.0 ml culture medium. In this preliminary work, cell cultivation experiments were limited to microspheres obtained from the three PLGA–polymers using concentrations of 0.10 g/ml $CHCl_3$ and a stirring velocity of 300 rpm. Vitality has been tested after 3 and 5 days of cultivation, cells were vital stained using a combination of fluoresceindiacetate and ethidiumbromide (FDA + EB) and imaged using a Leica DM 4000M microscope (Leica Microsystems) with Leica PL Fluotar objectives (5×, 10× and 20×) and a Leica N2.1 fluorescence filter. Image acquisition was performed using a Leica DFC 320 digital camera in combination with Leica IM1000 image acquisition software.

Adherency has been evaluated using neutral red (Roth) in a concentration of 25 mg/ml.

3. Results

3.1. Size distribution

Exemplary for the 85:15 PLGA the obtained size distribution of the microspheres is displayed in Fig. 1 showing the nine different histograms with the concentration plotted in the vertical columns and the stirring velocity plotted in the horizontal rows. The same diagrams were obtained for both 50:50 PLGA–polymers (data not shown). As a result, low concentrations result in smaller microspheres with a more narrow size distribution, while higher concentrations result in larger microcarriers with a wider size distribution. The influence of the stirring velocity on the other hand seemed to be relatively insignificant.

Further, both 50:50 polymers were comparable in their obtained size distribution and generally featured smaller microspheres then were obtained with the same parameter set and the 85:15 polymer, especially at high polymer concentrations. The three microsphere types featured size distributions