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Impact of implant composition of twin-screw extruded lipid implants on the release behavior

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ABSTRACT

The development of vaccine delivery systems that will remove or reduce the need for repeated dosing has led to the investigation of sustained release systems. In this context, the duration of antigen release is of great importance as is the requirement for concomitant adjuvant release. In this work, lipid implants consisting of cholesterol (CHOL), soybean lecithin, Dynasan 114 (D114), the model antigen ovalbumin (OVA) and the adjuvant Quil-A (QA) were produced by twin-screw extrusion. The release of antigen and adjuvant was investigated in vitro and we observed complete OVA release over a period of 7 days while QA was released in a linear fashion over a period of up to 12 days. In order to extend OVA release, lipid implants were subjected to post-extrusion curing at 45–55 °C. The OVA release could be extended to up to 14 days. Furthermore the influence of the implant composition on the release of the model antigen was investigated. It was shown that the percentage of cholesterol in particular plays an important role in modulating release.

1. Introduction

Vaccination often requires the administration of a prime and booster immunization to induce a strong memory immune response and long-term protection. Often subunit antigens such as proteins or peptides are used in modern vaccines, replacing whole pathogens, for purity and safety considerations (Arnon and Ben-Yedidia, 2003). In order to induce immunity, multiple doses of these subunit antigens, in combination with immunostimulatory adjuvants, are required as they are less immunogenic than whole-pathogen vaccines (Kersten and Crommelin, 1995). However, repeated administration of vaccines often compromises patient compliance. Therefore, a delivery system, which is able to release antigen in a sustained manner, could be a major advance in the development of vaccines. The general concept of sustained release of vaccines has already been investigated by Preis and Langer (1979) using ethylene–vinyl acetate copolymer pellets and by Lofthouse et al. (2002) using silicone based implants. The sustained antigen from both types of implants proved effective in eliciting prolonged antibody formation. It should be noted that such systems made from non-biodegradable polymers would require surgical removal (Gombotz and Pettit, 1995).

For this reason biodegradable polymers such as polylactic acid (PLA) and poly(lactic-*co*-glycolic acid) (PLGA), which can release drug continuously after parenteral administration (Putney and Burke, 1998; Rothen-Weinhold et al., 1998; Vogelhuber et al., 2003b), are increasingly being investigated today for drug and vaccine delivery as injectable or implantable depot formulations. However, an issue with these formulations is polymer degradation to smaller chain acids upon contact with water, leading to significant drops in the micro-environmental pH, which can result in a loss of activity with protein-based drugs (Brunner et al., 1999; Kreye et al., 2008). The use of synthetic polymer matrix materials has the additional problem that during manufacture irreversible changes in structure and activity of proteins can be induced by heating, high shear forces, exposure to organic solvents and increased osmotic pressure (Vogelhuber et al., 2003b).

An interesting alternative is to utilize lipid implants as parenteral controlled delivery systems. Lipids are considered to be safe for diverse types of applications and are widely used in the food and cosmetic industry. Lipids such as triglycerides (Vogelhuber et al., 2003b), mixtures of triglycerides with cholesterol (CHOL) and phospholipids (Myschik et al., 2008a), phospholipids or blends of phospholipids and CHOL (Demana et al., 2005) have

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been considered as alternatives to polymers for the development of controlled-release systems. Lipids are generally biocompatible and biodegradable and are normally not inherently immunogenic (Allababidi and Shah, 1998; Guse et al., 2006b; Koennings et al., 2007). Lipid implants have been thoroughly investigated as sustained delivery systems for protein and to a lesser extent for vaccine delivery (Even et al., 2014a; Guse et al., 2006a; Kreye et al., 2008, 2011c; Mohl and Winter, 2004; Myschik et al., 2008b; Sax et al., 2012; Yamagata et al., 2000). The studies also included in vivo biodegradation experiments on lipid extrudates. Typically, the release period is shorter than the degradation period and therefore the release kinetics are less affected by degradation than in most PLGA systems (Sax et al., 2012).

Recently, we reported that lipid implants for vaccine delivery can be prepared by twin-screw extrusion (Even et al., 2014a). They were solid, easy to handle and showed good mechanical properties. Administration of these implants to mice resulted in enhanced antigen-specific IgG titers when both an adjuvant (Quil-A, QA) and antigen (ovalbumin, OVA) were present in the implants. The release behavior of adjuvant used in sustained release implants will be an important aspect to consider as it has been reported that antigen and adjuvant must be released synchronously in order to obtain an optimal immune response (Kamath et al., 2012). Therefore the release of QA, a saponin derived from the tree *Quillaia saponaria,* and OVA from different implant formulations was examined. Both release behaviors were compared to ensure the release of adjuvant and antigen was simultaneous.

It has been reported that implants releasing OVA over a period of 7 days could induce immune responses similar in magnitude to two injections (Even et al., 2014a), this led to the hypothesis that longer antigen release may induce even stronger immune responses. In order to tune the release of antigen and adjuvant the effect of curing implants post-production was examined. Kreye et al. (2011b) had reported that curing lipid implants composed of Dynasan 120 sustained the release of propranolol hydrochloride. We wanted to investigate if similar results could be achieved for antigen release. Curing temperatures ranging from 45 °C to 55 °C were chosen for our implants. The curing temperatures were slightly below the melting temperature of the lipids in order to melt the outer surface of the implants and change the size of pores at the surface of the implant therefore influencing antigen release (Kreye et al., 2011b).

2. Materials and methods

2.1. Materials

Ovalbumin from chicken egg white (OVA) grade V, solubility 40 mg/mL, was purchased from Sigma–Aldrich. Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany) (T_m = 147 – 150 °C). Soybean Lecithin (approx. 90% phosphatidylcholin) was purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). PBS tablets from Oxoid Limited (Basingstoke, England). Purified Quil-A (QA), solubility 10 mg/L, was sourced from Brenntag Biosector (Frederikssund, Denmark) as a lyophilised powder and used as supplied. Dynasan 114 (D114) a triglyceride (known as trimyristin, $T_{m\beta}$ = 55 – 58 °C) was kindly provided by SASOL Germany GmbH (Witten, Germany). Ultrapure deionized water having a conductivity of less than 0.055 μS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

2.2. Preparation of lipid implants by twin-screw extrusion

Mixtures of soybean lecithin, CHOL, D114, with and without OVA and/or QA were used to prepare the implants. High grade stainless steel beakers for milling in a swing mill, Retsch® CryoMill (Retsch Technology, Haan, Germany) were filled with soybean lecithin and D114. The system was precooled with liquid nitrogen for 10 min at 5 Hz, the mixture was then ground for 1 min at 25 Hz. A plastic mortar and pestle were used to mix the obtained powder by hand with the remaining components. QA and OVA were then gradually blended with the lipid mixture and subsequently fed into a twin-screw extruder (Haake MiniLab® Micro Rheology Compounder, Thermo Haake, Germany). The implants were extruded at a rotation speed of 40 rpm at an extrusion temperature of 45 °C with closed bypass channel using an outlet of 2 mm diameter. The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 2.5 cm, resulting in an implant mass of about 0.08 g.

2.3. Curing of lipid implants prepared by twin-screw extrusion

Implants were cured using an oven (UM 400, Memmert GmbH + Co. KG, Schwabach, Germany) at 55 °C for 15 min (min), or at 40, 45 or 50 °C for 60 min, respectively. Implants were placed into Eppendorf tubes leaving the cap open. The tubes were then horizontally placed into the heating cupboard. To ensure that the complete surface of the implants was heated equally, implants were turned by turning the Eppendorf tube (every 5 min for incubation time of 15 min, every 15 min for 60 min incubation time). At 55 °C a curing time of 15 min instead of 60 min was chosen to avoid implant deformation.

2.4. Differential scanning calorimetry (DSC)

A DSC 204 Phoenix (Netsch, Selb, Germany) was used to analyses each lipid before extrusion. Thermograms of implants were recorded directly after the extrusion as well as after the post-treatment. Samples of about 4 mg were each weighed into aluminum crucibles. A heating and cooling rate of 5 K/min was used as between 20 °C and 160 °C. An empty crucible served as reference.

2.5. In vitro release of OVA from implants

The release of the model antigen OVA was investigated over a period of up to 15 days. Lipid implants of a length of 2.5 cm (n = 3) were incubated at 37 °C in a Heidolph 1000 Incubator in vials containing 1.8 mL phosphate buffered saline (PBS) (pH 7.4, 0.01 M, 0.05% NaN_3). At defined time points samples were taken and the release medium was exchanged completely. All samples were centrifuged at 14000 rpm (Mikroliterzentrifuge Z 160 M, Hermle Labortechnik, Wehingen, Germany) for 5 min to remove lipid particulates as described in literature (Sax and Winter, 2012). OVA was measured in the supernatant by UV (Agilent Technologies 8453) at a wavelengths of 280 nm. For each tested mixture an implant containing neither OVA nor QA was used as a blank for the UV measurements. Measurements were performed as long as OVA was released from the implants. Each implant was weighed before the release and the total amount of protein present in each implant was calculated individually using a standard curve prepared by an 11-fold 1:1 dilution starting from a sample of 3 mg OVA in 1 mL PBS. All measured samples lay within the linear part of the standard curve (3 mg/mL–5 μg/mL).

2.6. In vitro release of Quil-A from implants

Implants were cut in 3.5 cm lengths and weighed. All implants contained 55% cholesterol, 340–750 μg of QA and 145–2240 μg of OVA. Implants were placed into 5 mL tubes filled with 1.8 mL PBS buffer (pH 7.3) and incubated at 37 °C (Clayson incubator, New