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# Dual dye *in-vivo* imaging of differentially charged PLGA carriers reveals antigen-depot effect, leading to improved immune responses in preclinical models

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ABSTRACT

The present *in-vivo* study investigated the behavior and performance of differently charged poly(lactic-*co*-glycolic) acid microparticles (PLGA MP) as vaccination platform. For this purpose, particles loaded with ovalbumin (OVA) as model antigen were subcutaneously (s.c.) injected in SKH1 mice. The utilized SKH1 hairless mice exhibit a fully operative immune system and allow parallel imaging investigations due to the lack of hair. Usage of this species enabled the combination of two investigations within a single study protocol, namely noninvasive *in-vivo* imaging and immune responses directed towards the antigen. All treatments were well tolerated, no safety drop-outs occurred.

The fate of the model antigen OVA as well as the PLGA particles was monitored using a dual dye approach (CF660C & DiR) by multispectral fluorescence imaging (msFI). A depot effect for the OVA antigen adsorbed to the MP surface could be observed for the positively charged MPs. The immune response against OVA was then analyzed. OVA alone did not induce an immune response, whereas the positively charged as well as the neutral MP induced a strong and consistent humoral immune response with a clear favor of IgG1 over IgG2a subclass antibodies. In contrast, negatively charged MP were not able to induce measurable antibody responses. Cellular immune response was weak and inconsistent for all treated groups, which verifies previous *in-vitro* results conducted with the herein described microparticulate antigen platform.

In conclusion, the characterization of the *in-vivo* performance yielded valuable information about antigen and carrier fate after application. The presented adjuvant platform is capable of inducing strong $T_H2$ dominated immune responses characterized by enhanced IgG1 subclass titers which are critical for vaccines aimed at promoting induction of neutralizing antibodies.

## 1. Introduction

Conducting animal studies before testing possible new medications in humans is currently unavoidable. However, animal studies should be carefully designed to minimize the number of animals needed and reduce the pain possibly caused by the intervention to the lowest feasible extent without endangering the study outcome. This principle, completed by the question whether the study goal could also be achieved by replacing living animals with tissues or cells, is well known as 3R principle (reduce, refine, replace). Since its introduction by Russell and Burch in 1959 (Russell and Burch, 1959) the 3R principle gained increasing attention and can surely be regarded as gold standard when planning and conducting *in-vivo* studies nowadays. A recent approach to account for the 3R principle is *in-vivo* imaging, which can reduce the number of animals needed for a specific study and also helps in refining the experimental setup by being inherently non-invasive (Campbell et al., 2016).

For *in-vivo* imaging, several techniques are available which can be chosen based on the study specific question to be answered. A comprehensive overview about these techniques can be found in Campbell et al. (2016). Gamma-scintigraphy or positron emission tomography are suitable methods for the investigation of biodistribution (Toita et al., 2013) and immunologic responses (*e.g.* to a labeled antigen). However, a radioactive entity may hamper immune responses (Barsegian et al.,

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2015). Furthermore, the radioactive half-life of the utilized tracers may limit the observation period. Therefore, multi-spectral fluorescence imaging (msFI) can be an adequate non-radioactive alternative (Rahimian et al., 2015; Wang et al., 2014). In detail, Wang et al. (2014) investigated the fate of fluorescence labeled ovalbumin (OVA) after subcutaneous (s.c.) administration, either bound to well-known alum as adjuvant or by employing poly(lactic-*co*-glycolic) acid (PLGA) microparticles (MP). Although this study provided valuable information about the OVA fate, the degradation of the carrier could not be monitored due to the absence of a second label. Moreover, immune responses were measured in a different mice species in comparison to the imaging part of the study. Translating imaging results from one mouse model into immunological results obtained from another may be difficult, thus a combination of both parts of the study appears elegant.

The aim of the present study is to combine information on the *in-vivo* fate of the carrier, as well as OVA as a model antigen, with information about the immune responses elicited in the same mouse model. Such combination of all three parts in a single study is highly desirable in light of the 3R principle, because it reduces the overall number of animal studies. If both studies are equally powered in terms of investigated individuals, this corresponds to a strong reduction of required animals.

As carrier served differently charged PLGA MP which were manufactured employing following stabilizers: positively charged MP – *R*-1,2-dioleoyl-3-trimethylammonium-propane (*R*-DOTAP); neutral MP – polyvinylalcohol (PVA); negatively charged MP – docusate sodium (DOSS). PLGA itself was chosen due to its proven safety track record, which is reflected in its approval for intravitreal application for human use (Pacella et al., 2013).

In order to observe the fate of the PLGA carrier in parallel to OVA, a dual dye approach was employed. PLGA particles were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) and OVA with covalently bound CF660C (similar to Alexa Fluor 660, as employed by Wang et al. (2014)). DiR as surrogate for MP fate was chosen for its favorable fluorescence properties in the near-infrared range (NIR), allowing a deeper penetration of body tissues. Additionally, DiR has been found to be non-irritant on macrophages from murine origin (Eisenblaetter et al., 2009) and also on fibroblasts (Granot et al., 2007), minimizing the risk of interfering with the immunologic results. The utilized labels enabled the use of msFI over the course of 42 days.

SKH1 hairless mice were chosen as appropriate animal species due to their lack of fur, thus interfering less with fluorescence imaging (Benavides et al., 2009), and their immune competence and ability to evolve a complete adaptive immune response. The combination of imaging and immune readout would not have been possible by using for example the more common BALB/c nude mice, since they lack a thymus and therefore are not capable of inducing a complete cellular immune response. Humoral as well as cellular immune response could be evaluated by using blood and spleen samples of the animals.

## 2. Materials

PLGA (RG 503 H) as matrix polymer with intrinsic viscosity of 0.32–0.44 dL/g was obtained from Evonik (Darmstadt, Germany). *R*-1,2-dioleoyl-3-trimethylammonium-propane (*R*-DOTAP; chloride salt with molecular mass of 698.5, Merck & Cie Schaffhausen, Switzerland) was used as cationic stabilizer. Polyvinylalcohol (PVA) in excipient quality with 88% degree of hydrolysis and a nominal viscosity of 4.3–5.7 mPas (4% solution in water; Emprove PVA 5–88) was obtained from Merck KGaA (Darmstadt, Germany).

Docusate sodium (DOSS, purity > 97%), Dulbecco's phosphate buffered saline (PBS, 10× stock, article D1408, diluted 1:10 before usage, unless otherwise stated), OVA (lyophilized powder, purity > 98%, article A5503) and biotinylated chain-specific goat anti-mouse IgG were obtained from Sigma-Aldrich, St. Louis, USA. Fluorescence labels DiR and CF660C were from Molecular Probes (Eugene, USA) and Biotium Inc. (Fremont, USA), respectively. Dichloromethane (p.a.), acetonitrile (gradient grade), trifluoroacetic acid (spectroscopy grade), acetic-acidic 0.1% eosin solution and Entellan© were from Merck KGaA, Darmstadt, Germany. Milli-Q grade water was freshly taken from a Millipore Advantage A 10 with Q-Pod apparatus (Merck KGaA, Darmstadt, Germany).

RPMI 1640, complete medium was either purchased from Life Technologies, Carlsbad, USA or from Sigma-Aldrich Chemie GmbH, Munich, Germany (containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin).

Formalin was purchased from Carl Roth, GmbH + Co. KG, Karlsruhe, Germany. Hematoxylin solution was from DAKO Agilent Pathology Solutions, Santa Clara, USA.

Isoflurane (Forene©) was from AbbVie Deutschland GmbH & Co. KG, Ludwigshafen, Germany.

Biotinylated rat anti-mouse IgG1 and IgG2, peroxidase conjugated streptavidin and enzyme-linked immunospot (ELISPOT) kits were supplied by BD Pharmingen, San Diego, USA.

## 3. Methods

### 3.1. Preparation of the Particles

#### 3.1.1. Preparation of DiR-loaded MP

Particles were produced *via* an oil-in-water (O/W) emulsion - solvent evaporation method. Organic phase was 5% (m/V) PLGA (and 25 mg *R*-DOTAP for *R*-DOTAP particles) in 2 mL dichloromethane for *R*-DOTAP & PVA stabilized MP. For DOSS stabilized MP, the organic phase was 12.5% (m/V) PLGA in 2 mL dichloromethane. DiR as NIR probe was used in a concentration of 0.02% (m/m) in respect to polymer mass and added to the organic phase.

4 mL of the aqueous phase (0.8% DOSS or 4% PVA in water for DOSS or PVA particles or pure water for *R*-DOTAP MP) were added to the organic phase and thoroughly mixed by high-shear mixing (Ultra-Turrax T 8, IKA, Staufen, Germany) to form the pre-emulsion.

This pre-emulsion was transferred into a 100 mL beaker containing 50 mL of the aqueous phase used for the pre-emulsion under magnetic stirring. Afterwards, the emulsion was stirred overnight in the open beaker to allow dichloromethane evaporation. All MP were produced in a laminar flow cabinet (Herasafe KSP, Thermo Fisher Scientific, Asheville, US) under best-clean conditions.

After MP washing (Riehl et al., 2015), MP were aliquoted into 2R vials and freeze-dried. Mannitol was used as matrix forming agent. Placebo MP manufacturing was finished at this stage, verum MP were further processed by applying the OVA loading procedure described below.

#### 3.1.2. Loading of Verum MP With CF660C-coupled OVA

OVA was fluorescence labeled using the CF660C kits according to manufacturer's instructions.

An aliquot of MP (mass calculated to be sufficient for 5 animals + 20% overage) was incubated with CF660C-labeled OVA in 2R lyophilisation vials by adding 0.6 mL of OVA-CF660C solution (concentration: 0.45 mg/mL) for verum samples or 0.6 mL PBS (pH 7.4) for placebo samples. Depending on the MP species, OVA in PBS solution was adjusted to either pH 7.4 (*R*-DOTAP MP) or to pH 2–3 (DOSS & PVA MP) to account for the distinct particle surface charges. With OVA having an isoelectric point of pI ~ 5 (Winzor, 2004), this corresponds to a negatively charged protein used during incubation of positively charged *R*-DOTAP-MP and correspondingly in a positively charged protein for the negatively charged DOSS- and almost neutral PVA-MP. MPs were incubated at room temperature under light exclusion by aluminum foil for 90 min on a vortexer using gentle agitation.

Particles were transferred to brown centrifuge caps (2 mL) and washed 2× with 500 µL MilliQ (2 min centrifugation time, 6440 RCF).