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Note

Characterization of protein-adjuvant coencapsulation in microparticles for vaccine delivery

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ABSTRACT

Protein antigens encapsulated as vaccines in poly[(*rac*-lactide)-*co*-glycolide] (PLGA) microparticle carriers can induce immune responses. The intensity and directions of this response can be controlled by coloading the microparticles with immunomodulatory adjuvants, e.g., muramyl dipeptide (MDP) as adjuvant combined with ovalbumin (Ova) as protein antigen. In this study, methodologies for an individual quantification of both encapsulated substances should be reported, which comprise (i) a separation process to isolate and determine MDP as intact molecule and (ii) a simultaneous degradation of both analytes with subsequent specific quantification of Ova fragments. It was shown that coloading of both substances resulted in a substantially reduced encapsulation efficiency of MDP. This illustrates that correct conclusions on dose–response relationships in future vaccination studies can only be drawn, if a selective method for adjuvant and protein quantification will be applied.

1. Introduction

Degradable polymeric microparticles of phagocytizable size (<10 μm) have been explored as carrier systems for antigenic vaccine components. In most cases, increased immune responses compared to soluble antigen were observed, which may be partly attributed to the enhanced engulfment of these carriers by immune cells due to their resemblance to microorganisms in terms of size [1]. Furthermore, it was indicated that microparticles can modulate antigen presentation pathways of exogenous antigens towards a cross-presentation [2]. For further enhancing the immunogenic potency of such particles, immunomodulatory substances (adjuvants) can be coencapsulated with the antigenic proteins to elicit superior immune responses [2–4].

The specific combination of adjuvant/antigen in mixture finally present in the microparticles may crucially affect the cell activation status due to mutual interference and enhancement of responses. Therefore, it will be essential to quantify the final microparticle payload properly. Generally, the coencapsulation of the different types of molecules could occur at different efficiencies in case of different solubilities, hydrophilicities, and hydrodynamic radii. The capability to quantify the protein and the coloaded adjuvant independently depends strongly on the nature of the coencapsulated compounds. In the past, most of the coloaded vaccine microparticles contained nucleotide-derived adjuvants [2,3], which are chemically different from proteins and may be detected without interference. Recently, polymer microparticles encapsulating peptidoglycan-based agonists of nucleotide-binding oligomerization domain (NOD)-like receptors were reported, which illustrated their capacity to induce immunoactivation and thus should be combined with protein antigens in the future [5]. However, as peptidoglycans, NOD receptor agonists such as *N*-acetylmuramyl–*L*-alanyl–*D*-isoglutamine (muramyl dipeptide, MDP), might be co-detected by common quantification methods of protein antigens such as the BCA assay. Since the BCA assay involves the formation of a complex between peptide bonds and cupric ions in alkaline conditions [6], it may be sensitive to all peptide bonds irrespective of their origin from proteins or peptidoglycans.

The scope of this work was to identify methodologies that would allow an accurate, precise, and independent quantitative detection of the protein-adjuvant payload of microparticles and their individual entrapment efficiencies. Due to the small molecular weight of MDP (494 g mol^{-1}) compared to proteins, it was expected that this goal may be achieved by separation of the

Abbreviations: BCA, bicinchoninic acid; MDP, muramyl dipeptide, *N*-acetylmuramyl–*L*-alanyl–*D*-isoglutamine; MP, microparticles; NOD, nucleotide-binding oligomerization domain; Ova, ovalbumin; PLGA, poly[(*rac*-lactide)-*co*-gylcolide]; PVA, poly(vinyl alcohol).

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coloaded components prior to analysis. Poly[(*rac*-lactide)-*co*-glycolide] (PLGA) was selected as particle matrix based on its well-established use as pharmaceutical carrier system and to allow comparability with numerous experimental vaccine delivery studies based on PLGA carrier [2–5,7]. Ovalbumin with an average molecular weight of 45 kDa (45,000 g mol^{-1}) and a hydrodynamic radius of 3 nm served as a model antigen. This selection was based on the availability of ovalbumin (Ova) sensitized animal models, making it particularly interesting for future mechanistic immunological studies.

2. Materials and methods

2.1. Materials

PLGA (50 mol.% glycolide, carboxyl end groups, number average molecular weight M_n = 5 kDa, polydispersity PD = 3.2; Resomer® RG 503H) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) [Mowiol 4-88] (PVA) was from Kuraray Europe GmbH (Frankfurt, Germany), MDP was procured from Invivogen (San Diego, CA, USA), and Endograde Ovalbumin was from Hyglos GmbH (Bernried, Germany). The bicinchoninic acid (BCA) assay kit was purchased from Sigma–Aldrich (Taufkirchen, Germany). All other chemicals including HPLC solvent were of analytical grade.

2.2. Preparation and characterization of microparticle size and surface morphology

Microparticles (MP) were prepared in a biological safety cabinet under laminar air flow by the water-in-oil-in-water (w/o/w) double emulsion/solvent evaporation method as reported before [5]. Briefly, 75 μL 0.1% (*w*/*v*) MDP and/or 1.3% (*w*/*v*) Ova in water containing 1% (*w*/*v*) sodium bicarbonate and 5% (*w*/*v*) sucrose were added to a 19 wt.% PLGA solution in dichloromethane and emulsified by sonication. This emulsion was then added to 2% (*w*/*v*) PVA solution, homogenized by rotor–stator homogenization, and further treated as reported [5].

Particle size analysis was performed by laser diffraction using a Mastersizer 2000 (Hydro 2000S dispersion unit, Malvern Instruments, Herrenberg, Germany). The morphology of lyophilized microparticles was studied with a Gemini Supra™ 40 VP SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) without sputtering to avoid artefacts.

2.3. Determination of encapsulation efficiency

The MDP content of microparticles was determined after polymer extraction (3×) from 10 mg samples with 1 mL acetonitrile, dissolution of the pellet after centrifugation in 10 mg mL^{-1} aqueous SDS solution, and separation by Amicon Ultra-0.5 mL centrifugal filters (Millipore GmbH, Schwalbach am Taunus, Germany) at 14,000 g (Heraeus Biofuge Primo R, Hanau, Germany). MDP was quantified by HPLC using a Lichrosphere 100 RP 18 5 μm column (250 × 4 mm) with UV detection at 240 nm and 0.01 M phosphate buffer pH 3/methanol (98/2, *v*/*v*) as eluents (25 °C, 1.5 mL min^{-1}) [5].

The encapsulated protein was preferentially quantified by amino acid analysis. Briefly, 10 mg of microparticles and encapsulated protein were treated with 1 mL of 7.5 N NaOH at 106 °C for 12 h (PMC block heater, Germany) for basic hydrolysis. Subsequently, samples were neutralized and subjected to HPLC analysis on a C_{18} column (EC 250/4 Nucleosil 100–5 C_{18} HD) with pre-column derivatization using 50 μL sample and 50 μL o-phthaldialdehyde (10 mg mL^{-1} in methanol/ borate buffer (90/10, *v*/*v*)). Acetonitrile/water (80/20, *v*/*v*) was used as mobile phase (35 °C, 1 mL min^{-1}) with fluorescence detection (ex. 335 nm, em. 450 nm). Alternatively, the Ova content of only Ova loaded microparticles could be determined after polymer extraction by the BCA assay performed on microtiter plates (Carl Roth GmbH, Karlsruhe, Germany).

2.4. Determination of osmolality

The osmolality of the different w_1 phases was determined by freezing point depression using Knauer Semi-Micro Osmometer A-0300 (Knauer GmbH, Berlin, Germany).

3. Results and discussion

Microparticles were prepared using the water-in-oil-in-water (w_1/o/w_2) emulsion/solvent evaporation technique. Ova and MDP were coencapsulated by their dissolution in the w_1 phase, which also contained additives such as sodium bicarbonate to neutralize acidic polymer degradation products and sucrose as lyoprotectant [8]. The obtained microparticles, as exemplarily shown for coloaded microparticles (Ova−MDP MP) (Fig. 1A), showed a narrow particle size distribution in all cases with d(0.5) particle sizes below 10 μm (Table 1).

As illustrated in Fig. 1B, the microparticles were of spherical shape and exhibited small open pores. The pore formation can be assigned to the hydrophilic substances in the w_1 phase, which built up osmotic pressure in the nascent particles as indicated by the determined osmolality of the w_1 solutions (Table 1). For Ova, surface active properties in emulsions are known [9]. It may be speculated on whether its leaching to the w_2-phase, particularly in combination with MDP, supported the stabilization of the o/w_2 interphases in addition to PVA employed as steric stabilizer.

During the particle preparation process, not only the o-phase solvent but also the desired payload from the w_1 phase may partially diffuse to the external aqueous phase. This is supported by an influx of water into the polymer matrix due to the osmolality differences in the w_1-phase and w_2-phase.

The encapsulation efficiency of microparticles loaded with MDP only (MDP MP) was determined by (i) removing PLGA by repeatedly extracting the samples with acetonitrile, (ii) dissolving MDP into an aqueous medium, and (iii) quantifying MDP by reverse phase HPLC. Although being a small hydrophilic molecule, MDP was successfully loaded in the microparticles with 70 wt.% efficiency. Similarly, microparticles loaded only with Ova (Ova MP) could be characterized after similar sample pretreatment (polymer extraction, protein dissolution in aqueous medium) followed by protein quantification with the BCA assay method. Ova loaded microparticles showed high encapsulation efficiencies of 94 wt.%.

In principle, the individual encapsulation efficiencies may be altered for coloaded systems due to the increased osmotic pressure and different hydrodynamic radii of the two encapsulated substances. For coloaded particles, the previously applied standard strategy with PLGA extraction and direct analysis of Ova and MDP could not be followed, because: (i) Ova as a larger molecule would block HPLC columns suitable for MDP quantification, thus prohibiting the analysis of MDP + OVA mixtures, and (ii) MDP with its dipeptide segment interfered with the Ova quantification by the BCA assay as identified by a standard addition experiment (Fig. 2A). Therefore, a separation of the two hydrophilic molecules should be applied before individual analysis.

Based on the substantial differences in the number of amino acids in the protein and adjuvant (Ova: 385; MDP: 2), a selective precipitation behaviour using trichloroacetic acid was assumed. However, despite successful removal of the protein and subsequent qualitative detection of MDP in the supernatant, the peak