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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*-gylcolide] (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.

Keywords: amino acid analysis, co-encapsulation, microparticles, ovalbumin, poly[(*rac*-lactide)-*co*-gylcolide]

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 theses 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 mixtures 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 strongly depends 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 receptors sensing nucleotide and oligomerization domains (NOD) 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 agonists such as *N*-acetylmuramyl–*L*-alanyl–*D*-isoglutamine (muramyl dipeptide, MDP), might be co-detected during common protein quantification methods such as the BCA assay. Since the BCA assay involves the formation of a complex between peptide bond 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 $g \cdot mol^{-1}$) compared to proteins, it was expected that this goal may be achieved by separation of the coloaded components prior to analysis. Poly[(*rac*-lactide)-*co*-gylcolide] (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 $g \cdot mol^{-1}$) 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.

Materials and Methods

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.

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^{TM} 40 VP SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) without sputtering to avoid artefacts.

Determination of encapsulation efficiency

The MDP content of microparticles was determined after polymer extraction (3x) from 10 mg samples with 1 mL acetonitrile, dissolution of the pellet after centrifugation in 10 $mg \cdot mL^{-1}$ 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 x 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 $mL \cdot min^{-1}$) [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 $mg \cdot mL^{-1}$ in methanol/ borate buffer (90/10 v/v)). Acetonitrile/water (80/20 v/v) were used as mobile phase (35 °C, 1 $mL \cdot min^{-1}$) 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).

Determination of osmolality

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

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 Figure 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 build 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.

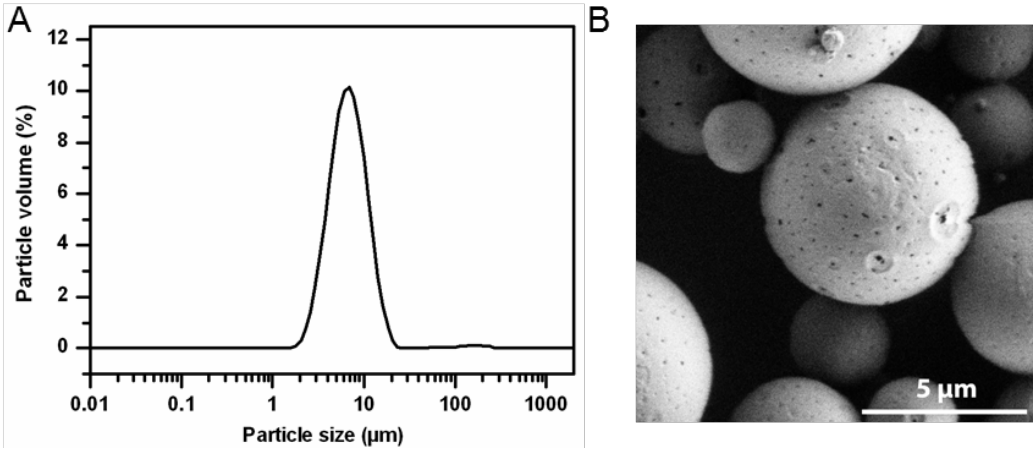

Figure 1: (A) Size distribution and (B) morphology of particles coencapsulating MDP and Ova.

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 of 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.%.

Table 1: Characteristics of different microparticle (MP) compositions.

Sample ID	Content	Particle size[b)]		w_1-phase osmolality	Theoretical payload	Encapsulation efficiency
		d(0.5)	Span			
	-	[µm]		[mOsm·Kg^{-1}]	[µg·mg^{-1}]	[wt.%]
MDP MP[a)]	Muramyl dipeptide	5.5	1.6	578	3	70 ± 1
Ova MP	Ova	6.7	1.1	614	40	94 ± 3
Ova–MDP MP	Ova MDP	4.2	1.6	720	40 3	95 ± 3 48 ± 4

[a)] Data reproduced from [5], Copyright 2012, with permission from Elsevier.
[b)] Data derived from an average of three measurements

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 (**Figure 2A**). Therefore, a separation of the two hydrophilic molecules should be applied before individual analysis.

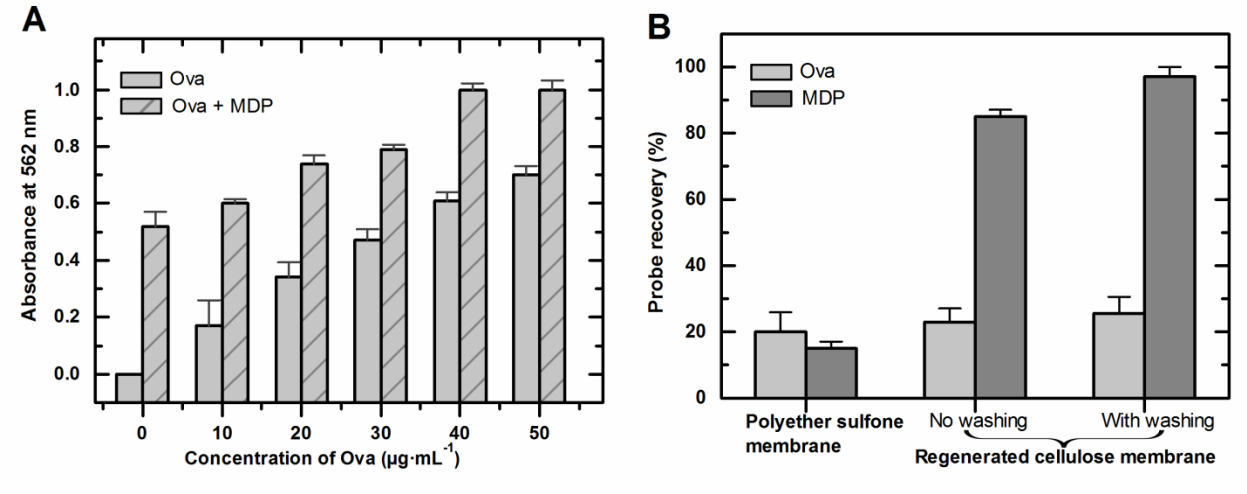

Figure 2: Evaluation of experimental methods and their shortcomings in the quantification of MDP and Ova. (A) Interference of MDP (20 µg·mL^{-1}) in the Ova determination by the BCA assay. (B) Effect of absorption on filter membranes on the individual recovery of Ova (250 µg·mL^{-1}; BCA assay) and MDP (25 µg·mL^{-1}; HPLC) standards subjected to size exclusion centrifugation for their separation.

Based on the substantial differences in the number of amino acids in the protein and adjuvant (Ova: 385; MDP: 2), a selective precipitation behavior using trichloroacetic acid was assumed. However, despite successful removal of the protein and subsequent qualitative detection of MDP in the supernatant, the peak of the α-anomer of MDP (see HPLC chromatogram, inset of **Figure 3**) could surprisingly not be detected by HPLC. This suggests that MDP may not have quantitatively remained in solution.

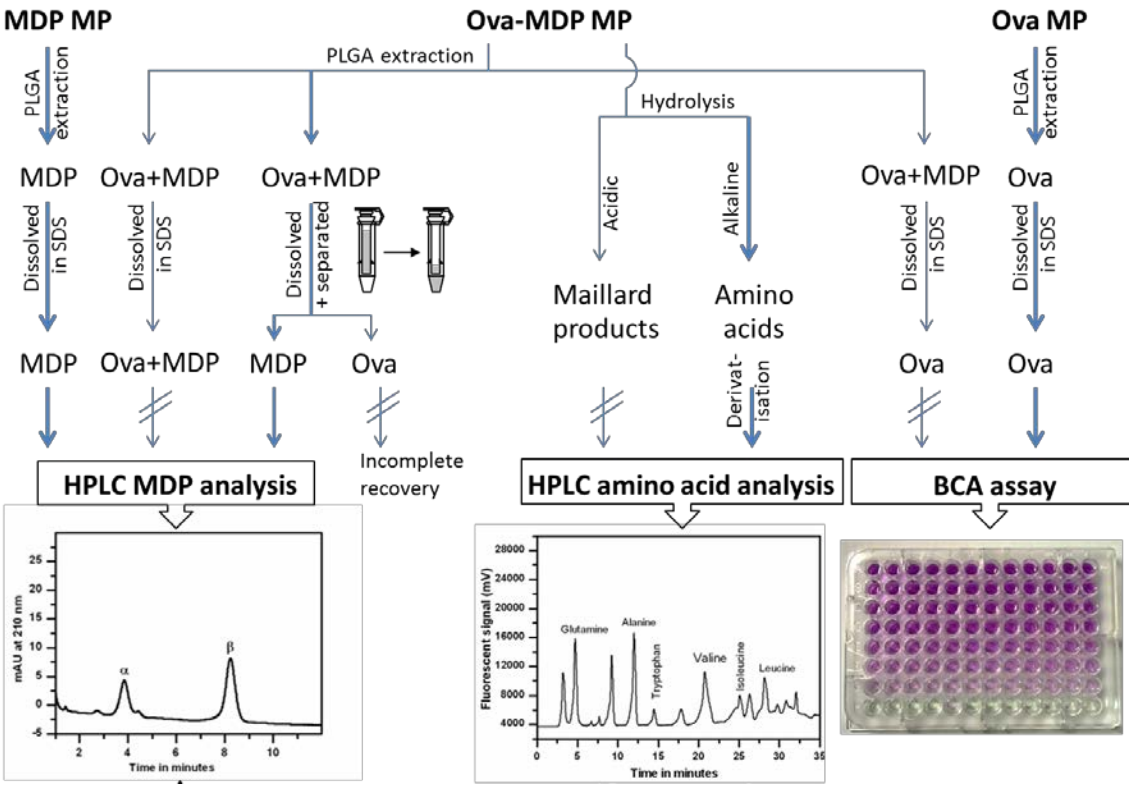

Figure 3: Scheme presenting the different analytical strategies followed for the quantification of the single and coencapsulated components.

As an alternative approach, the substantial differences of hydrodynamic radii of the analytes should be used to separate MDP from Ova by ultra-filtration. For this purpose, centrifugal filters with different types of membrane materials were explored. The centrifugal filter equipped with polyethersulfone membranes showed considerable adsorption of MDP, which was avoided with a subsequently employed low-binding regenerated cellulose membrane having a molecular weight cut-off of 3 kDa. Importantly, adsorption was reduced by first washing and hydrating the filters several times with water (**Figure 2B**). This methodology was found useful in case of MDP, which could be quantitatively recovered in control experiments with standard solutions.

By applying this method, the MDP encapsulation efficiency in Ova−MDP loaded particles was identified to be 48 wt.%, which is low compared to the MDP loaded particles (**Table 1**).

In contrast to MDP, Ova could not be quantitatively recovered in all cases and apparently remained partially adsorbed inside the centrifugal filter tubes, which may possibly assigned to its relatively higher hydrophobicity. Accordingly, the low encapsulation efficiency of Ova determined by this procedure (26 ± 5 wt.%) is erroneous and an artifact resulting from the unsuitable separation process.

Therefore, a method for Ova detection was required which would not be disturbed by the presence of MDP. In principle, antibody-based assays would be suitable, but always depend on the availability of the respective protein-specific antibody. Therefore, amino acid analysis was selected for Ova quantification since it may be widely applicable to different proteins. This method should be applied after hydrolysis of the entire microparticles rather than the multi-step extraction/separation of the protein. The resulting mixture of PLGA degradation products and amino acids should be quantified by HPLC analysis, where each amino acid in the mixture can be separated and quantified individually. In this way, alanine and isoglutamine partially originating from MDP can be separated from other amino acids and excluded in data evaluation for Ova quantification.

Acidic hydrolysis, especially using aqueous hydrochloric acid, is known as preferable method for peptide bond cleavage since it ensures complete hydrolysis of proteins with less destruction of amino acids [10]. However, when particles were subjected to acidic hydrolysis using 6 N HCl at 115 ºC for 24 hours, a brown discoloration of the hydrolysate was observed. In contrast, this was not observed for standards of the pure protein subjected to the same procedure. Apparently, Maillard products were formed by reaction of amino acids with reducing sugars. The same

products were observed when spiking blank microparticles with soluble ovalbumin, but not for pure PLGA with Ova. This observation suggested that sucrose used as lyoprotectant was converted under strong acidic conditions to glucose and fructose, which then can react with amino acids. The contribution of MDP with its *N*-acetylmuramyl moiety in this reaction was considered to be negligible.

Since glycosidic bonds should be more stable in basic conditions, the alkaline hydrolysis of the loaded PLGA particles in 7.5 N aqueous NaOH solution at 106 ºC for 12 h was evaluated. Under these conditions, the microparticles and Ova was completely hydrolyzed without interference by the Maillard reaction, thus allowing subsequent amino acid analysis for Ova quantification. The encapsulation efficiency of Ova in coloaded microparticles very well agreed with that of the particles encapsulating only Ova and showed high values of > 90 wt.% (Table 1).

Overall, it can be concluded that the presence of MDP as small molecule did not affect the encapsulation process of Ova as a large protein in the investigated particles. In contrast, the MDP encapsulation efficiency was diminished in coloaded microparticles. This may probably be due to its much smaller hydrodynamic radius, which makes it more prone to osmotic pressure mediated leakage during preparation such as through water-rich domains acting as diffusion channels. Ova and MDP did not appear to exhibit strong physical interactions, which could promote MDP entrapment in the polymer matrix. Importantly, the substantial differences of theoretical and final payload observed particularly for the immunostimulating adjuvant illustrated that correct conclusions on dose-response relationships in future vaccination studies demand the application of selective methods for adjuvant and protein quantification. In case of MDP and Ova studied in here, an accurate, precise and independent quantitative detection was possible by combining i) a separation process to isolate and determine MDP as intact molecule

and ii) a procedure of simultaneous fragmentation of both analytes with subsequent specific quantification of Ova.

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