International Journal of Pharmaceutics 466 (2014) 139–146

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Factors affecting alum–protein interactions

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ARTICLE INFO

Article history:
Received 13 November 2013
Received in revised form 18 February 2014
Accepted 2 March 2014
Available online 5 March 2014

Keywords:
Alum
Adjuvant
Protein
Adsorption
Desorption
Formulation

ABSTRACT

Alum (or aluminum-containing) adjuvants are key components of many vaccines currently on the market. The immuno-potentiation effect of alum adjuvants is presumably due to their interaction with antigens, leading to adsorption on the alum particle surface. Understanding the mechanism of antigen adsorption/desorption and its influencing factors could provide guidance on formulation design and ensure proper in-vivo immuno-potentiation effect. In this paper, surface adsorption of several model proteins on two types of aluminum adjuvants (Alhydrogel® and Adjuphos®) are investigated to understand the underlying adsorption mechanisms, capacities, and potential influencing factors. It was found that electrostatic interactions are the major driving force for surface adsorption of all the model proteins except ovalbumin. Alhydrogel has a significantly higher adsorption capacity than Adjuphos. Several factors significantly change the adsorption capacity of both Alhydrogel and Adjuphos, including molecular weight of protein antigens, sodium chloride, phosphate buffer, denaturing agents, and size of aluminum particles. These important factors need to be carefully considered in the design of an effective protein antigen-based vaccine.

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1. Introduction

Aluminum-containing compounds was first discovered to have adjuvant activity in 1926 when an alum-precipitated diphtheria vaccine showed better antigenic properties than the standard diphtheria vaccine (Glenny et al., 1926). Since then, aluminum-containing compounds have been used as effective adjuvants both in human and veterinary vaccines. In fact, due to their good record of safety, low cost and good adjuvanticity with various types of antigens, it has been the most commonly used vaccine adjuvant (Aprile and Wardlaw 1966; Gupta 1998; Kool et al., 2012). On the other hand, recent studies revealed the possible linkage between certain adverse effects to the use of aluminum in vaccines (Shaw and Petrik, 2009; Tomljenovic and Shaw, 2012). Therefore, optimization of aluminum adjuvant type and quantity in the design of an effective and safe vaccine is still a necessity (Clapp et al., 2011; Vecchi et al., 2012).

Even with the long history of use, the mechanism of aluminum-induced immune-potentiation is not completely understood. Several possible mechanisms have been demonstrated and/or proposed based on a number of studies. These include prolonged retention of antigens at the injection site, enhanced recruitment of dendritic cells, more efficient uptake of adsorbed antigens by dendritic cells at the injection site and promotion of a local pro-inflammatory environment (Iyer et al., 2003a,b; Morefield et al., 2005b; Hem and Hogenesch 2007; Tritto et al., 2009). Some of these mechanisms are clearly linked to antigen adsorption on aluminum adjuvants. Thus, it has been generally accepted that antigen should be largely adsorbed by the aluminum adjuvants to be effective. Likely for this reason, the World Health Organization (1976) recommends adsorption of 80% or more of tetanus and diphtheria toxoids to aluminum adjuvants. More recent studies suggest that the degree of adsorption of antigen in interstitial fluids rather than in the vaccine product correlated with the immune response, due to the fact that the degree of antigen adsorption to an aluminium adjuvant may change after its administration in vivo (Chang et al., 2001). Therefore, it appears that the antigen binding to an aluminum adjuvant in interstitial fluid determines the immune response in mice after subcutaneous injection (Iyer et al., 2003b).

Nonetheless, antigen adsorption on the surface of aluminum adjuvants in-vitro has been a key consideration in the design of a vaccine product. Antigen adsorption can take place generally by one or two major mechanisms, that have been demonstrated for many antigens – electrostatic interaction (attraction) and ligand exchange. While electrostatic attraction requires presence of

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http://dx.doi.org/10.1016/j.ijpharm.2014.03.015
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Table 1
Molecular properties of the model proteins used in this study.

Protein	Lysozyme	hGH	Ovalbumin	DT	IgG	PEG-hGH
Molecular Weight (kD)	14	22	45	62	150	62
pI	11.0	4.9	4.6	5.2	8.0	4.4

opposite charges on the antigen and aluminum adjuvants under a particular solution condition, ligand exchange requires presence of phosphorus in antigens, exchanging with the hydroxyl group in aluminum adjuvants. Many factors have been shown to influence antigen adsorption, and potentially vaccine efficacy. These include solution pH (Rinella et al., 1998; Burrell et al., 2000; Wolff et al., 2009), presence of salt, if electrostatic interaction is the main adsorption mechanism, (Wolff et al., 2009), presence of phosphate in solution (Iyer et al., 2003a,b; Jendrek et al., 2003), and consistency in aluminum adjuvant quality during the manufacturing process (Yau et al., 2006). Understanding these and other factors potentially affecting the interaction between antigens and aluminum adjuvants is critical for the design of an effective and safe aluminum-based vaccines. Therefore, this study was carried out to evaluate the adsorption capacity of several model proteins – ovalbumin (OVA), lysozyme (LYS), human growth hormone (hGH), PEGylated growth hormone (PEG-hGH), diphtheroid toxoid (DT), and a monoclonal antibody (IgG). These model proteins were chosen to cover different molecular sizes and pI's (Table 1), Several product formulation-related factors, which potentially affect the adsorption capacity of these model proteins, were examined, including use of a phosphate, a commonly-used buffering agent, sodium chloride, a tonicity/ionic strength modifier, and PEG, a potential protein stabilizer. Two other factors, use of a denaturing agent and size of aluminum particles were also examined to understand the protein adsorption strength and capacity.

2. Material and methods

2.1. Materials

Materials—Aluminum hydroxide adjuvant (Al(O)(OH); Alhydrogel® 2% and Alhydrogel® '85') and aluminum phosphate adjuvant ($AlPO_4$; Adjuphos®) were purchased from Accurate Chemical and Scientific Corporation (Westbury, NY) and the manufacturer is Brenntag, Denmark. Ovalbumin (OVA) and lysozyme (LYS) were purchased from Sigma (St Louis, MO) and used without further purification. Human growth hormone (hGH), PEGylated growth hormone (PEG-hGH), diphtheroid toxoid (DT), and IgG were all produced and purified in house within Pfizer. Linear PEG 35 kDa was purchased from Sigma–Aldrich. All other chemicals were of analytical reagent grade purity. Distilled deionized water was used for the preparation of all solutions.

Protein stock preparation—Protein stock solutions were prepared in either 20 mM phosphate or histidine buffer (pH 6.5). Protein concentration was determined using an established extinction coefficient and UV absorption at 280 nm using Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE).

Protein adsorption—A series of alum–protein solutions were prepared at room temperature by mixing different amounts of protein stock solution, the respective stock buffer (20 mM histidine buffer, pH 6.5 or 20 mM phosphate buffer, pH 6.5) and aluminum stock. The final aluminum (Al) concentration was 1.0 mg/ml and protein concentration was varied from 0 to less or equal to 5 mg/ml. Al content of the original aluminum stock was provided by the manufacturer's specification sheet and was used to calculate the final Al concentration. Proteins and aluminum in the presence of selected buffer was gently mixed by end-over-end rotation at room temperature for around 2 h. The samples were centrifuged for 10 min at 10,000 rpm. The supernatant of the sample was determined for protein concentration by measuring the absorbance at 280 nm using Nanodrop UV spectrometer (Thermo Scientific, Wilmington, DE). The amount of protein adsorbed on alum was determined by subtracting the amount of protein in the supernatant from the initial input.

Point of zero charge (or isoelectric point, pI)—The point of zero charge (or isoelectric point, pI) of alum and protein were measured by Malvern Zetasizer Nano ZS™ equipped with an autotitrator (MPT-1) attachment. Briefly, aluminum (1.0 mg/ml Al content) or protein (1.0 mg/ml) diluted in water were titrated by 0.25 and 2.5 M sodium hydroxide or HCl in the range of pH 4–10. Zeta potential was measured every step of 0.2 pH unit change using disposable capillary cells at a temperature of 25 °C. The zeta potential was calculated from the measured electrophoretic mobilities using the Smoluchowski approximation (Malvern.com, 2014). The pH, where the measured zeta potential is 0, is considered as pI of proteins or point of zero charge of aluminum adjuvant.

Protein phosphorylation measurements—The extent of protein phosphorylation was measured using the commercial Phosphoprotein Phosphate Estimation Kit from Pierce Biotechnology (IL, USA).

Particle size measurements—Alum adjuvant particles size was measured by dynamic light scattering using Malvern Zetasizer Nano ZS™ at 25 °C. The instrument is operated at a wavelength of 633 nm and the scattered light is detected at an angle of 173°. Alum was diluted to 1.0 mg/ml and particle size was measured using 1 ml clear disposable cuvette. Three measurements were averaged to obtain the mean particle size.

Inductively coupled plasma mass spectroscopy (ICP-MS)—Half milliliter of Alhydrogel was diluted with 4.5 ml of buffer at different phosphate strengths; and was gently mixed for a predetermined incubation time. For the phosphate exchange kinetic study, the suspension in 10 mM phosphate was gently mixed for 5 min, 30 min, 2 h, 8 h, 24 h and 48 h. The samples were centrifuged for 10 min at 10,000 rpm. The supernatant was then removed. The pellet was resuspended and washed with 10 ml of water before spinning at 10,000 rpm for 10 min. The supernatant was removed. The pellet was then dissolved in nitric acid and analyzed by ICP-MS.

3. Results

In this study, we chose several model proteins to evaluate their interaction with two major alum adjuvants – alumimum hydroxide (Alhydrogel) and aluminum phosphate (Adjuphos). The model proteins, covering a wide range of molecular weights, include lysozyme (14 kD), hGH (22 kD), ovalbumin (45 kD), PEG-hGH, DT (62 kD), and IgG (150 kD). Their respective pI's and PZC of the alum adjuvants were determined by zeta potential titration to understand their charge at a particular solution pH. The results are shown in Fig. 1 and Table 1 (protein results only). Alhydrogel has a pI of 9.4 while Adjuphos has a pI of about 4.3. The model protein antigens hGH, ovalbumin and DT all showed similar pI's in the range of 4–5, while lysozyme and IgG had a pI of about 11 and 8, respectively. It is expected that hGH, ovalbumin, and DT are negatively charged and lysozyme and IgG are positively charged at pH 6.5. Based on these results, a series of protein adsorption studies were conducted at pH 6.5, a weakly acidic condition suitable for stability of many proteins.

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