PREPARATION AND CHARACTERIZATION OF NANOSTRUCTURED FERRIC HYDROXYPHOSPHATE ADJUVANTS

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Abstract: *This article describes part of the results obtained during the development of a new generation of vaccine adjuvants based on nanostructured hydroxyphosphates of tunable composition and physicochemical characteristics. Colloidal gels of ferric hydroxyphosphates of various iron/phosphate ratios were prepared by precipitation techniques, sterilized by autoclaving and analyzed by transmission electron microscopy (TEM) and dark-field optical microscopy. The obtained materials were composed of a network of amorphous nanoparticles (<20 nm in size) that were aggregated into micron-sized structures in physiological saline. Preliminary adsorption experiments indicated the ability of the obtained materials to adsorb protein substances, which is an important prerequisite for their potential application as vaccine adjuvants and further optimization of the production process to achieve reproducibility of the physicochemical characteristics.*

Keywords: ADJUVANT, IRON(III), HYDROXYPHOSPHATE, MORPHOLOGY, ADSORPTION

1. Introduction

Iron is vital for the majority of organisms by participating in the structure of many different enzymes (catalase, lipoxygenases, various oxidoreductases, etc.) and in metabolic reactions, including electron transfer, processes of transport, storage and use of oxygen [1]. Its importance for health had been recognized by the ancient inhabitants of the Balkan region, who used iron-containing red stones (the so-called *Argilla rubra*) prescribed to weak and anemic people [2]. Nowadays, nanosized colloidal dosage forms of ferric hydroxide have found clinical application as formulations for parenteral administration in the treatment of severe iron-deficiency anemia [3-5].

Interestingly, injectable suspensions of the sparingly soluble ferric hydroxide and ferric phosphate have been found to potentiate the immune response against protein antigens and therefore have been proposed for vaccine adjuvant use. The preparation of ferric-based adjuvants and their use in adjuvanted vaccines have been described mostly in patents [6-8] and rarely in scientific articles [9]. It has been found that colloidal iron hydroxide behaved comparably to aluminium hydroxide with respect to supporting induction of an antibody response to tetanus toxoid and also induced long-lasting antibody responses, which protected animals from tick-borne encephalitis virus (TBEV) infection even one year after vaccination [9]. It should be noted that the use of colloidal iron hydroxide as adjuvant had the additional advantage to reproducibly support induction of HIV-1 envelope-specific cytotoxic T lymphocytes (CTL), when used as an adjuvant for a HIV-1 env-carrying recombinant fowlpox virus and being applied via the subcutaneous route, while aluminium hydroxide was much less active in this respect [9]. The ferric phosphate has also been demonstrated to be a good adjuvant; as regards the IgG1, the results obtained have been clearly superior to those obtained when the antigen was administered alone, even though the results were not quite as good as those obtained with aluminium hydroxide; as regards the IgG2, the titers obtained were as high as those obtained with aluminium hydroxide [8]. Also, it has been found that ferric phosphate is a good adjuvant for tetanus toxoid, clearly better than ferric hydroxide under the same conditions [8].

Previous studies on aluminium hydroxyphosphate adjuvants have demonstrated that the metal/phosphate molar ratio has a significant effect on some physicochemical properties [10,11], while similar studies on their ferric-based analogues could not be found in the available literature. Here, we present our research on the preparation of ferric-based hydroxyphosphates of variable iron/phosphate molar ratio as potential candidates for adjuvant use. We studied the effects of the iron/phosphate molar ratio on the ultrastructural morphology and the formation of micron-sized aggregates in physiological saline solution. Preliminary experiments on the electrokinetic properties and protein adsorption were also performed.

2. Materials and Methods

2.1. Reagents

For the preparation of ferric hydroxide and the various ferric hydroxyphosphates, we used iron(III) chloride-6-hydrate (>99%), sodium hydroxide (>98%) and sodium phosphate tribasic dodecahydrate (>98%), purchased from Sigma-Aldrich, Germany.

2.2. Preparation of adjuvant gels

Ferric chloride-6-hydrate (1.45 mmol; 390 mg) was dissolved in distilled water (3.5 ml) and diluted with 20 ml of distilled water. Then, a solution of sodium phosphate tribasic dodecahydrate (1.45 mmol; 550 mg) in distilled water (3.5 ml) was added dropwise with stirring (600 rpm) to prepare the $FePO_4$ dispersion; for preparation of the $Fe(OH)_3$ dispersion, NaOH (4.35 mmol; 175 mg) was used instead of sodium phosphate. Three different hydroxyphosphates were also prepared with initial Fe/P molar ratio 100/75, 100/50 and 101 100/25 by keeping the total amount of iron equal to 1.45 mmol. The hydroxyphosphates were precipitated from the ferric chloride solution by using a mixed solution of sodium hydroxide and sodium phosphate with amounts of regents calculated to obtain the expected composition. For example, in order to obtain the composition $FePO_4.Fe(OH)_3$ (Fe/P = 100/50), 87 mg (2.175 mmol) of NaOH and 275 mg (0.725 mmol) of $Na_3PO_4.12H_2O$, dissolved in 3.5 ml of distilled water, were used. The pH of dispersions should not exceed 7. The precipitates formed were stirrered for 5 minutes at room temperature and were autoclaved for 30 min (121 °C).

2.3. Physicochemical characterization

The particle morphology and ultra-structure were observed by transmission electron microscope JEM-2100 (JEOL) at acceleration voltage of 200 kV, equipped with a micro-analyzer X-Max 80T (Oxford Instruments). Micron-sized aggregates in the gels were visualized directly in 0.9% NaCl by using an optical microscope (Optika B-180, Italy) with a dark-field condenser.

3. Results and discussion

3.1. Physicochemical properties

The ultrastructural morphology of the obtained ferric phosphates prepared by using different initial Fe/P (iron/phosphate) molar ratios showed a network of nanoparticles of average sizes up to about 20 nm that were aggregated into micro-sized structures (up to about 30 μm), as seen from the transmission electron images shown in Fig. 1.

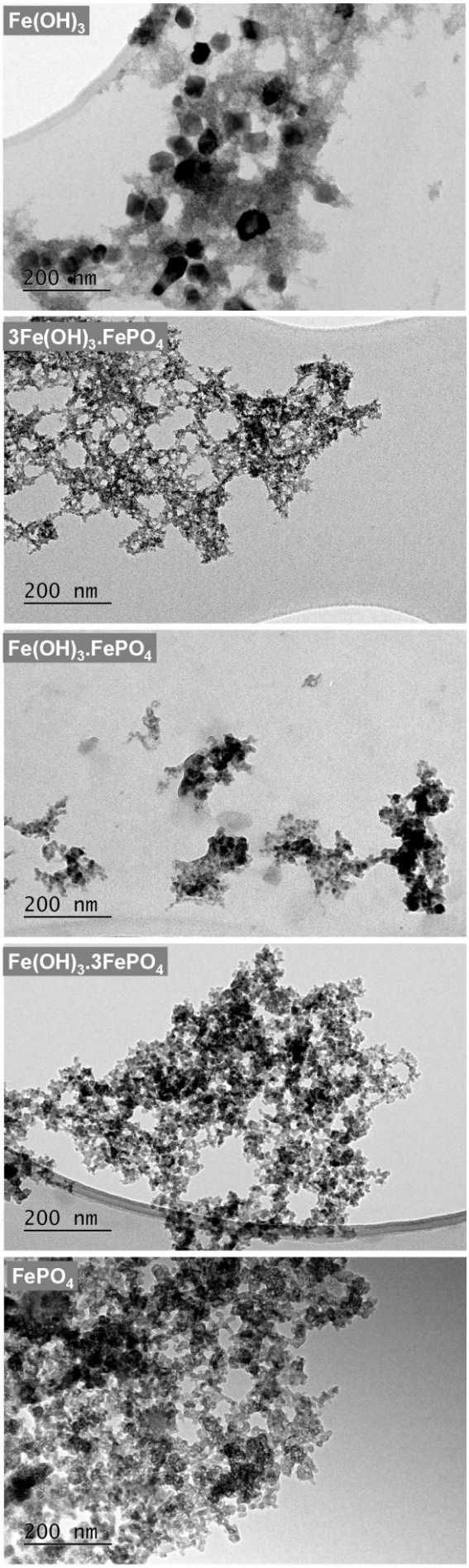

***Fig. 1**. TEM images at magnification of x25k of $Fe(OH)_3$, $FePO_4$ and different ferric hydroxyphosphates (given in the figure legends).*

This morphology was very similar to that of aluminium phosphate currently used in human vaccinations, although the primary nanoparticles of aluminium phosphate are larger, about 20-50 nm in size [12,13]. The increasing hydroxide/phosphate ratio (that corresponds also to increased Fe/P ratio) resulted in the formation of ferric hydroxyphosphates with even smaller primary nanoparticles of sizes <20 nm. These primary nanoparticles were aggregated into clusters via "bridges" of amorphous material.

Analysis by energy dispersive spectrometry (EDS) showed the presence of iron, phosphorous and oxygen. A representative scanning TEM (STEM) image and maps of element (Fe, P and O) distribution in the ferric hydroxyphosphate with an initial molar ratio of Fe/P = 100/50 is shown in Fig. 2. The signals for the elements Fe, P and O are localized in the same areas, indicating the formation of hydroxyphosphate particles (but not separate hydroxide and phosphate particles). Data from quantitative EDS measurements of the Fe/P/O ratio in the obtained materials showed that it was close to that was used in their preparation. However, these values should be interpreted with care, because we found that they may depend on the time for count accumulation probably as a result of changes in the material upon interaction with the electron beam of the microscope. The morphology of the sample can also change upon longer observation times. It should be noted that the interaction with the electron beam can result in heating of the observed sample, dehydration and evaporation of material thus affecting both the morphology and the elemental composition. For that reason, TEM images (Fig. 1) were taken with minimum time of exposure of the material to the electron beam (within less than few minutes).

The electron diffraction analysis showed that the obtained hydroxyphosphates with different hydroxide/phosphate ratio were structurally amorphous. The ultrastructure of the sample formally referred to "ferric hydroxide" or "$Fe(OH)_3$" (prepared by using sodium hydroxide instead of phosphate) was quite different from that of the ferric hydroxyphosphates prepared at similar conditions. TEM observation revealed that it contained nanocrystals of size up to ~50 nm that were dispersed within an amorphous matrix. Under high-resolution mode (HRTEM) the matrix appeared granular, containing vary small particles, about 3 nm in size. The composition of the sample according to EDS analysis corresponded to Fe/O molar ratio of 1.0/2.4, while the nanocrystalline phase was confirmed to be hematite.

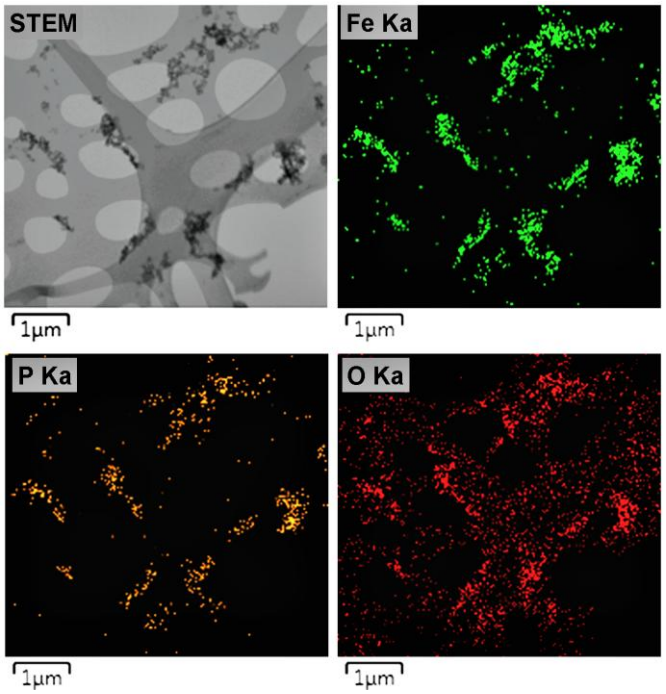

***Fig.2**. STEM image and maps of element (Fe, P and O) distribution of ferric hydroxyphosphate (Fe/P = 100/50), $Fe(OH)_3.FePO_4$.*

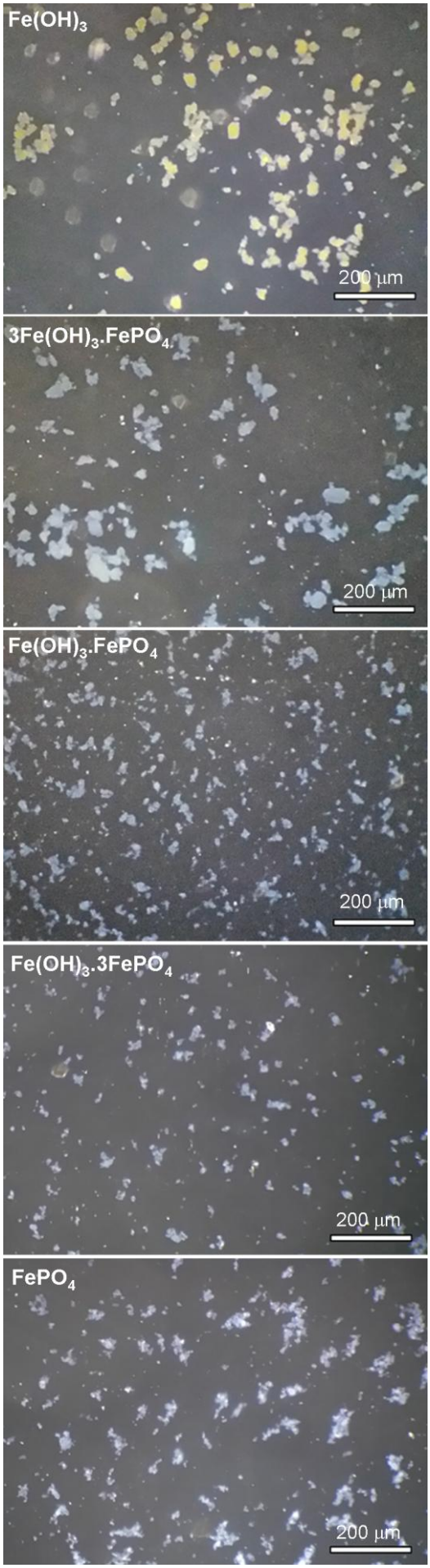

***Fig.3**. Dark-field optical microscopy images of the micron-sized aggregates of ferric hydroxyphosphates in physiological saline.*

Analysis by X-ray powder diffraction (XRD) confirmed the amorphous structure of the obtained materials and the presence of a nanocrystalline hematite phase in the $Fe(OH)_3$ sample (data not shown). It should be noted that the hematite phase was formed during the autoclaving, since the non-autoclaved $Fe(OH)_3$ sample was structurally amorphous.

We used a dark-filed optical microscope to observe the micron-sized aggregates (secondary particles formed by the aggregated primary nanoparticles) in 0.9% NaCl (Fig. 3). There were different structures – raging in size from few microns to about 20-30 μm. The ferric hydroxide sample contained the largest particles, which were colored in yellow. These samples were also prone to relatively faster sedimentation forming a fine precipitate upon standing, while all ferric hydroxyphosphates appeared as gel-like suspensions that formed a gel sediment upon mild centrifugation. The aggregation state of adjuvants in vaccines depends also on its concentration and interactions with proteins and other components of the formulation [14]. The size of secondary adjuvant particles (aggregates) appears to be of importance for both the effective adsorption of antigens and phagocytosis of adjuvant particles by antigen-presenting cells [15,16]. The relatively larger particle size in the case of ferric hydroxide may be a reason to expect a lower rate of phagocytosis compared to the hydroxyphosphates although it can be revealed only by detailed future experiments on the intracellular fate of these adjuvant systems.

3.2. Potentials for adjuvant use

Inorganic adjuvants, such as aluminium oxyhydroxide and hydroxyphosphate, which are currently used in many human and veterinary vaccinations, are known to serve as enhancers of antigen phagocytosis and activation of antigen-presenting cells [17], as well as stimulators of inflammatory reactions that appear to play a key role in mediating adjuvanticity and subsequent development of specific immunity [18]. It is currently known that the immune potentiation requires phagocytosis of the adjuvant/antigen by dendritic cells [19]. Similar mechanisms of adjuvanticity can be assumed also for the ferric-based adjuvants, since it is well-established that particulate ferric hydroxide is rapidly phagocytosed by macrophages upon parenteral administration [20,21]. The ferric-based adjuvants can potentially serve as antigen carriers to the phagocytic antigen-presenting cells. In preliminary experiments, we found that all investigated ferric hydroxyphosphate adjuvant gels had isoelectric points between 3.5 and 4.5, and could adsorb albumin (as a model of protein antigen), about 30 mg/mmol Fe(III) at pH 7, which is an important prerequisite for their potential application as protein antigen carriers (details on zeta-potential measurements and protein adsorption will be reported elsewhere).

Among the most important issues in the development of inorganic adjuvants besides efficacy are their safety [22], immunotoxicity [23] and toxicokinetics [24]. High loading of insoluble adjuvant particles in phagocytic cells without immediate cytotoxicity might predispose to their subsequent transport throughout the body, while on the other hand, heightened solubility of adjuvants and potentially the generation of metal ions in the endosomal environment have been positively correlated with an increase in cell mortality *in vitro* [25]. Although it has been demonstrated that ferric phosphate could be dissolved in citrate solutions, similarly to aluminium phosphate [12], the *in vivo* degradation of the ferric-based adjuvants is still unknown. It might be expected that endosomal degradation of ferric phosphates would result in the release of ferric ions inside phagosomes. Phagocytic cells have the ferroportin transmembrane transporter that facilitates transportation of ferric ions out of the cell [21]. Once exported into the extracellular environment, most of the ferric ions are expected to bind with transferrin (ferric-specific transporter protein in blood plasma) and to be included in the normal iron metabolism. Also, adverse effects from the relatively small amount of iron (few milligrams) applied during vaccination are less likely to occur since iron is present in humans in relatively large amounts (35-50 mg iron/kg body weight). However, detailed toxicokinetic experiments

must be performed in order to evaluate the exact safe doses of the different ferric-based adjuvants.

4. Conclusions

Ferric hydroxyphosphates of various Fe/P molar ratios as potential adjuvants have been prepared and characterized. The hydroxyphosphate gels consisted of networks of primary amorphous nanoparticles of average sizes about <20 nm, smaller in size compared to those of aluminum phosphate adjuvants (20-40 nm). The ferric hydroxide obtained at similar conditions consisted of hematite nanocrystals dispersed into amorphous matrix. These primary nanoparticles formed micron-sized aggregates (secondary particles) in physiological solution. Preliminary experiments indicated the ability of the obtained adjuvant gels to adsorb protein substances, which is an important prerequisite for their potential application as vaccine adjuvants and further optimization of the production process.

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