Needle-Free Dermal Delivery of a Diphtheria Toxin CRM$_{197}$ Mutant on Potassium-Doped Hydroxyapatite Microparticles

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Injections with a hypodermic needle and syringe (HNS) are the current standard of care globally, but the use of needles is not without limitation. While a plethora of needle-free injection devices exist, vaccine reformulation is costly and presents a barrier to their widespread clinical application. To provide a simple, needle-free, and broad-spectrum protein antigen delivery platform, we developed novel potassium-doped hydroxyapatite (K-Hap) microparticles with improved protein loading capabilities that can provide sustained local antigen presentation and release. K-Hap showed increased protein adsorption over regular hydroxyapatite ($P < 0.001$), good structural retention of the model antigen (CRM$_{197}$) with 1% decrease in $\alpha$-helix content and no change in $\beta$-sheet content upon adsorption, and sustained release in vitro. Needle-free intradermal powder inoculation with K-Hap–CRM$_{197}$ induced significantly higher IgG1 geometric mean titers (GMTs) than IgG2a GMTs in a BALB/c mouse model ($P < 0.001$) and induced IgG titer levels that were not different from the current clinical standard ($P > 0.05$), namely, alum-adsorbed CRM$_{197}$ by intramuscular (i.m.) delivery. The presented results suggest that K-Hap microparticles may be used as a novel needle-free delivery vehicle for some protein antigens.

The number of immunizations rises annually with population growth, vaccine availability, and sophistication of immunization schedules. Consequently, the risk associated with needles as a vector of infection is contributing to the global burden of disease (1–3). Of the 16 billion therapeutic injections administered with needles and syringes in 2004 globally, 800 million were prophylactic inoculations, and approximately 30 million of the 16 billion (or 0.19%) resulted in needlestick injuries, some of which have transmitted blood-borne pathogens, such as HIV, hepatitis B, or hepatitis C (4, 5).

While a plethora of needle-free inoculation devices exists, most needle-free immunization technologies require the reformulation of vaccines that are routinely administered with a hypodermic needle and syringe (HNS) (6–8). In the case of protein antigens, dry-powder manufacture for intradermal or mucosal delivery is largely empirical and hence particularly costly, which presents a barrier to the widespread use of needle-free inoculation (9–13).

Protein antigens are often poorly immunogenic and generally require the addition of an adjuvant to elicit robust antibody responses (14, 15). Colloidal adjuvants, such as aluminum salts, are routinely used to boost vaccine immunogenicity (16). However, previous efforts to reformulate vaccines for use in needle-free intradermal injection have linked aluminum salt coformulation with an increased aggregate content and the denaturation of the protein antigen after lyophilization or spray lyophilization (17–21). Furthermore, alum has been reported to induce granuloma formation when injected intradermally (22). While alum adjuvantation is problematic in combination with lyophilization and dermal inoculation, a novel dermal delivery vehicle may not require alum adjuvantation.

The potential use of calcium phosphate-based scaffolds as a drug delivery vehicle has been extensively researched and is well documented (23, 24). In particular, hydroxyapatite (Hap), a biocompatible mineral that is naturally found in bone and largely comprises of calcium and phosphate, has been shown to strongly adsorb proteins and to provide sustained release of the bioactive molecule (25, 26). Recently, it was reported that a commercial calcium phosphate adjuvant consisted of nanosized Hap needles (27). Therefore, Hap has demonstrated its capability to act as a drug and vaccine delivery substrate and has a documented track record of biocompatibility and safety and may potentiate the immune response to antigens.

Here, we investigate whether intradermal delivery of Hap microparticles can elicit antibody titers to the adsorbed CRM$_{197}$ protein antigen, using the Venturi needle-free ballistic injection device (28). The diphtheria toxin mutant CRM$_{197}$ protein is a 58-kDa, two-subunit, nontoxic protein that is frequently used as a polysaccharide carrier protein in commercial glycoconjugate vaccines, but it is poorly immunogenic without adjuvantation (29–31). To our knowledge, this is the first report of needle-free ballistic intradermal immunization with CRM$_{197}$ using Hap microparticles.

MATERIALS AND METHODS

K-Hap particle manufacture. Potassium-substituted hydroxyapatite (K-Hap) microparticles were manufactured by molten salt synthesis. The K-Hap particle manufacture.

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Hap and K-Hap BSA-TRITC loading. Hap and K-Hap 10-mg powder samples were incubated at room temperature (RT) for 3 h with 5 mg of bovine serum albumin (BSA)-tetramethyl rhodamine isothiocyanate (TRITC) (Sigma-Aldrich, Dorset, United Kingdom) in 1 ml of phosphate-buffered saline (PBS) (Sigma-Aldrich, Dorset, United Kingdom) at pH 7.4 on a VWR rotating shaker (VWR, Dublin, Ireland). Protein loading on Hap and K-Hap was determined by micro-bicinchoninic acid (micro-BCA) assay kit according to the manufacturer's recommendations (Sigma-Aldrich, Dorset, United Kingdom). In addition to the bulk average protein loading measured by BCA assay, the protein loading on individual particles was characterized using fluorescence imaging. Briefly, protein loading of albumin-TRITC on dry-powder Hap and K-Hap was assessed using fluorescence imaging at 80-ms exposure and ×4 magnification on a Nikon Eclipse Ti light microscope and NIS Elements AR 3.0 software. Albumin-TRITC was excited at 550 nm, and fluorescence was monitored at 600 nm. Fluorescence intensity was averaged over the respective particle area using ImageJ analysis software and reported from low to high on an 8-bit (0 to 255) gray-scale intensity spectrum.

K-Hap CRM197 dry-powder formulation. The model antigen CRM197 (Novartis Vaccines and Diagnostics, Siena, Italy) was provided in 10% (wt/vol) sucrose−10 mM KH2PO4 at pH 7.2 and was dialyzed against PBS (pH 7.4) for 12 h using a Mini Slide-A-Lyzer (Fisher Scientific, Loughborough, United Kingdom) with a 10-kDa-molecular-size cutoff prior to use. Dialyzed CRM197 was adsorbed onto 10 mg of K-Hap particles in 1 ml of PBS (pH 7.4) for 3 h at 1.6 mg/ml on a VWR rotating shaker. The slurry was centrifuged for 3 min at 2,000 × g (MSE, London, United Kingdom), and the supernatant was subsequently decanted. The K-Hap–CRM197 particles were washed thrice with isopropanol (Fisher Scientific, Loughborough, United Kingdom). The wet powder was then transferred into lyophilization vials (Adelphi Healthcare Packaging, Haywards, United Kingdom) and vacuum dried for 24 h at 13.3 Pa and 20°C with a FTS Lyostar 1 Instrument (SP Industries, Warminster, PA, USA). The vials were sealed, crimped, and stored at room temperature until further use.

K-Hap CRM197 adsorption and in vitro release. The total protein loading on K-Hap (Ct0) was determined as the percentage of the mass difference between CRM197 in solution before and after adsorption measured by micro-BCA assay (Sigma-Aldrich, Dorset, United Kingdom) and divided by the mass of K-Hap. The Langmuir isotherm is described by the following equation:

\[ y = \frac{bx^{1/2}}{(1 + bx^{1/2})} \]

and was iteratively fitted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The dependent variable y (in milligrams per gram) is the amount of CRM197 per mass of K-Hap, the independent variable x (in milligrams per liter) is the CRM197 concentration in solution. The variable a is the adsorption capacity, while b and c are model constants. The approximate surface area of particles was obtained on a Malvern Mastersizer S analyzer (Malvern Instruments Ltd., Malvern, United Kingdom), in a small-volume dispersion cell. A 300W lens with backscatter detector with an active beam length of 14.3 mm was used to obtain an average of 6 times 6,000 measurements according to Mie theory. Particles were added until 10 to 13% detector obscuration was achieved at a stirring rate of 2,000 rpm. Refractive indices (RI) for HA and K-Hap particles (RI, 1.6501) dispersed in deionized (DI) water (RI, 1.3300) were used in the method.

CRM197 release from K-Hap in PBS was quantified by micro-BCA assay (Sigma-Aldrich, Dorset, United Kingdom) after 0, 0.5, 1.5, 6, and 24 h of incubation in PBS (pH 7.4) at 37°C on a rotating shaker (n = 3). Absorbance at 562 nm was measured on a Cary 50 Bio UV-visible spectrophotometer (Varian, CA, USA). Concentrations were plotted as percent release relative to total protein loading (Ct/Ct0).

Structural characterization of surface-adsorbed CRM197. The secondary structure of CRM197 when adsorbed on K-Hap or aluminum phosphate (Adju-Phos; Brenntag Biosector, Frederikssund, Denmark) was determined using a LN2-cooled Fourier transform infrared (FT-IR) Tensor 37 spectrometer, equipped with the attenuated total reflection
BioATR II sample compartment (Bruker Optics, Ettlingen, Germany). Over the range 4,000 to 800 cm\(^{-1}\), 128 scans were averaged at a 4-cm\(^{-1}\) resolution. The attenuated total reflection (ATR) unit was temperature controlled at 20°C. OPUS 6.5 software data postprocessing included atmospheric compensation, vector normalization, baseline correction, and integration of the amide I and II band regions from 1,720 cm\(^{-1}\) to 1,480 cm\(^{-1}\). The spectra were evaluated against the Quant II structural database provided by Bruker Optics.

**Immunization.** Six- to 8-week-old female BALB/c mice (Harlan, Oxfordshire, United Kingdom) were fully anesthetized with isoflurane gas. Before needle-free administration of K-Hap–CRM\(_{197}\), the murine pinna was sterilized with 70% ethanol solution. The in-line Venturi device (Particle Therapeutics Ltd., Yarnton, United Kingdom) was placed onto the dorsal pinna for injection. Medical-grade helium pressured at 55 \(\times\) 10\(^5\) Pa was used to accelerate the K-Hap powder containing 10 \(\mu\)g CRM\(_{197}\) into the skin. Intramuscular injection with 12.5 \(\mu\)l of aqueous CRM\(_{197}\) was administered into the medial thigh of each hind leg using a 29-gauge needle. The total volume of 25 \(\mu\)l contained 10 \(\mu\)g CRM\(_{197}\), with or without alum (\(n = 8\)). Briefly, CRM\(_{197}\) was formulated with Adju-Phos at a ratio of 10 \(\mu\)g CRM\(_{197}\) to 85 \(\mu\)g alum in 10 mM Tris buffer (pH 7.0 to 7.5). For 10 doses, 30 \(\mu\)l of saline was added to 170 \(\mu\)l of Adju-Phos containing 850 \(\mu\)g of aluminum under aseptic conditions. After 15 min at room temperature, 50 \(\mu\)l of a 2-mg/ml CRM\(_{197}\) solution was added and incubated at room temperature for 1 h prior to injection. Naïve mice served as a negative control (\(n = 4\)). Mice were primed on day 0 and boosted on day 28 with the same formulations and antigen dose. Blood was collected from the tail on days 28 and 42.

**ELISA.** Antibody responses were assessed by enzyme-linked immunosorbent assay (ELISA) after coating plates (Fisher Scientific, Loughborough, United Kingdom) with 5 \(\mu\)g of CRM\(_{197}\) antigen per well in carbonate-bicarbonate buffer (Sigma-Aldrich, Dorset, United Kingdom). The reactions in the wells were blocked with PBS containing 5% BSA (Sigma-Aldrich, Dorset, United Kingdom). Serum samples were serially diluted in PBS containing 0.05% Tween 20 (PBS–Tween) with 5% BSA at a starting dilution of 1:200. After incubation at 25°C for 2 h, an antibody detecting goat anti-mouse antibody (Southern Biotech, Cambridge, United Kingdom) was added at a dilution of 1:2,000 in PBS–Tween with 5% BSA. After 1-h incubation of the plates, the reactions in the wells were developed with 1,128 scans were averaged at a 4-cm\(^{-1}\) resolution, and surface charge of individual particles. Although particle morphology varied between prismatic and spheroidal, there was no apparent effect of morphology on protein adsorption.

**Secondary structure of surface-adsorbed CRM\(_{197}\).** Immunogenic epitopes may be adversely affected by changes in the secondary structure of the antigen, which should be avoided during dry-powder formulation. FT-IR analysis of surface-adsorbed CRM\(_{197}\) suggested that the amide I band peak position of K-Hap-adsorbed CRM\(_{197}\) was observed at 1,648 cm\(^{-1}\) (Table 2). This was also the amide I band peak position of CRM\(_{197}\) when CRM\(_{197}\) was suspended in phosphate buffer. Adsorption onto K-Hap caused a 1% reduction in \(\alpha\)-helix content but no detectable increase in \(\beta\)-sheet content relative to the solution. This suggests that adsorption onto K-Hap largely conserved the native secondary structure of CRM\(_{197}\). On the other hand, alum-adsorbed CRM\(_{197}\) induced a 5% decrease of \(\alpha\)-helical content along with a 5% increase in \(\beta\)-sheet structures (Table 2). These results suggest that a form of CRM\(_{197}\) that is more similar to the native state may be presented to the immune system on K-Hap than on alum.

**Protein adsorption onto Hap and K-Hap.** An increased surface adsorption propensity can increase the antigen payload that is delivered per microparticle and thereby reduce the extraneous material delivered per dose. Using fluorescence microscopy, it was determined that the mean adsorption of albumin–TRITC in physiological PBS was significantly greater (\(P < 0.001\)) on K-Hap than on Hap (Fig. 2b). These results were confirmed by micro-BCA assay (data not shown). Fluorescence images suggested some variability in protein loading within the Hap and K-Hap particle groups (Fig. 2a). This may be attributable to variation in particle surface properties such as surface roughness, material composition, and surface charge of individual particles. Although particle morphology varied between prismatic and spheroidal, there was no apparent effect of morphology on protein adsorption.

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mass of CRM197 per mass of K-Hap. Figure 3a suggests that CRM197 adsorption on K-Hap is well described by the Langmuir isotherm ($R^2 = 0.988$). Computational extrapolation estimates the adsorption capacity at 5.9 mg of CRM197 per g of K-Hap as the maximum amount of CRM197 per gram of substrate. When related to the particle surface area obtained from the light-scattering particle size distribution, the maximum CRM197 adsorption capacity corresponds to a surface protein loading density on K-Hap of 9.7 mg/m$^2$.

**Time-dependent CRM197 release.** Colloidal adjuvants retain the antigen at the injection site, which has been linked to immune response potentiation. K-Hap microparticles released approximately 28% of their maximum 5.9 mg of CRM197 per g of K-Hap payload within the first 90 min in PBS buffer as an initial burst but provided sustained release of the antigen thereafter (Fig. 3b). Therefore, K-Hap may similarly retain CRM197 at the injection site, like aluminum adjuvants, while providing a slow release of the antigen.

**Serum IgG antibody response.** Pathogen opsonization, agglutination, and toxin neutralization are facilitated by the presence of IgG in serum. It was investigated whether K-Hap–CRM197 could induce IgG levels similar to that of the current clinical standard (e.g., alum-CRM197). As depicted in Fig. 4a, anti-CRM197 serum IgG geometric mean titers (GMTs) were not different for alum-adsorbed CRM197 and K-Hap-adsorbed CRM197 28 days after prime ($P > 0.05$), although the mean IgG GMT for K-Hap-adsorbed CRM197 was higher on average. On day 42, 14 days after the booster dose, alum-adsorbed CRM197 GMTs were higher on average than in the K-Hap-adsorbed CRM197 group, but the difference was not significant ($P > 0.05$). Both alum and K-Hap conditions showed significantly higher mean GMTs than the naive control ($P < 0.001$). An approximately 1.4-fold increase in IgG GMT levels was observed for K-Hap–CRM197 and a 1.7-fold increase in the alum-adjuvanted CRM197 condition was seen between days 28 and 42. A nonsignificant trend suggests that K-Hap may induce a stronger initial response than alum-CRM197 (administered intramuscularly [i.m.]), but alum-adsorbed CRM197 may have a stronger booster response.

**IgG subclass responses.** It was investigated whether the pre-
sentation of CRM$_{197}$ in the K-Hap microparticle surface-adsorbed state could influence the IgG1/IgG2a balance. The alum-adjuvanted and needle-free K-Hap groups both showed a 2-fold IgG1/IgG2a-biased response. These results suggest that alum and K-Hap may similarly act as adjuvant and induce an IgG1-biased response. Two weeks after the booster dose (day 42), anti-CRM$_{197}$ IgG1 GMTs were higher for the alum-adjuvanted group than for the needle-free K-Hap group ($P < 0.05$). Both the alum-adjuvanted and K-Hap-adjuvanted ($P < 0.001$) groups induced significantly higher GMTs than the naive control. These results suggest that K-Hap-adsorbed CRM$_{197}$ injected needle-free induces a similarly IgG1-biased response as alum-adsorbed CRM$_{197}$ by HNS and that K-Hap may act in a similarly adjuvanting manner as alum.

**DISCUSSION**

While needle-free immunization has clear advantages over inoculation with HNS, the latter is less technically demanding (6). The three pivotal aspects of successful needle-free intradermal vaccination are the antigen payload deposition in the correct dermal layer, the structural preservation of the antigen during dry-powder formulation, and the magnitude and type of immune response that can be elicited compared to HNS inoculation (33). This paper addresses these three aspects. The data presented show successful targeting of the needle-free vaccine to the epidermis, structural retention of the CRM$_{197}$ protein in the K-Hap-adsorbed state, and comparable immunogenicity of the needle-free delivery approach with CRM$_{197}$-adsorbed K-Hap relative to the alum-adjuvanted condition by HNS.

Needle-free powder delivery to the optimal dermal layer reportedly presents a challenge in part due to highly variable skin characteristics (34, 35). Previous ballistic delivery studies have emphasized the influence of VED and SC thickness on particle penetration depth, with an increase in layer thickness translating to reduced penetration into the skin (36). In this study, we did not observe this effect on K-Hap penetration depth. A possible reason for this may be the varied K-Hap particle shape factor (spherical to prismatic) that could influence penetration properties or the relatively large particle size relative to the dermal layers. However, the thicker SC and VED layers in the pinna allowed for good colocalization of K-Hap particles with the epidermis, whereas particles penetrated too deep at the other injection sites. Interestingly, while differences in VED and SC thickness are also thought to impact the particle distribution in the dermis, the obtained results show an almost identical variance about the mean despite significantly different VED and SC dimensions (Table 1). This suggests that while VED and SC thickness can impact the powder penetration depth, they may contribute less to the particle distribution in the skin than other factors, such as the underlying particle characteristics, the injection device, and the propulsion pressure.

An alternative antigen delivery platform should preserve the structure of the antigen and have sufficient antigen-carrying capacity to minimize the codelivery of extraneous material. On solid particles, the antigen-carrying capacity is restricted to surface adsorption. Protein sorption propensity on surfaces is affected by the protein-loading capacity of the negatively charged BSA-TRITC with CRM$_{197}$-adsorbed K-Hap relative to the alum-adjuvanted powder CRM$_{197}$-adsorbed K-Hap (Fig. 2a and b). Alternatively, the ionic sorbent properties of K-Hap may similarly act as adjuvant and induce an IgG1-biased response. Two weeks after the booster dose (day 42), anti-CRM$_{197}$ IgG1 GMTs were higher for the alum-adjuvanted group than for the needle-free K-Hap group ($P < 0.05$). Both the alum-adjuvanted and K-Hap-adjuvanted ($P < 0.001$) groups induced significantly higher GMTs than the naive control. These results suggest that K-Hap-adsorbed CRM$_{197}$ injected needle-free induces a similarly IgG1-biased response as alum-adsorbed CRM$_{197}$ by HNS and that K-Hap may act in a similarly adjuvanting manner as alum.

**FIG 4** All treatment groups ($n = 8$) received 10 µg CRM$_{197}$ on days 0 (prime) and 28 (boost). Mice were bled on days 28 and 42. Naive mice served as the control group (four mice). Preimmunization serial titers were less than 200. CRM$_{197}$ alone in PBS (CRM$_{197}$) and CRM$_{197}$ adjuvanted with 85 µg alum (CRM$_{197}$ + Alum) were injected i.m. with HNS. K-Hap–CRM$_{197}$ powder was administered intradermally (i.d.) needle-free (CRM$_{197}$ + K-Hap). (a) The IgG GMT and 95% confidence interval (95% CI) were indicated for all treatment groups on day 42. Two-way ANOVA compared the categorical variables (a) “treatment group” and “time point” and (b) “treatment group” and “IgG subclass” as predictors of the continuous-variable CRM$_{197}$ GMT and was conducted at $\alpha = 0.05$ significance level. Values that are significantly different ($P < 0.001$) are indicated (***). $P$ values above the $\alpha$-level were considered not significant (NS).
of CRM197 per g of K-Hap and was well-described by a Langmuir isotherm ($R^2 = 0.988$). This suggests that CRM197 adsorbed onto K-Hap as a single layer, which correlates with the minor changes in secondary structure observed by FT-IR. When related to the particle surface area, which was obtained from the light-scattering particle size distribution, the maximum CRM197 adsorption capacity corresponds to a K-Hap surface protein-loading density of 9.7 mg/m². The protein-loading density of BSA on pure hydroxyapatite has been reported as 1.5 mg/m², but buffer strength, protein type, and substrate can affect the loading density and may therefore not be compared directly to the results presented in this paper (40). Additionally, light scattering can give an estimate of the surface area, but it cannot account for the surface roughness. Therefore, the average surface loading density of CRM197 on K-Hap can be regarded only as a rough estimate. The cumulative CRM197 release from the K-Hap particles after 24 h was less than 40% of the total payload, which is less than 2.4 μg of CRM197 per 1 mg of K-Hap. Therefore, the possible soluble aggregate content of this formulation can be considered minimal at any given time in situ and hence does not pose a serious concern (41, 42). Furthermore, the strong antigen retention on K-Hap suggests a similar antigen depot function as alum, and therefore, K-Hap may be considered an adjuvant, more so than either an immune modulator or an inactive formulation excipient. This theory is supported by the elicited IgG and IgG subclass GMTs.

The magnitude and quality of the induced immune response can provide insight into the effect of a novel antigen formulation (43). In this study, needle-free immunization with K-Hap–CRM197 induced geometric mean IgG antibody titers (IgG GMT) that were not different ($P > 0.05$) from alum-adjuvanted CRM197 by HNS 4 weeks after prime and 2 weeks after boost. The GMT ratios of IgG1/IgG2a were approximately 2-fold IgG1 biased for the alum-adjuvanted CRM197 and K-Hap-adsorbed CRM197 groups. Based on the observed IgG and IgG subclass responses, the immune activation with K-Hap-adsorbed CRM197 resembled the adjuvant effect observed with alum. The adjuvanticity of alum is partially ascribed to the formation of an antigen depot, as well as to the noncovalently bound surface presentation of the antigen. Based on the physical characterizations of CRM197 sorption and release from K-Hap, the adjuvant effect of K-Hap on CRM197 immunogenicity may be achieved in a fashion similar to that of aluminum salts.

While immunization with CRM197 alone is not of direct clinical applicability, the use of the CRM197 protein in this study presents a good model to probe the capability of the K-Hap formulation approach. For example, Haemophilus influenzae type b (Hib), pneumococcal, and meningococcal vaccines contain oligosaccharides that are covalently linked to CRM197 (30). From a formulation standpoint, the structural preservation of CRM197 is the most challenging component of a dry-powder K-Hap vaccine, as it is the protein that likely denatures (44). In vivo and FT-IR results suggest that the structure of CRM197 was sufficiently preserved. This finding motivates the investigation of the K-Hap platform as a needle-free delivery vehicle for a variety of CRM-based conjugate vaccines.

In conclusion, the data presented suggest that needle-free intradermal vaccination with CRM197 antigen-loaded K-Hap microparticles induces an IgG1-biased antibody response with strong IgG GMTs that are not different from the alum-adjuvanted CRM197 by HNS. Simple coinoculation of the antigen with the K-Hap carrier microparticles followed by vacuum desiccation was sufficient to reformulate the antigen, whereas conventional pharmaceutical dry-powder formulation processes require the need for largely empirical excipient selection and time-consuming spray lyophilization, which can degrade proteins. In the surface-adsorbed state, CRM197 largely retained its secondary structure according to FT-IR measurements. These findings encourage the investigation of the in vivo immunogenicity of needle-free glycoconjugate formulations using the K-Hap vehicle.

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**REFERENCES**

1. Varmus H, Klausner R, Zerhouni E, Achariy T, Daar AS, Singer PA. 2003. Grand challenges in global health. Science 302:398–399. http://dx.doi.org/10.1126/science.1091769.

2. Guo YL, Shiao J, Chuang YC, Huang KY. 1999. Needlestick and sharps injuries among healthcare workers in Taiwan. Epidemiol Infect 122:259–265. http://dx.doi.org/10.1017/S0950268899002186.

3. Rapiti E, Pruss-Ustun A, Hutin Y. 2005. Sharps injuries: assessing the burden of disease from sharps injuries to healthcare workers at national and local levels. World Health Organization, Geneva, Switzerland.

4. Hopkins C. 2012. Needlestick injuries. Nurs Stand 27(3):59.

5. World Health Organization. 2004. Safety of injections: global facts and figures. World Health Organization, Geneva, Switzerland.

6. Mitragotri S. 2005. Immunization without needles. Nat Rev Immunol 5:905–916. http://dx.doi.org/10.1038/nri1728.

7. Sullivan SP, Koutsoumanos DG, Del Pilar Martín M, Lee JW, Zarnitsyn V, Choi SO, Murthy N, Compans RW, Skountzou I, Prausnitz MR. 2010. Dissolving polymer microneedle patches for influenza vaccination. Nat Med 16:915–920. http://dx.doi.org/10.1038/nm.2182.

8. Prausnitz MR, Langer R. 2008. Transdermal drug delivery. Nat Biotechnol 26:1261–1268. http://dx.doi.org/10.1038/nbt.1504.

9. Fang WJ, Qi W, Kinzell J, Prestrickl S, Carpenter JE. 2012. Effects of excipients on the chemical and physical stability of glucagon during freeze-drying and storage in dried formulations. Pharm Res 29:3278–3291. http://dx.doi.org/10.1007/s11095-012-0820-z.

10. Wang W. 2000. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm 203:1–60. http://dx.doi.org/10.1016/S0378-5173(00)00423-3.

11. Prausnitz MR, Langer R, 2005. Protein aggregation and its inhibition in biopharmaceuticals. Int J Pharm 289:1–30. http://dx.doi.org/10.1016/j.ijpharm.2004.11.014.

12. Kersten G, Hirschberg H. 2007. Needle-free vaccine delivery: Expert Opin Drug Deliv 4:459–474. http://dx.doi.org/10.1517/17425247.4.5.459.

13. Weissmueller NT, Schiffter HA, Pollard AJ. 2013. Intradermal powder immunization with protein-containing vaccines. Expert Rev Vaccines 12:687–702. http://dx.doi.org/10.1586/erv.13.48.

14. Snape MD, Perrett KP, Ford KJ, John TM, Pace D, Yu LM, Langley JM, McNeil S, Dull PM, Ceddia F, Anemona A, Halperin SA, Dobson S, Pollard AJ. 2008. Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial. JAMA 299:173–184. http://dx.doi.org/10.1001/jama.2007.29-2.

15. Pollard AJ, Perrett KP, Beverley PC. 2009. Maintaining protection against invasive bacteria with protein-poly saccharide conjugate vaccines. Nat Rev Immunol 9:213–220. http://dx.doi.org/10.1038/nri2494.

16. Lindblad EB. 2004. Aluminum compounds for use in vaccines. Immunol Invest 33:759–805. http://dx.doi.org/10.1081/MI-200037594.

17. Chen D, Erickson CA, Endres RL, Periwal SB, Chu Q, Shu C, Maa YF, Payne LG. 2001. Adjuvantaion of epidermal powder immunization. Vaccine 19:2908–2917. http://dx.doi.org/10.1016/S0264-410X(01)00544-2.

18. Chen DX, Kristensen D. 2009. Opportunities and challenges of developing thermostable vaccines. Expert Rev Vaccines 8:537–557. http://dx.doi.org/10.1586/erv.09.20.

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[cvi.asm.org](http://cvi.asm.org)
19. Maa YF, Ameri M, Shu C, Payne LG, Chen D. 2004. Influenza vaccine powder formulation development: spray-freeze-drying and stability evaluation. J Pharm Sci 93:1912–1923. http://dx.doi.org/10.1002/jps.20104.

20. Maa YF, Shu C, Ameri M, Zuleger C, Che J, Osorio JE, Payne LG, Chen D. 2003. Optimization of an alum-adsorbed vaccine powder formulation for epidermal powder immunization. Pharm Res 20:969–977. http://dx.doi.org/10.1023/A:1024493719236.

21. Maa YF, Ameri M, Shu C, Zuleger CL, Che J, Osorio JE, Payne LG, Chen D. 2007. Hepatitis-b surface antigen (hbsag) powder formulation: process and stability assessment. Curr Drug Deliv 4:57–67. http://dx.doi.org/10.2174/156720107779314758.

22. Marsee DK, Williams JM, Velazquez EF. 2008. Aluminum granuloma after administration of the quadrivalent human papillomavirus vaccine. Report of a case. Am J Dermatopathol 30:622–624. http://dx.doi.org/10.1097/DAD.0b013e318185a691.

23. Verron E, Khairoun I, Guicheux J, Bouler JM. 2010. Calcium phosphate biomaterials as bone drug delivery systems: a review. Drug Discov Today 15:547–552. http://dx.doi.org/10.1016/j.drudis.2010.05.003.

24. Ginebra MP, Canal C, Espanol M, Pastorino D, Montufar EB. 2012. Calcium phosphate cements as drug delivery materials. Adv Drug Deliv Rev 64:1090–1110. http://dx.doi.org/10.1016/j.addr.2012.01.008.

25. Yin G, Liu Z, Zhan J, Ding FX, Yuan NJ. 2002. Impacts of the surface charge property on protein adsorption on hydroxyapatite. Chem Eng J 87:181–186. http://dx.doi.org/10.1016/S1385-8947(01)00248-0.

26. Shen JW, Wu T, Wang Q, Pan HH. 2008. Molecular simulation of protein adsorption and desorption on hydroxyapatite surfaces. Biomaterials 29:513–532. http://dx.doi.org/10.1016/j.biomaterials.2007.10.016.

27. Jiang DP, Premachandra GS, Johnston C, Hem SL. 2004. Structure and adsorption properties of commercial calcium phosphate adjuvant. Vaccine 22:693–698. http://dx.doi.org/10.1016/j.vaccine.2004.06.029.

28. Costigan L, Liu Y, Brown GL, Carter FV, Bellhouse BJ. 2005. Evolution of the design of Venturi devices for the delivery of dry particles to skin or mucosal tissue. p 719–724. In Jiang Z (ed). Shock waves. Proceedings of the 24th International Symposium on Shock Waves, Beijing, China, 11 to 16 July 2004. Springer-Verlag, Berlin, Germany.

29. Malito E, Bursulaya B, Chen CN, Lo Surdo P, Picchiante M, Balducci E, Biancuzzi M, Brock A, Berti F, Bottomley MJ, Nissum M, Costantino P, Rappuoli E, Spraggon G. 2012. Structural basis for lack of toxicity of the diphtheria toxin mutant CRM197. Proc Natl Acad Sci U S A 109:5229–5234. http://dx.doi.org/10.1073/pnas.1201964109.

30. Shinesfeld HR. 2010. Overview of the development and current use of crm197 conjugate vaccines for pediatric use. Vaccine 28:4335–4339. http://dx.doi.org/10.1016/j.vaccine.2010.04.072.

31. Kanra G, Viviani S, Yurdakok K, Ozmert E, Anemona A, Yalcin S, Demiralp O, Bilgili N, Kara A, Cengiz AB, Mutlu B, Baldini A, Marchetti E, Poddà A. 2003. Effect of aluminum adjuvants on safety and immunogenicity of Haemophilus influenzae type b-crm197 conjugate vaccine. Pediatr Int 45:314–318. http://dx.doi.org/10.1046/j.1442-200X.2003.01706.x.

32. Viswanath B, Raghavan R, Guraos NP, Ramamurty U, Ravishankar N. 2008. Mechanical properties of tricalcium phosphate single crystals grown by molten salt synthesis. Acta Biomater 4:1448–1454. http://dx.doi.org/10.1016/j.actbio.2008.02.031.

33. Dean HJ, Fuller D, Osorio JE. 2003. Powder and particle-mediated approaches for delivery of DNA and protein vaccines into the epidermis. Comp Immunol Microbiol Infect Dis 26:373–388. http://dx.doi.org/10.1016/S0147-9571(03)00021-3.

34. Kendall M, Mitchell T, Wrighton-Smith P. 2004. Intradermal ballistic delivery of micro-particles into excised human skin for pharmaceutical applications. J Biomech 37:1733–1741. http://dx.doi.org/10.1016/j.jbiomech.2004.01.032.

35. Kendall M, Rishworth S, Carter F, Mitchell T. 2004. Effects of relative humidity and ambient temperature on the ballistic delivery of micro-particles to excised porcine skin. J Invest Dermatol 122:739–746. http://dx.doi.org/10.1111/j.0022-202X.2004.02230.x.

36. Kendall MAF, Chong Y-F, Cock A. 2007. The mechanical properties of the skin epitherm in relation to targeted gene and drug delivery. Biomaterials 28:4968–4977. http://dx.doi.org/10.1016/j.biomaterials.2007.08.066.

37. Hartvig RA, van de Weert M, Ostergaard J, Jorgensen L, Jensen H. 2011. Protein adsorption at charged surfaces: the role of electrostatic interactions and interfacial charge regulation. Langmuir 27:2634–2643. http://dx.doi.org/10.1021/la104720n.

38. Weissmueller NT, Schifferer HA, Pollard AJ, Cuneyt TA. 2014. Molten salt synthesis of potassium-containing hydroxyapatite microparticles used as protein substrate. Mater Lett 128:421–424. http://dx.doi.org/10.1016/j.matlet.2014.04.154.

39. Fink AL. 1998. Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des 3:R9–R23. http://dx.doi.org/10.1016/S1359-0278(98)00002-9.

40. Mavropoulos E, Costa AM, Costa LT, Achete CA, Mello A, Granjeiro JM, Ross AM. 2011. Adsorption and bioactivity studies of albumin onto hydroxyapatite surface. Colloids Surf B Biointerfaces 83:1–9. http://dx.doi.org/10.1016/j.colsurfb.2010.10.025.

41. den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarah S, Seidl A, Hainzl O, Jiskoot W. 2011. Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharm Res 28:920–933. http://dx.doi.org/10.1007/s11095-010-0297-1.

42. Rosenberg AS. 2006. Effects of protein aggregates: an immunologic perspective. AAPS J 8:ES01–ES07. http://dx.doi.org/10.1208/saaps080359.

43. Plotkin SA. 2010. Correlates of protection induced by vaccination. Clin Vaccine Immunol 17:1055–1065. http://dx.doi.org/10.1128/CVI.00131-10.

44. Peters GH, van Aalten DM, Edholm O, Toxvaerd S, Bywater R. 1996. Dynamics of proteins in different solvent systems: analysis of essential motion in lipases. Biophys J 71:2245–2255. http://dx.doi.org/10.1016/S0006-3495(96)79428-6.