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REVIEW

Potential of nanoparticles for allergen-specific immunotherapy – use of silica nanoparticles as vaccination platform

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ABSTRACT

Introduction: Allergen-specific immunotherapy is the only curative approach for the treatment of allergies. There is an urgent need for improved therapies, which increase both, efficacy and patient compliance. Novel routes of immunization and the use of more advanced vaccine platforms have gained heightened interest in this field.

Areas covered: The current status of allergen-specific immunotherapy is summarized and novel routes of immunization and their challenges in the clinics are critically discussed. The use of

nanoparticles as novel delivery system for allergy vaccines is comprehensively reviewed. Specifically, the advantages of silica nanoparticles as vaccine carriers and adjuvants are summarized.

Expert opinion: Future allergen-specific immunotherapy will combine engineered hypoallergenic vaccines with novel routes of administration, such as the skin. Due to their biodegradability, and the easiness to introduce surface modifications, silica nanoparticles are promising candidates for tailor-made vaccines. By covalently linking allergens and polysaccharides to silica nanoparticles, a versatile vaccination platform can be designed to specifically target antigen-presenting cells, render the formulation hypoallergenic, and introduce immunomodulatory functions. Combining potent skin vaccination methods, such as fractional laser ablation, with nanoparticle-based vaccines addresses all the requirements for safe and efficient therapy of allergic diseases.

Keywords: allergy, immunotherapy, nanoparticles, silica, skin vaccination

Article highlights box

- Next generation vaccines for allergen specific immunotherapy have to combine hypoallergenic vaccine formulations with antigen targeting and novel delivery routes, such as the skin.
- Linking allergens to polysaccharides renders them hypoallergenic, specifically targets antigen presenting cells, and has immunomodulatory properties.
- Nanoparticles can serve as a defined “core particle” to which allergens and immunomodulatory compounds such as polysaccharides can be covalently linked, resulting in more defined vaccine formulations compared to conventional conjugation approaches.
- Silica nanoparticles present an attractive platform for next generation allergy vaccines due to their physicochemical characteristics, biodegradability, and adjuvant properties.

1. Introduction

Type I allergic diseases have reached epidemic proportions in highly developed countries and their incidence is still increasing. Basically, type I allergic diseases comprise inappropriate immune reactions against common environmental proteins (allergens) derived from tree and grass pollen, dust mites, animal dander, stinging insects, and foods. The allergic immune response is characterized by production of high levels of allergen-specific IgE antibodies and T helper 2 (TH2) type associated cytokines. Mostly, atopic individuals, i.e., genetically predisposed to mount exaggerated IgE responses, suffer from type I allergic reactions, including rhinoconjunctivitis (“hay fever”), asthma, sinusitis, food allergy, atopic dermatitis, angioedema and urticaria, anaphylaxis, and insect and drug allergy. The first step in the progress of allergic diseases is the uptake of allergens by professional antigen presenting cells (APCs), particularly dendritic cells (DCs), which present peptides thereof to naïve T cells and bias them towards a TH2 phenotype. In turn, T cells upregulate expression of the cytokines IL-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF. These cytokines are responsible for isotype-switching of B cells to generate IgE antibodies, recruitment of mast cells, and maturation of eosinophils and basophils, the hallmarks of allergic immune responses ¹.

As a second step, following sensitization, repeated contact with the respective allergen leads to crosslinking of IgE bound to the high-affinity IgE receptor FcεRI on mast cells and basophils, causing release of inflammatory mediators such as histamine and leukotrienes, which promote vascular permeability, smooth muscle contraction, and mucus production, the symptoms of the early allergic response. Additionally, cytokines and chemokines are produced, which attract mainly eosinophils and TH2 cells that are responsible for the late phase response. Finally, progression of the disease to asthma is characterized by airway remodeling and goblet-cell metaplasia, driven by IL-13 and a variety of growth factors ¹.

1.1 Allergen-specific immunotherapy

In contrast to short-term relief from allergic symptoms, which can be achieved by treatment with corticosteroids, anti-histamines, anti-leukotrienes, or agonists of adrenergic receptors, allergen-specific immunotherapy (AIT) has been used for more than 100 years as a treatment capable to modify the inappropriate immune response against a certain allergen. Particularly for treatment of allergic rhinoconjunctivitis with or without moderate asthma and venom hypersensitivity, AIT has proven long-term clinical efficacy¹. The immunological mechanisms underlying improved symptoms after allergen exposure depend on re-direction of allergic TH2 responses towards a TH1-type characterized by increased IFN- γ production (immunodeviation) and/or T regulatory (Treg) type (immunosuppression) with IL-10 and TGF- β as key cytokines². Consequently, after a transient increase, allergen-specific serum IgE levels drop during the course of the treatment, whereas IgG1, IgG4, and IgA antibodies increase. IgG4 has been shown to compete with IgE bound on the surface of mast cells and basophils (blocking antibodies), thereby preventing their activation³. Furthermore, AIT is able to reduce the recruitment of basophils, mast cells and eosinophils to the sites of allergen encounter, i.e. the skin as well as the bronchial and nasal mucosae⁴.

Commonly, AIT is performed by subcutaneous injections (SCIT) of increasing doses of allergens, starting with weekly intervals (build-up phase), followed by a maintenance phase, during which monthly injections are given. Taking into account that 3 to 5 years of treatment are recommended and, in some cases, life-long treatment might be required, this procedure is extremely laborious and time-consuming, leading to considerable drop-out rates⁵. Moreover, SCIT is associated with side effects ranging from local reactions to anaphylaxis and even near fatal reactions⁶. Moreover, some patients reject this treatment due to fear of needles. Thus, in the 1980s, sublingual immunotherapy (SLIT) has been established as a non-invasive alternative to SCIT, which has meanwhile gained acceptance due to its proven efficacy for treatment of respiratory allergies caused by pollen or house dust mites⁷. This type of therapy has certain advantages over SCIT, as it avoids the use of needles, is especially suitable for children, and avoids systemic reactions. However, as uptake of allergen via mucosal surfaces is quite limited, 50-100-fold the doses applied during SCIT are used for SLIT, usually

as drops or fast dissolving tablets, which have to be kept under the tongue for 1-2 minutes, before being swallowed. Because this can cause severe localized reactions, SLIT is only recommended for patients with relatively mild allergic symptoms and has to be avoided for food allergies. Moreover, this therapy requires daily administration over a period of 3-4 years ⁸.

To overcome the problems associated with standard immunotherapies, recently novel allergy vaccines, application routes, and adjuvants have been explored.

1.2 Novel allergy vaccines

Classical SCIT and SLIT is performed with natural allergen extracts, which contain a variety of allergens with huge variations in amounts and potency, depending on the manufacturer ^{9,10}. To circumvent problems associated with poor quality of allergen extracts, purified recombinant allergens produced under good manufacturing practices are used. For birch and grass pollen allergens there is evidence that they are equally effective compared to extracts ¹¹; however, observed side effects are similar to allergen extracts as the molecules retain their allergenicity. Reduction of side effects can be achieved via denaturation of allergen extracts with aldehydes, leading to impaired recognition by IgE antibodies (allergoids) ¹². Moreover, by genetic engineering, hypoallergenic derivatives such as allergen fragments, mutants, or mosaic molecules have been developed, which retain the immunodominant T cell epitopes, but display reduced IgE reactivity due to removal of major B cell epitopes ¹³. However, hypoallergenic derivatives have been shown to induce systemic late phase adverse reactions, which are independent of IgE, but mediated by T cells ¹⁴. AIT with recombinant allergens in their native or hypoallergenic form requires the same laborious procedure including up dosing and maintenance as with extracts, and causes comparable side effects (native) or late-phase reactions (hypoallergen).

Another promising approach for AIT based on reduced allergenicity is peptide immunotherapy using synthetic T cell epitope-containing peptides from major allergens. Following this treatment, induction of IgG is avoided as the peptides used are too short to induce antibody responses. The

huge MHC diversity among patients requires a large number of different peptides to be included into this vaccine type. Peptide immunotherapy has been shown to require fewer injections but initially has been associated with the induction of late-phase adverse reactions, which could be overcome by modifying peptide length and dosing regimens ¹⁵.

More recently, B-cell epitope-based vaccines have been employed for AIT. This vaccine type contains peptides of a length between 20 and 40 amino acids. Though these peptides are derived from the IgE binding sites of major allergens, they themselves do not have intrinsic IgE-binding capacity. For induction of strong IgG responses, which potentially block binding of IgE to the respective allergen, the allergen peptides have to be bound to protein carriers. This can be achieved by chemical coupling ¹⁶ or production of recombinant fusion proteins ¹⁷. The T helper cell response required for induction of allergen-specific IgG antibody production is directed against the protein carrier. Even if the wild-type allergen as such is a weak immunogen, strong antibody responses can be induced due to this anti-carrier reaction. As B-cell epitope-based allergy vaccines largely avoid allergen-specific T cell epitopes, the risks for developing late phase reactions are reduced ¹⁸. Moreover, because of the reduced allergenic activity of the peptides, high doses of these vaccines are well tolerated, and few injections might be sufficient for successful therapy.

Furthermore, nucleic acid-based vaccines, either as plasmid DNA and mRNA encoding major allergens have been employed for AIT in numerous animal studies ^{19, 20}, and only recently also in clinical studies ²¹.

Notably, none of these “second generation” allergy vaccines has been approved for human use so far.

1.3 Novel routes

Classical AIT via subcutaneous injection or oral ingestion targets tissues with low abundance of immunocompetent cells, i.e. the subcutaneous fat tissue and the oral mucosa, potentially leading to poor immunogenicity and hence to low efficacy of allergy treatment. As early as 1929, the first report

on successful AIT via the skin was published. Only recently, the skin has been re-discovered as an alternative target tissue for AIT, which is rich in immunocompetent cells, and efficiently drained by local lymph nodes²². Concomitantly, the upper layers of the skin lack vascularization, a prerequisite for safe immunotherapy, as systemic spread of high allergen doses is prevented. The main challenge regarding skin immunization in general is to circumvent the outermost layer, the stratum corneum. This can be achieved by disrupting the skin barrier (e.g. via tape stripping)²³ or by using microneedles²⁴. Also, application of allergen as a powder to intact skin, followed by covering the area with an occlusive chamber, which leads to maceration of the skin and uptake of the allergen, has been shown to be a useful approach for skin-based AIT²⁵. However, localized and even systemic side effects have been reported as a consequence of cutaneous AIT²⁶. We have recently employed an infrared laser device for generating micropores in the skin for transcutaneous immunotherapy²⁷. This technique allows for well controlled and adjustable targeting of different skin layers, depending on the number of laser pulses and the energy used.

It is obvious that for AIT using the cutaneous route, hypoallergenic vaccines will be a prerequisite to avoid the side effects caused by local mast cell degranulation. Moreover, efficacy should be increased by including adjuvants, which specifically address skin resident immunocompetent cell types.

1.4 Novel adjuvants

Whereas in North America classical subcutaneous AIT is performed with soluble allergens, in Europe allergen extracts are usually adjuvanted with aluminium hydroxide or calcium phosphate. None of the commercially available SLIT vaccines contains any adjuvant²⁸.

An ideal immunopotentiator for vaccination against allergy has to meet several criteria: As allergy is a TH2-driven response, the adjuvant has to re-direct the immune reaction towards TH1 or a regulatory response. Moreover, besides being well tolerated, adjuvants for allergy vaccines should increase

efficacy, allowing for shortened therapy schedules and at the same time improve the safety profile by reducing the necessary allergen doses.

Being the adjuvant most frequently incorporated into licensed human vaccines and used in AIT since 1935, the mechanisms underlying the immune potentiating effect of aluminium hydroxide have only recently been elucidated. Originally, a depot effect at the injection site, providing a slow release of the antigen and its prolonged exposure to the immune system, has been suggested as mechanism of action²⁹. Aluminium hydroxide has also been proposed to reduce immediate systemic side effects by keeping allergens locally bound at the injection site³⁰. In 2008, it has been demonstrated that the particulate nature of the aluminium gel leads to activation of the Nalp3 inflammasome, and, in the long run, to production of pro-inflammatory cytokines and strong antibody responses^{31, 32}.

Additionally, aluminium hydroxide induces potent CD4-mediated cellular responses, which are TH2-biased³³ and hence are problematic for allergen immunotherapy. Furthermore, aluminium-based adjuvants may cause local granuloma formation following intradermal or subcutaneous injection. Aluminium compounds have also been associated with neurotoxicity and autoimmunity³⁴. As for AIT the total number and the frequency of administrations far exceeds that of normal vaccinations, new adjuvants with increased safety and TH-1 promoting potential are urgently needed for alternative immunization routes such as mucosa or skin.

In murine models, several TH2-downregulating compounds such as toll-like receptor (TLR) ligands, living or heat-killed bacteria, and small molecules such as dihydroxyvitamin D3 have been administered via the subcutaneous or other parenteral routes²⁸, and monophosphoryl lipid A (MPL) and CpG oligodeoxynucleotides have also been tested in human patients^{35, 36}. Bacterial toxins (cholera toxin, heat-labile enterotoxin from *E. coli* as well as genetically modified versions thereof) have been evaluated as mucosal immunopotentiators in pre-clinical models, either by co-administration with allergens, or conjugated or fused to them. Furthermore, TLR ligands have been shown to re-direct the allergic immune response when administered as adjuvants via nasal or sublingual routes. Another approach, using a “regulatory” adjuvant to stimulate the cytokine IL-10 by

mucosal dendritic cells was performed with co-administration of probiotics, i.e. commensal bacteria with the potential to trigger a regulatory immune response. Similarly, but in an antigen-independent approach, different lactic acid bacteria have been tested as stand-alone compounds for their preventive and therapeutic anti-allergic potential ²⁸.

2. Nanoparticles in allergen-specific immunotherapy

Besides adjuvants, which counteract the TH2-driven allergic reaction, a multitude of different particulate delivery systems (summarized in Table 1) has been employed for targeting allergen (or allergen-encoding plasmid DNA) to antigen-presenting cells at the site of administration. The immunopotentiating effect of these vectors is mediated by their particulate nature, which facilitates uptake and transport to secondary lymphoid organs. Additionally, tailored immune response types can be promoted by specifically addressing certain receptors on specialized APCs.

Chitosan, a biodegradable polysaccharide derived from Chitin, has been shown to have immunostimulatory capacities and to promote production of TH1/Treg associated cytokines ³⁷. Subsequently, chitosan nanoparticles have been extensively tested in animal models for their applicability in prevention and/or therapy of allergic diseases. Oral administration of allergen-encoding plasmid DNA complexed with chitosan resulted in TH1 responses, which protected from subsequent challenge with allergen ³⁸. This response type was also observed after an intramuscular booster immunization with the allergen ³⁹. Protection was associated with a shift towards IgG2a antibody production (representative of TH1 polarization in mice) and mucosal IgA ⁴⁰. Chitosan nanoparticles have also been used for intranasal treatment of sensitized mice with allergen extract and led to improvement of several lung parameters upon re-challenge ⁴¹.

With regards to sublingual application, chitosan particles delivering the model allergen ovalbumin have been demonstrated to enhance uptake by oral dendritic cells. As a result IL-10 secreting regulatory T cells (Tr1) were primed in cervical lymph nodes which led to a significant decrease of airway inflammation in asthmatic mice ⁴². A similar effect could be achieved with a mucoadhesive

formulation based on polymerized maltodextrin, which binds to epithelial cells providing prolonged contact of the allergen with the mucosa ⁴³.

Poly(D,L-lactic-co-glycolic) acid (PLGA) particles represent another vehicle, which has been utilized for delivery of allergen via both, the oral as well as the subcutaneous route ^{44, 45}. Immunization with allergens encapsulated into PLGA microparticles has been demonstrated to trigger TH1-biased immune responses in mice ⁴⁶. Furthermore, the efficacy of this particle type has been evaluated not only in a therapeutic setting, but also in a prophylactic approach, using mouse models of birch pollen allergy ^{44, 45}. In follow up studies, the same group demonstrated the immunomodulatory effect of targeting PLGA particles loaded with birch pollen extract to enterocytes or M-cells by functionalization with wheat germ agglutinin ⁴⁷ and *Aleuria alantia* lectin ⁴⁸, respectively.

Polyanhydrides, such as copolymers between methyl vinyl ether and maleic anhydride (PVMA), allow for uncomplicated production of nanoparticles due to the presence of reactive anhydride groups. PVMA nanoparticles are suggested to be interesting candidates for oral immunotherapy as they are able to interact with components of the intestinal mucosa ⁴⁹. Pre-treatment with ovalbumin nanoparticles by oral gavage confirmed their anti-allergic properties ⁵⁰. However, also intradermal injection with the same particles or particles loaded with grass pollen extract adjuvanted with LPS prevented sensitized animals from anaphylactic shock upon allergen challenge ^{51, 52}.

Biodegradable nanoparticles with anti-allergic potential have also been produced from poly(γ -glutamic acid) (γ -PGA), a high molecular weight polypeptide, which consists of γ -linked glutamic acid units and α -carboxylate side chains. γ -PGA nanoparticles loaded with grass pollen extract were efficiently taken up by monocyte-derived dendritic cells from allergic patients, which triggered increased IL-10 production and proliferation by autologous CD4⁺ memory T cells in vitro ⁵³.

Protamine, a cationic peptide of 4kDa, has been used to design biodegradable nanoparticles. When incubating protamine with oligodeoxynucleotides, nanoparticles are spontaneously formed ("proticles"). Such protamine-based, CpG-ODN-adjuvanted particles have been employed as carrier

system for the peanut allergen Ara h 2. Following subcutaneous injection, a favorable TH1-biased response could be shown in a mouse model of allergy⁵⁴.

Similarly, intramuscular injection of plasmid DNA encoding mite allergen Der f 1 formulated with the amphiphilic block copolymer poloxamine Tetronic 704 elicited a strong TH1 biased immune response polarization. In a therapeutic setting, administration of these particles led to a reduction of airway hyperresponsiveness and a significant decrease of the level of inflammatory cytokines in the bronchoalveolar lavage of asthmatic animals⁵⁵.

A particle type, which has already made its way to clinical application, is non-replicating virus-like-particles (VLP). Such particles consisting of capsid proteins from phage Q β coupled to peptides from dust mite allergen Der p 1 proved to be safe and immunogenic in healthy subjects⁵⁶. The TH1 promoting potential of these particles has been attributed to the fact that during self-assembly in bacteria, host RNA is incorporated into the particles, providing ligands for TLRs. A significant reduction of asthma symptoms was observed in mite allergic patients after six weekly injections of these VLPs adjuvanted with CpG-ODN³⁶. Moreover, by conjugation of the cat allergen Fel d 1 to such VLPs in a highly repetitive manner hypoallergenic molecules could be formed, as proven in a mouse model and with human basophils from allergic patients⁵⁷.

Another approach to reduce allergenicity of proteins and develop hypoallergenic molecules is to utilize carbohydrates in order to mask IgE epitopes^{58,59}. Moreover, carbohydrates can be applied for both, specific targeting of antigen presenting cells as well as modulating the resulting immune response⁶⁰⁻⁶². In a mouse model using subcutaneous immunization of the grass pollen allergen Phl p 5, conjugation to sepharose particles enhanced TH1 responses⁶³. Carbohydrate-modified ultrafine ceramic core based nanoparticles (aquasomes) were also studied for their potential to counteract allergic responses. Sensitized mice were treated by intradermal immunizations with OVA-adsorbed aquasomes compared to alum-adsorbed OVA. Following allergen challenge, lower levels of IgE and of serum histamine were observed as well as weaker symptoms of anaphylaxis⁶⁴. Recently, our group

coupled mannan, dextran, and maltodextrin to allergens with the aim to combine hypoallergenicity with specific cell targeting. The data clearly indicate a reduction of the allergenicity of the conjugates, as shown by a significant reduction of their IgE binding capacity. Intradermal immunization of these neoglycoconjugates showed that antigen presenting cells of the skin can be addressed via carbohydrate-specific receptors and thereby increase the immunogenicity of the vaccine. Moreover, this approach does not induce novel IgE production, but instead triggers strong production of IgG, which can act as so-called blocking antibodies^{3, 59}.

Several of the aforementioned approaches utilize highly complex mixtures of carbohydrate-protein conjugates which are difficult to characterize. Lack of batch to batch reproducibility is a common handicap of these methods due to the stochastic nature of the chemical approaches employed, and turned out to be a major hurdle for the development of such allergy vaccines. Therefore, coupling allergens and other moieties to nanoparticle cores of defined size in an oriented and specific way represents a feasible approach to generate standardized, well defined vaccines. Silica is considered an especially attractive material for such core nanoparticles, as discussed in the following section.

3. Silica nanoparticles as therapeutic agents

At present, the use of inorganic nanoparticles, consisting of a solid core to which antigen(s) of interest are conjugated, is widely discussed as potential vaccination strategy for the next decades^{65, 66}. A declared goal of this approach is to trigger a superior immunity by delivery of antigen-associated nanoparticles compared to immunization with soluble antigen alone. Therefore, a major prerequisite is thermal and chemical stability of the particles, thus preventing premature release as well as protection of the respective antigen from degradation. Silica nanoparticles (SNPs)⁶⁷ meet these requirements, and moreover, they are synthetically well accessible. Their size can be adjusted from 3 to several 100 nm,, their surface can be “functionalized” with a huge variety of different organic, biological or other functional moieties, (reservoir) pores of various size can be included in the particle core, and they are biocompatible and – depending on the synthesis conditions – also

biodegradable^{68, 69}. Ultrasmall silica nanoparticles (C dots) have been already approved by the FDA for melanoma diagnostics^{70, 71}. In addition to solid or porous particles, hollow particles as well as core shell particles or even hierarchical structures can be produced quite easily. Figure 1 gives an overview of the properties of silica nanoparticles that can be controlled and tailored in a very predictive manner to ideally fit the needs for the specific applications.

3.1 Preparation of silica nanoparticles

For the preparation of silica-based nanoparticles either gas phase processes, such as flame pyrolysis or solution-based processes, such as the Stöber method or synthesis in reverse microemulsions is applied^{72, 73}. Stöber processing involves the hydrolysis and condensation of silicon alkoxides (e.g. tetraethoxysilane) in the presence of a basic, aqueous catalyst (ammonium hydroxide) and a solvent (ethanol), generally also termed sol-gel chemistry. Careful control of the reaction conditions, such as concentrations of the precursors, pH value, temperature, stirring rate, etc., allows the deliberate synthesis of spherical, non-porous and non-aggregated particles with a rather monomodal size distribution from sub-10 nm up to several 100 nanometers. In reverse microemulsions – a water-in-oil (W/O) emulsion – surfactant molecules are dissolved in organic solvents forming spherical micelles that provide a water-filled nanometer-sized core. In principle these cores can be considered as very small nanoreactors, in which sol-gel processes of silica precursors can occur resulting in the formation of spherical particles limited in growth by the size of the aqueous core. Depending on the synthesis conditions, such as surfactant/ water ratio monodisperse silica particles can be obtained. A very common, industrially applied method for the synthesis of silica nanoparticles is the high temperature flame pyrolysis. In a typical process, silicon tetrachloride, SiCl_4 , is reacted with hydrogen and oxygen. However, the particle size and degree of agglomeration as well as morphology is very difficult to control. In contrast to the above-mentioned solution-based approaches, agglomerated or even aggregated particles are obtained with a larger variation in their sizes.

3.2 Functionalization of silica nanoparticles

In principle, one has to distinguish between physical adsorption of molecules on i) the particles surface or ii) inside the particles cores from chemical anchoring of functional units to the particles via abundant silanol groups (see Fig. 2). From a synthetic viewpoint different strategies can be considered. On the one hand, functional moieties, e.g. luminescent dyes, or membrane-anchoring molecules, can simply be added into the synthesis mixture such as a W/O microemulsion, resulting in e.g. monodispersed nanoparticles composed of an inorganic luminescent dye physically adsorbed inside the silica matrix, to name just one example ⁷⁴. In addition, functionalization can be done postsynthetically by reaction of the preformed silica nanoparticles with the respective functional moiety. A major drawback of simple physical adsorption is the facilitated leaching of the adsorbed molecules. Stronger chemical interactions can be formed by reacting the nanoparticles surface exposed silanol groups via different chemical procedures ⁷⁵. A commonly used method is the co- or postsynthetic condensation with chloro- or alkoxy silanes, e.g. introducing chemical linkers with different functions, such as amine, thiol, carboxyl, or methacrylate ^{76, 77}. Also azido groups have been successfully grafted to bromopropyl-modified silica particles, functionalizing them for copper mediated click chemistry ⁷⁸. Due to its high selectivity and yield, and mild reaction conditions ⁷⁹, click chemistry is a powerful approach to couple immunomodulatory molecules to SNPs. Meanwhile, various polysaccharides or TLR ligands are commercially available with azide- or alkyne-modifications. We have previously used click chemistry to covalently link hen egg lysozyme (HEL) and the beta-glucan laminarin to SNPs as a proof of concept for generating a multifunctional vaccination platform for AIT (Fig. 3A). Both molecules have been successfully linked to the silica core, resulting in a homogeneous size distribution of the conjugated particles (Fig. 3B). The different functionalization strategies have recently been reviewed in detail ⁷³.

3.3. Mesoporous silica nanoparticles (MSNs)

Compared to SNPs, mesoporous particles provide add-on functionalities, such as a higher specific surface area (up to 1000 m²g⁻¹) resulting from mesopores that yield increased loading capacities and allow for the delivery of cargo molecules of specific sizes ^{80, 81}.

According to IUPAC⁸² a mesoporous material is defined as a solid, containing pores in the diameter range from 2 and 50 nm^{83, 84}. In particular, the reports by Kresge and Beck on the family of silica materials containing periodically arranged mesopores, including MCM-41 and MCM-48 exhibiting 2D hexagonal and 3D cubic arrangements of pores, respectively, have quickly paved the way to diverse types of ordered MSNs. The range of porous materials synthesized by combining surfactant micellar aggregates with reactive silica precursors has been extended tremendously in the last 20 years and nowadays a precise tailoring of particle sizes, morphology and pore size and connectivity is possible by adjusting the reaction parameters such as concentrations, pH value, chemical nature of the surfactants, temperature, and time⁸³⁻⁸⁵. MSNs have tunable nanoscale sizes, different shapes ranging from spheres to rods, uniform cylindrical mesopores, high surface areas, and similar as for SNPs easily functionalizable surfaces⁸⁶⁻⁸⁸. Figure 4 shows typical examples of TEM images of SNPs, MSNs and hollow silica capsules.

Based on these properties, it is obvious to use them as a delivery vehicle by adsorbing the desired entity onto the particles followed by release by dissolution in the target medium. In this case, the rate of release only depends on the solubility of the adsorbed molecule in the surrounding environment. More elegant approaches are based on chemical modifications with so-called gate keepers. Gate keepers are molecules that block the pore entrances and open only by internal or external triggers. With that stimuli-responsive nanoscale particles are easily accessible^{89, 90}.

Utilizing a broad spectrum of physical and chemical properties enables an elegant strategy to functionalize SNPs and MSNs and thus rationally design the optimal release of biomolecules. For example, positively charged functional groups enhance endocytosis and enable increased binding of negatively charged proteins to the particles⁹¹. In addition to their potential to act as antigen carriers for directed and/or controlled release and for co-delivery of adjuvants, MSNs have displayed inherent adjuvant properties in vaccine formulations⁹²⁻⁹⁷. Several studies have therefore investigated the potential of MSNs and SNPs as vaccine carriers. Encapsulation of the *E. coli* bacterial protein

*Int1β*⁹⁴ or the model antigen bovine serum albumin (BSA)⁹² into MSNs resulted in more potent antibody responses compared to the same protein emulsified with complete Freund's adjuvant, especially in low-responder mouse strains. Similarly, a protein from Porcine Circovirus Type 2 was more immunogenic when adsorbed to MSNs and induced long-lasting antibody responses as well as increased secretion of IFN-γ by splenocytes⁹³. Enhanced cell-mediated immune responses with a mixed TH1/TH2 profile were also observed after vaccination with bovine viral diarrhea virus E2 protein adsorbed to MSNs⁹⁸. A possible explanation for the immunostimulatory property of SNPs is their activation of the Nalp3 inflammasome, which seems to be similar to aluminium salts⁹⁹. This assumption is in agreement with a study comparing SNPs with alum upon immunization with hepatitis B core antigen with and without MPL¹⁰⁰.

SNPs and MSNs have also been used for the delivery of antigens via the mucosal route. Oral immunization with SNPs functionalized with BSA led to mixed TH1/TH2 IgG responses and secretion of mucosal IgA⁹⁶. In a recent study, the combination of SNPs as delivery vehicle together with the mucosal adjuvant c-di-GMP proved to induce strong systemic and mucosal antibody production against an H1N1 influenza vaccine after intratracheal application¹⁰¹.

Depending on their size, SNPs are able to permeate human skin, e.g. via hair follicles of cyanoacrylate tape-stripped skin (which leads to opening of the hair follicle orifice), and can be taken up by Langerhans cells¹⁰². The size of the SNPs used in this study was 42±3 nm. In contrast, in another study, SNPs with 55 ± 6 nm did not penetrate the skin of SKH1 mice with or without normal tape-stripping, or after induced contact dermatitis¹⁰³. This indicates that topical application of nanoparticles for AIT will have to be combined with techniques highly efficient in disrupting the skin barrier, e.g. using infrared laser ablation¹⁰⁴.

In contrast to the majority of studies, which observed TH1 or mixed TH1/TH2 immune type responses after immunization with SNP vaccine delivery systems, a recent publication showed that house dust mite extract was more allergenic in an atopic dermatitis mouse model when agglomerated with SNPs

¹⁰⁵, and similarly, lung inflammation was exacerbated in an allergic mouse model when SNPs and ovalbumin were co-applied during the inhalation phase ¹⁰⁶.

4. Conclusion

Nanoparticles are attractive vaccine carriers for tailor-made allergen-specific immunotherapy. Several promising approaches have provided proof of concept in preclinical models for this approach. Of the numerous possible nanoparticle materials, SiO₂ based nanoparticles have gained increasing interest for vaccine development due to their biocompatibility, ease of functionalization and their inherent adjuvant property. First preclinical studies have confirmed the high potency for SNPs as general vaccine carriers and adjuvants, and they are also promising candidates for the development of versatile vaccination platforms for the treatment of allergies. However, it has to be kept in mind that depending on the type of particle, the use of additional adjuvants, and also the route of administration, adverse reactions cannot be excluded. In the final section of this review, we will therefore discuss the pre-requisites for a SiO₂ nanoparticle based vaccination platform for AIT.

5. Expert opinion

Future AIT will combine novel vaccine formulations with more immunogenic routes and patient-friendly application forms. Especially the skin has gained a lot of attention for AIT due to its richness in antigen presenting cells, its low degree of vascularization and efficient lymphatic drainage, and the prospect for painless vaccine application. Several methods have been developed to disrupt the stratum corneum, the major barrier for topical application of vaccines ¹⁰⁷. One especially promising method is fractional laser ablation, which can introduce micropores of precise shape and depth to target specific skin layers ¹⁰⁴, and additionally induce an adjuvant effect through the induction of damage associated molecular patterns (DAMPs) ^{108, 109}. We have previously demonstrated that this method shows higher efficacy and less side effects compared to classical immunotherapy in a mouse model of grass pollen allergy ²⁷. However, previous attempts in the clinics have shown that application of natural allergen extracts to severely barrier-disrupted skin may have intolerable side

effects due to the crosslinking of allergen-specific IgE on skin resident mast cells²³. Attempts have been made to reduce the allergenicity of allergens by modifying their surface through glutaraldehyde or formaldehyde crosslinking (allergoids) or changing their structure to destroy conformational B cell epitopes (hypoallergens)³⁰. More recently, nanoparticle formulations have been evaluated for their potential to enhance the immunogenicity of allergens and at the same time reduce the allergenicity of the protein. Such formulations are therefore promising candidates for allergen immunotherapy via the skin.

We have recently demonstrated that nanoparticles generated by crosslinking of allergens with carbohydrates fulfill the most important requirements for safe and efficient immunotherapy⁵⁹. For example mannan, a biodegradable linear polymer of 1,4-linked mannose residues is a promising candidate for drug nanocarrier systems¹¹⁰. Mannosylated particles can target antigen presenting cells via carbohydrate recognizing receptors such as DC-SIGN, Dectin -2, and mannose receptor⁶⁰ and thus exert immune stimulatory effects, e.g. through binding to mannan-binding-protein, initiation of the complement cascade, or interaction with TLRs⁶¹. Conjugates based on the crosslinking of the major birch pollen allergen Bet v 1 with mannan have an average hydrodynamic radius of approximately 9-10nm, a >100.000-fold reduced capacity to crosslink Bet v 1-specific IgE on basophils and to target APCs by a receptor-mediated mechanism (Fig. 5). Thus, these conjugates fulfill the requirements for AIT via skin. Indeed, we could recently show that application of Bet-Mannan to microporated skin using a fractional infrared laser (P.L.E.A.S.E. Professional, Pantec Biosolutions) induces stronger antibody responses compared to intradermal injection of the same formulation (manuscript in preparation). Based on these results we consider the combination of a hypoallergenic APC-targeting formulation together with an application method, which deposits the compound in the upper most skin layers as a highly promising approach for future AIT.

One caveat for the use of carbohydrate/protein conjugates based on crosslinking is the heterogeneity of the carbohydrate used, which results in polydisperse mixtures of carbohydrate-protein complexes that are difficult to characterize. Also, based on the number of reactive groups in

both the carbohydrate and the protein, coupling efficacy is highly variable requiring extensive case to case optimization and characterization of conjugate vaccines.

Due to these considerations we postulate that SNPs resemble an optimal biodegradable vaccine carrier of defined size, which can be easily functionalized to bind antigens and immunomodulatory compounds to target APCs and polarize immune responses in a desired way. In terms of AIT, one functional group can be used to covalently attach an allergen of interest, while a second group can link a polysaccharide. Based on the polysaccharide of choice, different immune polarizing properties can be incorporated. For example, mannans are bioactive and biodegradable¹¹⁰ and have been successfully used as adjuvant to generate high IgG titers and TH1/TH17 biased immunity¹¹¹⁻¹¹⁴. Beta glucans, such as laminarin, are known to be strong agonists of the C-type lectin receptor Dectin-1 which can drive strong TH1 polarization independent of TLR signaling¹¹⁵. More recently, it has been shown that Sialyl-lactose modified antigens are able to promote tolerance by inhibiting T cell proliferation and promoting *de novo* generation of regulatory T cells by establishing a regulatory program in dendritic cells dependent of Siglec-E¹¹⁶. Thus, based on the carbohydrate, different immunomodulatory functions can be incorporated into silica particles to address specific requirements for vaccination. Further functions can be incorporated into the linkers used to covalently attach the carbohydrate or proteins to the nanoparticles, such as delivery of the antigen to the endolysosomal compartment of APCs, by pH dependent release¹¹⁷ or specific cleavage by endolysosomal proteases.

These combinatorial possibilities enable to develop vaccine formulations based on the same SNP platform that simultaneously address different levels of anti-allergic immunological mechanisms of AIT, such as the induction of high levels of IgG (blocking antibodies), immune deviation (induction of TH1) or immune suppression (induction of Tregs). Such a vaccination platform will not only allow for a rational design of novel vaccines for AIT, but also has broader implications concerning tailor-made vaccines against infectious diseases, cancer, and autoimmunity.

Declaration of interest

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Fig. 1: Silica nanoparticles can be modularly assembled with different physical and chemical properties and functionalized with a huge variety of ligands for biological targeting

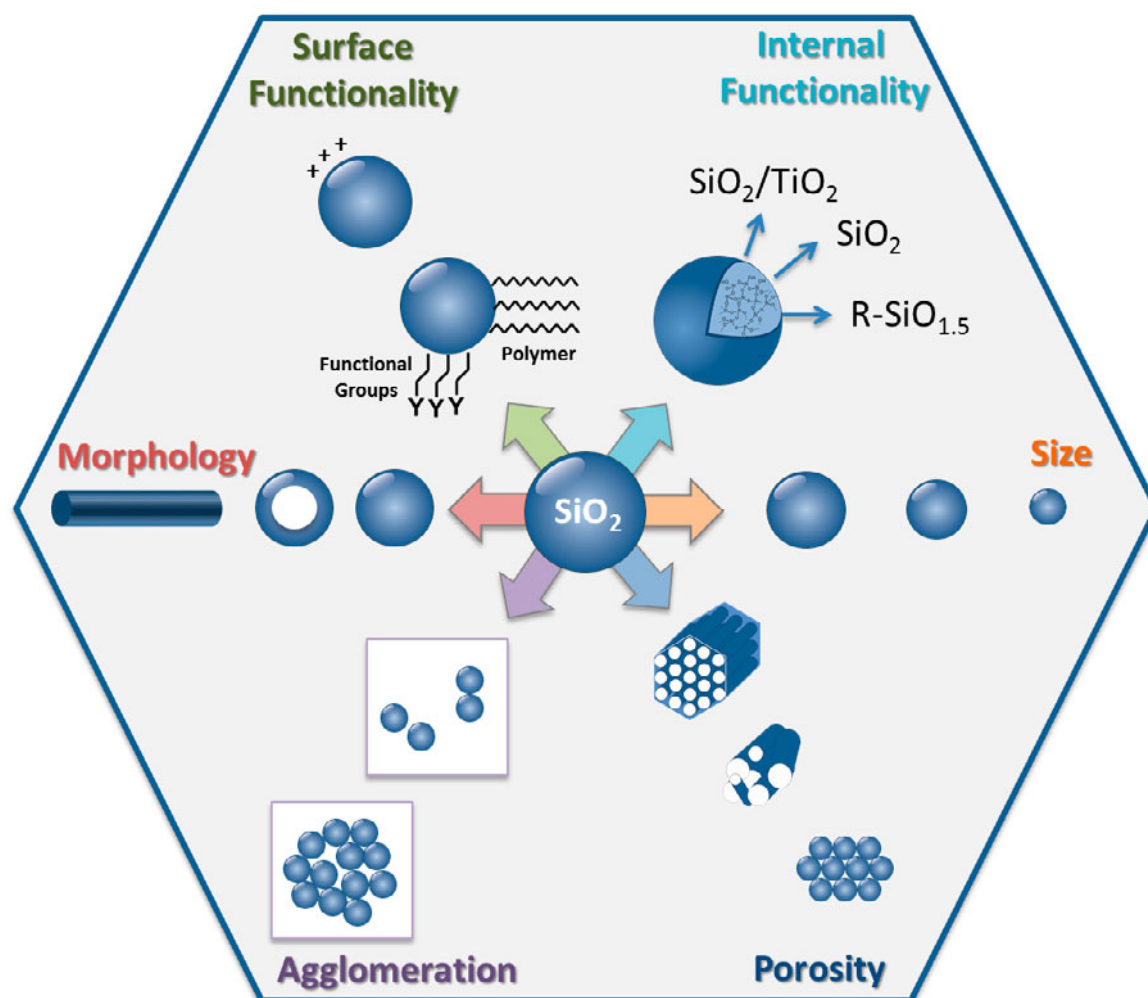


Fig. 2: Possible interaction schemes of SNPs with an antigen of interest

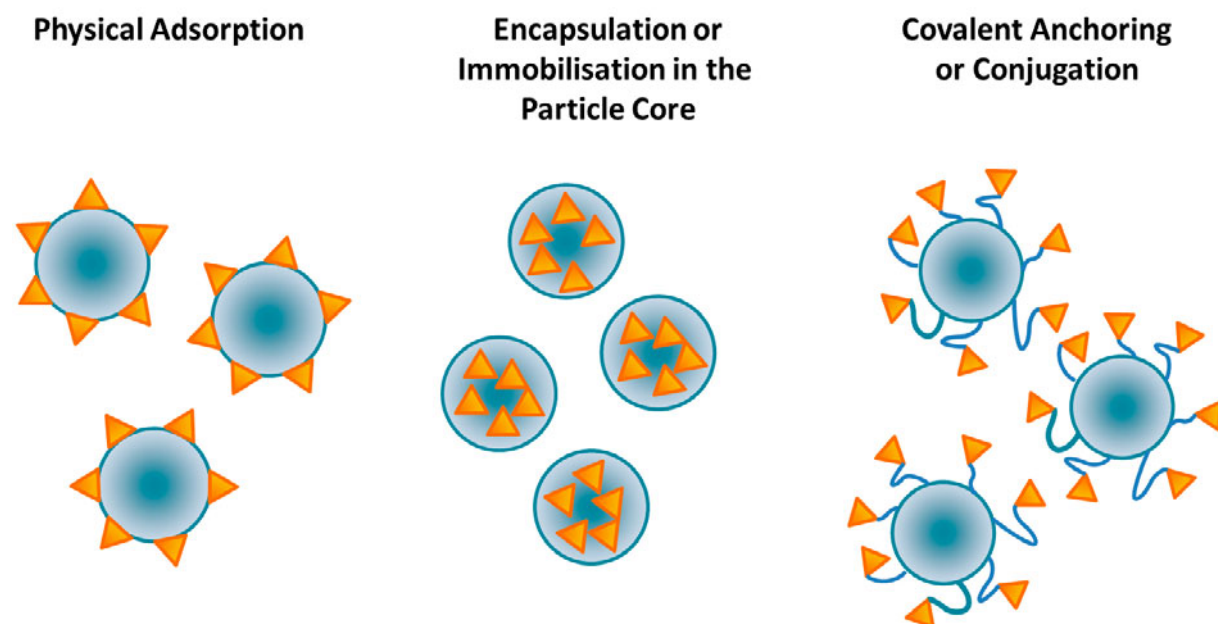


Fig. 3. SiO₂ nanoparticle (SNP) based vaccination platform for AIT. A) SNPs can be used as core particles with defined physical parameters such as size and surface charge. Using chemically functionalized linkers, different proteins and polysaccharides can be covalently linked to the same core particles, e.g. by click chemistry. Polysaccharides of sufficient length can interfere with the binding of IgE to the allergen linked to the SNP surface, thereby eliminating immediate phase reactions. B) Proof of concept for click chemistry based conjugation of hen egg lysozyme (HEL) and Laminarin to azido functionalized SNPs. Hydrodynamic radius before conjugation (85nm) and after conjugation (275nm) were analyzed by dynamic light scatter.

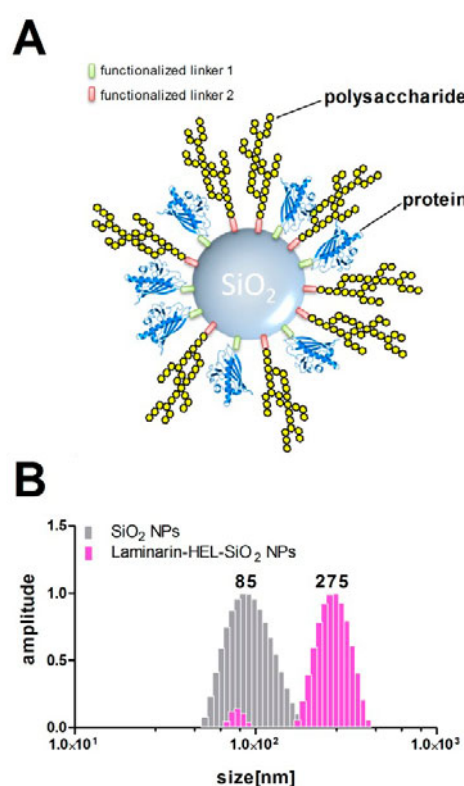


Fig. 4: Left: TEM image of solid SiO_2 spheres of about 17nm in diameter prepared via a modified Stöber approach; middle: mesoporous silica particles of about 100-200 nm in diameter and right: Hollow silica particles from an emulsion synthesis.

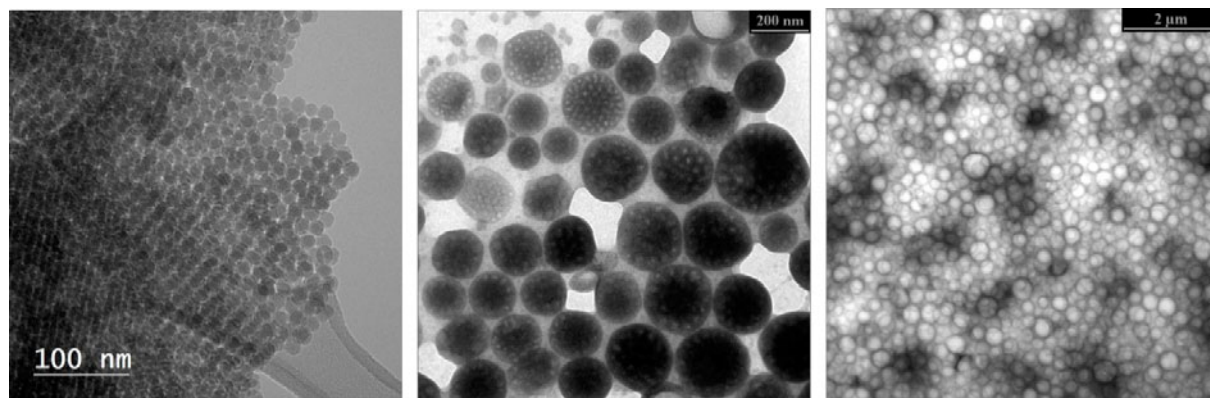
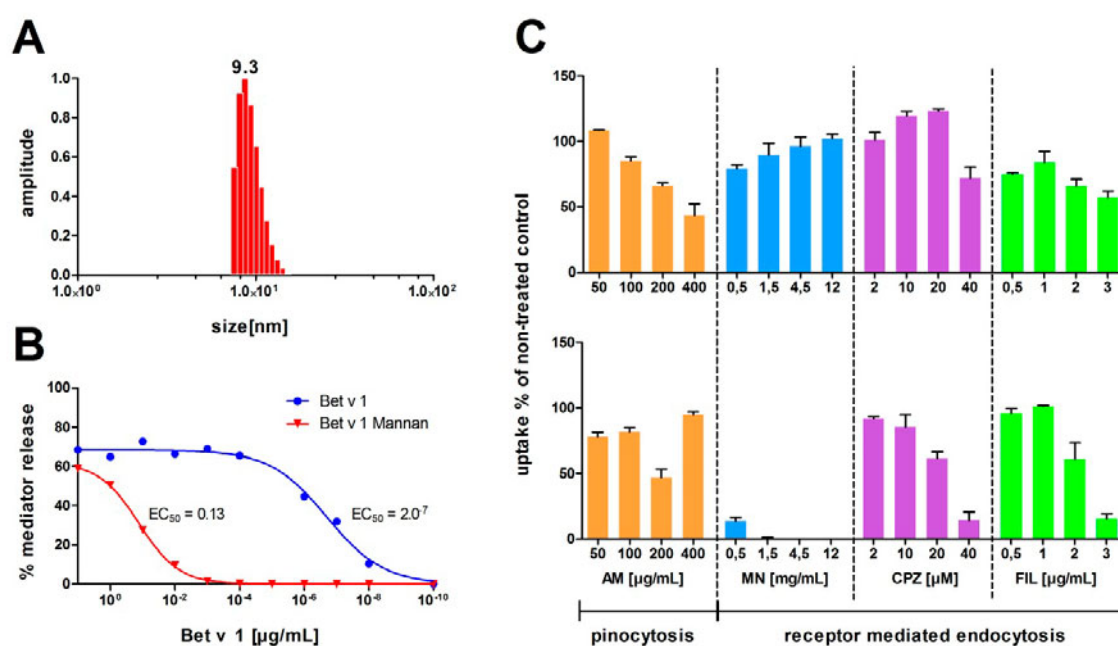


Fig. 5. Characteristics of Bet-Mannan neoglycoconjugates. A) Conjugation of Mannan to the major birch pollen allergen Bet v 1 resulted in conjugates (Bet-MN) with an average hydrodynamic radius of 9.3nm as determined by dynamic light scatter. B) Hypoallergenicity of Bet-MN was determined by loading rat basophil leukemia (RBL) with Bet v 1-specific IgE followed by incubation with decreasing concentrations to induce β -hexosaminidase mediator release. The effective concentration needed to induce 50% degranulation (EC_{50}) was calculated from the curves. C) Uptake mechanism of Bet-MN was studied by incubating bone marrow derived dendritic cells (BMDCs) with 20 μ g/mL of Bet or Bet-MN in the presence or absence of specific inhibitors. Amiloride (AM) is an inhibitor of pinocytosis. Mannan (MN) competes for binding to MN-specific receptors on the surface of BMDCs. Chlorpromazine (CPM) and Filipin (FIL) inhibit receptor mediated uptake via clathrin coated pits and caveolae, respectively.



Nanoparticle type	Delivered allergen(s)/Source	Route	Therapy	Reference
Chitosan-DNA	Der p 2 / mite	oral	-	38
Chitosan-DNA	Der p 1 / mite	oral	-	39
Chitosan-DNA	Ara h 2 / peanut	oral	-	40
Chitosan	Dermatophagoides farinae extract	i.n.	+	41
Chitosan	Ovalbumin / egg white	sublingual	+	42
Maltodextrin conjugate (mucoadhesive)	Ovalbumin / egg white	sublingual	+	43
PLGA	Bet v 1 / birch	s.c., oral	+	44, 45
PLGA (functionalized)	Birch pollen extract	oral	+	47, 48
PVM-MA	Ovalbumin / egg white	oral, i.d.	+	50, 51
PVM-MA	Lolium perenne extract	i.d.	+	52
γ -PGA	Phleum pratense extract	<i>in vitro</i>	-	53
Protamine	Ara h 2 / peanut	s.c.	-	54
block copolymer-DNA	Der f 1 / mite	i.m.	+	55
VLP	Der p 1 peptide / mite	i.m., s.c.	-	56
VLP	mite extract	s.c.	+	36
VLP	Fel d 1 / cat	s.c.	+	57
aquasomes	Ovalbumin / egg white	i.d.	+	64
neoglycoconjugates	Ovalbumin / egg white Papain / papaya	i.d.	-	59
neoglycoconjugates	Grass pollen allergoids	s.c.	-	58

Table 1. Nanoparticle formulations used for delivery of allergens or allergen-encoding plasmid DNA for prophylactic and therapeutic approaches against type I allergic diseases. PLGA: poly(D,L-lactic-co-glycolic) acid; PVM-MA: Poly(methyl vinyl ether-co-maleic) anhydride; γ -PGA: poly(γ -glutamic acid); VLP: virus-like particle