

Review

Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy

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Abstract

For the treatment of infectious diseases, cancer and allergy, the directed induction of an appropriate immune response is the ultimate goal. Therefore, with the development of pure, often very small proteins, peptides or DNA by molecular biology techniques, the research for suitable adjuvants or delivery systems became increasingly important. Particle formulations are made of a variety of materials, including lipids, proteins or amino acids, polysaccharides, polyacrylic substances or organic acids. Microparticles serve as vehicles and provide a depot for the entrapped or coupled antigen. The release occurs in a pulsatile or continuous manner, a feature, which is well controllable for many particulate systems. Particles attract antigen presenting cells to the administration site, thereby guaranteeing the efficient presentation of the antigen to the immune system. Importantly, particles also protect the entrapped substance. This is especially necessary after oral application to avoid gastric or tryptic breakdown. In this article, the design and construction of different antigen delivery systems and their immune effects, with special focus on the suitability for allergy treatment, are discussed.

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1. Introduction

During type I allergy, IgE antibodies (in mice IgE and IgG1) are formed against harmless antigens. These antibodies bind to the high-affinity IgE-receptor on effector cells, such as mast cells and basophils. Upon allergen contact mediator release is triggered from these cells, which is responsible for the symptoms (hay fever, asthma, urticaria, food allergy, anaphylactic shock). T-helper (Th) lymphocytes of the Th2-type as well as mast cells are predominantly responsible for interleukin (IL)-4 and IL-13 production and the isotype switch towards IgE [1]. The resulting immune reactivity pattern in allergy is, therefore, called Th2 response. The only causative treatment is the immunotherapy, which consists of repetitive allergen applications and results in permissiveness of the allergen. Allergen immunotherapy is described to induce a so-called modulated Th2 response, which is characterized by antibodies of the IgG4 subclass [2,3]. These antibodies trap the allergen before triggering of effector cells can occur (blocking antibodies). In murine models, a shift towards Th1-type antibody IgG2a is indicative for successful therapy.

Most protein antigens and allergens (especially when used in small quantity) need to be administered with an adjuvant to assure an immune response of high

quality and long-lasting memory [4]. In addition, smaller polypeptides (<10 kDa), haptens and non-protein antigens have to be conjugated to immunogenic carriers to become complete immunogens. To date, the only acknowledged adjuvants for the usage in humans are aluminum salts and MF59, a squalene microemulsion [5]. In the treatment of type I allergy, exclusively aluminum hydroxide (alum) is applied for the formulation of allergen extracts. Paradoxically, alum supports a Th2 response [6,7]. As a consequence, strong boosters of IgE are seen during the onset phase of allergen immunotherapy, later followed by IgG4. In this context, IgE-induction towards non-relevant proteins in the extract has been observed. Alum as adjuvant deposits the antigen only for 2–3 weeks [4]. Therefore, repeated injections are needed to achieve a sufficient immune response. As alum is no natural metabolite of the human organism, side reactions (e.g., granulomas) at the injection sites may be observed [8,9].

2. Antigen delivery systems

The obvious need for improved and optimized adjuvants led to the development of controlled release systems, which in most cases were invented for

antigens or drugs before their application in the field of allergy was considered (Table 1). In general, the adjuvant effect can be divided into two principal components: (1) antigen depot and delivery and (2) immune potentiation by targeting antigens to antigen presenting cells (APC), for instance, macrophages and

Table 1
Historical overview of the usage of particulate systems

Material	First report on antigen/drug delivery (year [reference])	First used for allergen delivery	Used in humans (×) or animals (#); approved by FDA (+)
Alum	1926 [210]	1937 [211]	×, #, +
Acrylic microspheres	1982 [13]	1982 [212]	× (not approved as pharmaceutical)
Alginate	1978 [213]	1967 [214]	×
Amino acid-based particles	1981 [40]	1981 [215]	×, + (polylysine)
Archaeosomes	1997 [216]	–	
Aspasomes	2004 [42]	–	
Bacterial ghosts	1999 [44]	–	
Chitosan	1988 [217]	1999 [78]	×, + (for human dietary consumption)
Cochleates	1989 [218]	–	×
Collagen	1973 [219]	–	×, + (for wound healing)
Gelatin	1977 [220]	1986 [221]	×, + (gelatin capsules)
ISCOMS	1984 [105]	1993 [110]	×, #
Liposomes	1975 [222]	1982 [223]	×, + (for liposomal injections of different drugs)
Niosomes	1986 [224]	–	
PLGA	1981 [225]	1994 [175]	×, + (as polymer for implants, and as drug delivery system)
Protamine	1996 [226]	–	×, + (protamine sulfate as adjuvant)
Proteosomes	1988 [227]	–	×
Sepharose	1997 (as agarose) [228]	2002 [203]	+ (for plasmapheresis filter membranes)
Starch particles	1990 [229]	2000 [230]	+ (as cyclodextrin-based formulations)
Transfersomes	1992 [231]	–	
Vesosomes	1997 [152]	–	
Virosomes	1992 [232]	–	×
Virus-like particles	1995 [233]	2002 [209]	

Table 2

The mechanism of immune stimulation depends on the size and administration route of particles

Particle size	Route	Effect
200 nm	i.v.	phagocytosed by Kupffer cells (macrophages), reach the lysosomal compartment in liver tissues [11,182,183,234]
1–10 µm	s.c.	phagocytosed by macrophages, which are recruited to the site of administration after s.c. injection⇒intracellular delivery of the antigen for processing by *the MHC II exogenous antigen pathway⇒TH-response, antibody production, or *MHC I endogenous pathway⇒CTL-response, cytotoxic lymphocytes [182,183]
>10 µm	s.c.	act as a depot, release the antigen in a second step
<5 µm	orally	effectively taken up by M-cells of PP and carried by macrophages to the mesenteric lymph nodes and spleen [182,183]
5–10 µm	orally	remain in the PP during 35 days [182,183]
>10 µm	orally	not absorbed at any point in the gastrointestinal tract [11]

dendritic cells [10]. Vaccine delivery systems are generally particulate, e.g., emulsions, microparticles, immune stimulating complexes (ISCOMS) and liposomes. The particulate characteristics facilitate uptake by APCs and transport to secondary lymph organs, resulting in the induction of immune responses (Table 2) [4,10]. They are recruited to the injection site or, upon oral application, adhere to the Peyer's patches (PP), which could facilitate the antigen presentation [11]. An amazingly high number of particulate antigen delivery systems has come up in the last two decades (Fig. 1). The most promising novel tools are reviewed here in alphabetical order with focus on the resulting type of immune response. Our major interest is the development of vaccines for the treatment of IgE-mediated hypersensitivity and, therefore, we discuss the possible application of the various particles for the entrapment of allergens.

2.1. Acrylic microspheres

Polyacrylamide is not classified as biodegradable, as it is only slowly metabolized in vivo. Although the monomers used for the preparation are toxic, the

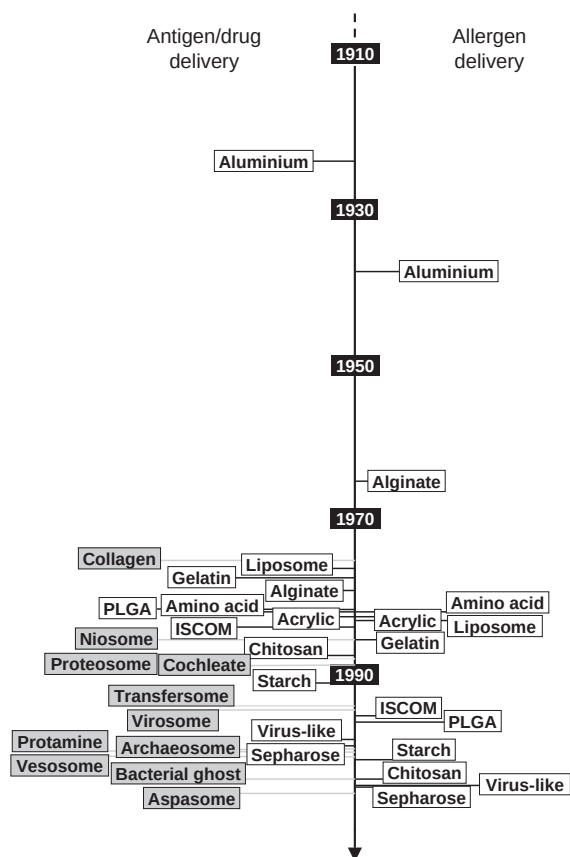


Fig. 1. The introduction of the different particle formulations for antigen/drug delivery or allergen delivery mirrors the rapid progress in the development of these formulations during the last two decades. In contrast, the application of alum as adjuvant started already in 1926, and since then, it is broadly used in vaccination. Grey panels: formulation only used for antigen delivery, white panels: also used for allergen delivery.

polymer is neither toxic nor immunogenic [12]. In mice it has been shown that polyacrylamide in particle form is able to elicit a significantly higher IgG antibody response to the immobilized enzyme L-asparaginase than to the soluble antigen. The delivery via the intravenous (i.v.) or intraperitoneal (i.p.) route was more effective than antigen administered intramuscularly (i.m.) or subcutaneously (s.c.), which induced only low antibody titers [13].

Polymethacrylic microspheres with ovalbumin (OVA) were used for oral immunization of rats. They were capable of sustaining a memory secretory IgA immune response, which was significantly higher than in the control group fed with soluble antigen alone

[14]. Particles of the polymethacrylate type were also able to break an established tolerance to OVA in BDF1 as well as in BALB/c mice [15]. Feedings with OVA in polymethacrylate particles before i.p. injection of OVA in alum could abolish this suppression (total serum anti-OVA antibody titers up to eight times higher). In contrast, the injection of OVA adsorbed to alum in tolerized mice induced only marginal levels of anti-OVA antibodies. In the OVA-polymethacrylate mice the proliferative response from splenocytes and the production of IL-4 (52 pg/ml compared to 11 pg/ml) and interferon (IFN)- γ (3500 pg/ml compared to 937 pg/ml) was less suppressed. The authors concluded that Th1 as well as Th2 cell responses could be restored after feeding tolerized mice with OVA in water-soluble, biodegradable polymethacrylic acid co-polymer particles (Eudragit L30D®, Röhm, Darmstadt, Germany).

Not only Th1 and Th2, but also CTL responses from CD8⁺ T-cells for vaccination against viruses and cancer have been evoked by polymethacrylic microparticles (Eudragit E100) [16]. The CD8⁺ response is dependent on antigen presentation by MHC I on APCs. An efficient way to reach MHC I presentation seems to be the direct targeting of phagosomes by microparticles. To release the antigen in a reasonable time period, pH-sensitive particles can be chosen, which will be destroyed by the acidic pH of the phagosome. By this method, the incorporation of MHC I-restricted peptide antigen from influenza A matrix protein into the methacrylate particles led to the induction of a peptide-specific cytolytic activity in human-HLA-A*0201 transgenic mice. The cytotoxicity was 42% after s.c. vaccination with the entrapped protein, which was significantly greater than cytotoxicity induced by injection of peptide alone (16% lysis).

The extract of short ragweed, which plays a role as an inhalative allergen, has been used as model allergen in the formulation of polymethacrylic acid co-polymer microcapsules [17–19]. The extract was solubilized in a solution containing polyvinylpyrrolidone, spray-coated onto a sugar/starch core, and the particles were enterocoated with methacrylic acid co-polymer (Fig. 2). The resulting capsules had a mean diameter of 500 μ m and proved to be stable at pH 1.2 for at least 6 h. These features made them suitable for oral application. A phase I clinical trial in human patients was performed. The treated patients had high titers of

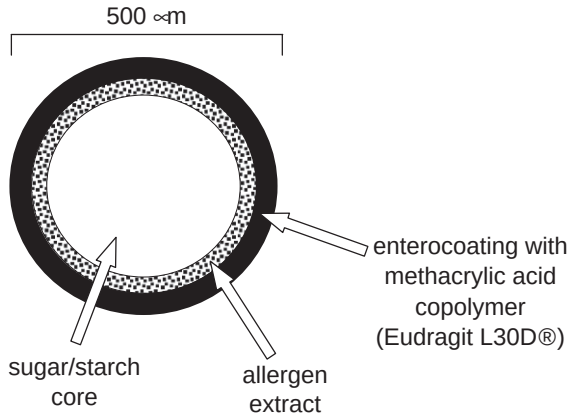


Fig. 2. Schematic presentation of an enterocoated polymethacrylic acid co-polymer microparticle. The allergen extract is solubilized and spray-coated onto the sugar core. Enterocoating is performed with an aqueous dispersion of methacrylic acid co-polymer (Eudragit L30D®). The mean diameter size of such a particle is 500 μm (adapted from Ref. [19]).

serum IgG (especially IgG4, up to 6 $\mu\text{g}/\text{ml}$ compared to <2 $\mu\text{g}/\text{ml}$ in the placebo group) [17] and nasal IgA (6.7 ng/ml vs. 4.2 ng/ml) [19]. The increase of seasonal IgE was less pronounced, and the symptom scores were better in the verum group (3.9 vs. 7.7). In these patients, side effects such as gastrointestinal symptoms were observed with increasing doses of allergen. Diarrhea was seen in 16 events in five patients of the actively treated group compared to only 7 events in six patients of the placebo group [17].

As a further example of an allergen, grass pollen extract has been enterocoated by methacrylic acid [20]. In s.c. primed guinea pigs, this preparation induced a greater secondary antibody response after oral administration (hemagglutinating antibody titer increased from 1 to 8) than the soluble allergen (from 2.5 to 3.5). Besides these immune effects in animals, the principal suitability for oral hyposensitization in humans was proposed in the same study: 75% of the entrapped allergen were protected against breakdown by gastric fluid, in contrast to only 5% of the soluble grass pollen extract [20]. Furthermore, tolerance studies were performed in grass pollen allergic human volunteers. It could be demonstrated that capsules containing enteric-coated allergen extract produced neither gastrointestinal nor allergic side effects.

Another research group used polyacryl starch particles, which are prepared by the polymerization

of acryloylated starch in an emulsion, achieving a mean diameter of 2–3 μm [21,22]. In these studies, the humoral immune response was not monitored, but feedings of mice with OVA encapsulated in microparticles resulted in the induction of antigen-specific proliferative and cytotoxic cellular immune responses ($\text{CD4}^+\text{CD8}^+$ T-lymphocytes). The effects were comparable to those achieved by s.c. immunization with OVA in connection with the more traditional adjuvant DETOX-PC with an antigen-specific lysis of target cells by T-cell lines around 50%. Incorporation of the formalin-inactivated rotavirus into these polyacryl starch particles elicited antigen-specific IgA and neutralizing antibodies in orally immunized mice. During challenges with the virus, these antibodies protected all mice from diarrhea [23]. However, one has to admit that the oral administration of free virus in this study induced two times higher IgA titers and was also capable of 100% protection.

Covalent coupling of another model antigen, human serum albumin (HSA), to polyacryl starch microparticles led to the induction of specific immune responses after parenteral (i.v., i.m., i.p.) as well as after oral immunization of mice [24]. The most pronounced increase after oral application occurred in the IgM and IgG classes. Predominantly IgG1 but also IgG2a increased to approximately 40 ng/ml and 9 ng/ml, respectively, in contrast to the controls, where feedings of free HSA did not induce any detectable antibody levels. These humoral data are supported by splenocyte proliferation studies. The IgG1/IgG2a ratio continuously decreased from 25 to 3.3 during 30 days after polyacryl starch particle administration. Therefore, the authors stress the increasing influence of the Th1 response. In addition, the delayed type hyperreactivity was elevated to 172% in ear thickness tests.

To sum up, the type of immune modulation achieved with acrylic particles is strongly dependent on size, administration route and the way of packing the antigen. Their capability to induce Th1 responses to allergens (and to antigens from *Salmonella* [25]), at least in mice, makes them in principle suitable for allergy vaccination. However, it should be considered that i.v. injection of polyacryl starch particles induced alterations of rodent livers, like vacuolization of the liver cells, necrosis and formation of granulomas [26].

2.2. Alginate conjugates

Alginates are derived from brown seaweed, e.g., *Laminaria hyperborea*, and are the salts of alginic acid, a part of the algae cell membrane with stabilizing functions. Sodium alginate preparations (Conjuvac[®], SmithKline Beecham, Uxbridge, UK) were used to conjugate grass pollen extract. They were compared in s.c. immunizations of BALB/c mice with extract alone and extract adsorbed to alum [27]. Higher allergen-specific total IgG (antibody titer of 40960 vs. 2560), but much lower amounts of IgE (antibody titer assessed by passive cutaneous anaphylaxis, PCA, of 160 vs. 1280) were induced with the alginate-conjugated extract than with unmodified extract. Nevertheless, in an ongoing Th2 response, no suppression of specific IgE could be achieved by immunization with alginate conjugates. The properties of alginate were also investigated in different animal species [28]. In rabbits as well as in guinea pigs, immunizations with grass pollen extract in alginate revealed similar levels of allergen-specific Th2-antibodies as the treatment with alum. These levels of IgG1 were four times higher than after immunization with aqueous pollen extract. On the other hand, immunizations with these particles did not elicit Th2-type antibodies in rats. Conjuvac showed even a suppressive effect, as IgE antibody titers in sensitized animals treated with Conjuvac were below 5 (determined as serum dilution in PCA), whereas in saline-treated rats they were about 160. Histologically, on the Conjuvac injection sites, material could be observed up to 4 weeks with infiltrations of neutrophils and mononuclear cells. In the alum treated group, polymorphonuclear leucocytes as well as large numbers of macrophages were present and injected material could be detected throughout the whole 6 months observation period [29].

In a multicenter hyposensitization study of allergic patients, the efficacy of grass pollen extract conjugated to sodium alginate was evaluated [30]. Nearly all treated patients benefited from the treatment regarding the symptom/medication score, especially running nose and sneezing. Alginate treated patients also showed 96% reduced reactivity to allergen in skin prick test, and 50% in nasal as well as conjunctival provocation. Although about 20

times higher specific IgG could be induced in alginate treated patients than in the placebo group, no significant changes in IgE levels were achieved. As a drawback, 6 of 18 actively treated patients showed side effects like local swelling at the injection site, 5 had rhinitis and 1 urticaria.

Alginate-coupled allergen extract was also used for injection of patients allergic to house dust mites (*Dermatophagoides pteronyssinus*) [31]. In this case, specific IgE levels decreased significantly in alginate treated patients, whereas simultaneously IgG1 and IgG4 levels increased in serum and IgG in nasal secretions. In this study, no linkage between the serological data and the clinical outcome was seen, but in a consecutive study the development of asthmatic symptoms in rhinitis patients allergic to house dust mite could be prevented by immunotherapy with alginate-conjugated extract. In bronchial challenges, a 2.88-fold higher dose was tolerated by the verum group than by the placebo group [32].

For evoking mucosal immunity, this preparation was shown to be effective in oral immunizations of BALB/c mice with entrapped cholera toxin B subunit plus a pneumococcal capsular polysaccharide PS19, or a capsular streptococcal polysaccharide [33,34]. During these feedings, high levels of anti-PS19 mucosal IgA in small intestine wash samples and systemic IgM in sera were induced in animals treated with up to 25 µg antigen, whereas higher amounts of antigen possibly induced tolerance. Interestingly, there was a lack of antigen-specific IgG in serum.

Murine studies with microencapsulated plasmid DNA for bacterial β -galactosidase (LacZ) and/or bovine adenovirus type 3 (BAd3) showed that the route of administration is a critical factor for the type of immune response also with this particle material [35]. In general, IgG titers were higher after parenteral (i.m., s.c., i.p.) than after mucosal applications. On the other hand, the specific IgA titers in serum, lung lavages and feces were two times higher after i.n. administration compared to the parenteral treatment. The observed results indicate that the alginate preparations can be used for mucosal as well as for parenteral treatment. This makes these particles interesting for application in diseases like viral infections, cancer or allergy.

2.3. Amino acid-based particles

Protamine is a cationic peptide of about 4 kDa [36,37]. It is a potential penetration enhancer for phosphodiester antisense oligonucleotides (ODNs). From this material, complexes form spontaneously into nanoparticles (proticles, diameter 90–150 nm). ODNs can be bound to proticles and thereby be protected from DNase I digestion. In this form, ODNs against the *c-myc* proto-oncogene showed an increased cellular uptake into human histiocytic lymphoma cells in vitro. They reduced the cell proliferation to 59.6%. This indicates the usefulness of these proticles in treatment of cancer. Unfortunately, allergic reactions to protamine sulfate have been observed earlier [38].

Other amino acid-based particles of poly(L-leucine-block-L-glutamate) with spontaneous self-assembling characteristics were introduced for the drug delivery, e.g., of human insulin [39]. This amphiphilic material forms a stable, low-viscosity colloidal solution of nanoparticle platelets with a diameter of 200 nm, which are able to spontaneously adsorb proteins, such as insulin, directly from a solution.

Amino acid-based particles have also been examined for their effectiveness in allergen immunotherapy. Polymers of A:D-glutamic acid:D-lysine were linked to short ragweed extract and used for parenteral immunotherapy of ragweed-allergic patients [40]. The clinical outcome was determined by skin test sensitivity and symptom scores. Both remarkably decreased in the groups treated with aqueous extract or with allergen incorporated into particles. The concentrations of allergen necessary to evoke positive reactions in skin tests increased in the verum group 10- to 100-fold. However, besides these positive effects, IgE (in parallel to IgG) increased in serum. Local as well as mild systemic adverse reactions were observed, for example, swelling at the injection site or urticaria.

2.4. Aspasomes

In their first approach, Gopinath et al. used amphiphilic L-O-alkyl glycerols for bilayer vesicle formation (Algosomes), but these particles were stable only for a few days [41]. Recently, scientists used ascorbyl palmitate for formation of bilayer vesicles

(aspasomes) in combination with cholesterol and negatively charged lipid (dicetyl phosphate) [42]. These particles are prepared by film hydration. In the aqueous regions, soluble substances can be encapsulated, e.g., azidothymidine. The release is dependent on the cholesterol content of the particles, although there is no linear correlation between the two factors. The antioxidant potency of the ascorbyl moieties remained even after preparation of the vesicles. This was shown by the in vitro prevention of lipoperoxidation of human blood. In addition, the transcutaneous permeation of the azidothymidine entrapped in aspasomes could be enhanced to a threefold magnitude. These results point towards skin permeation enhancing properties of ascorbyl palmitate. So far, no data about possible immune modulating properties are available.

2.5. Bacterial ghosts

Bacterial ghosts are envelopes of different species of non-living Gram negative bacteria. They are separated from their cytoplasmic content by using lysis gene E, leading to the formation of a transmembrane tunnel. Through this tunnel, the cytoplasmic content of the bacteria is expelled osmotically [43]. In the resulting empty internal space, no bacterial nucleic acids, ribosomes or other constituents should be left. The resulting ghosts share functional and antigenic determinants of the envelope with their living counterparts. Their natural surface make-up provides them with bioadhesive features and targeting functions of their bacterial origin. Thus, they are able to bind to and/or be taken up by specific cells or tissues of animal, human or plant origin.

Native as well as foreign antigens can be cloned into the bacteria before they are lysed. Antigens, drugs or DNA of different origin can, by this way, be presented simultaneously to the immune system. In addition, the bacterial ghosts can be filled with proteins such as streptavidin [44] and other water-soluble substances through the lysis hole [45,46]. In vitro studies revealed that non-covalently loaded drugs (e.g., Doxorubicin, an anthracycline antibiotic for cancer therapy) are released from ghosts in a slow manner [47]. The simple production method, their safety and their versatility as a combination vaccine make bacterial ghosts interesting tools. Freeze-dried

bacterial ghosts are stable without the requirement of a cold chain. They can be effectively administered perorally (p.o.) or by aerosol. Ghosts per se are capable of eliciting Th1 humoral as well as cellular immune responses against cell envelope constituents, including protective (mucosal) immunity against challenge infections. This is due to their immune stimulating compounds, such as lipopolysaccharide (LPS) or lipid A and peptidoglycan [48]. Correspondingly, the i.m. delivery of the major outer membrane protein of *Chlamydia trachomatis* induced a systemic Th1 response in BALB/c mice. The measurement of IL-4 in spleen cells of these animals revealed only low levels [49].

On the other hand, i.m. as well as i.d. immunizations of BALB/c mice with ghosts loaded with the gene for β -galactosidase induced a 1.5 times increment of Th2-antibodies IgG1 and a reduction of IgG3 (four times i.m. and two times i.d.) compared to the groups immunized with naked DNA [50]. Noteworthy, also IFN- γ producing cells were higher in ghost-treated animals, which was explained as a ghost-dependent improvement of the processing and presenting capacity of APC for MHC I-restricted antigens.

In summary, bacterial ghosts enhance the immune response against the transported antigen and thus comprise adjuvant features [51]. Despite their endotoxin content, ghosts of *Escherichia coli* have not induced significant fever responses. This system is described as having a higher specificity for targeting specific tissues than, for instance, liposomes [46]. From these studies it is tempting to speculate that they may also be suitable carrier molecules for allergy therapy.

2.6. Chitosan/chitin microparticles

Chitin is a polymer of *N*-acetyl-D-glucosamine, which naturally occurs in crustaceans (crabs, lobster, shrimp), insects, mushrooms and microorganisms. Chitosan, a polysaccharide, is derived by deacetylation of chitin by boiling it in sodium hydroxide after decolorization with potassium permanganate [52]. Similar to cellulose, it has no mammalian counterpart, but it can be completely biodegraded by lysozymes and *N*-acetyl-D-glucosamidase, which are produced by macrophages [53]. It shows low toxicity, and no

irritating or sensitizing properties have been found so far [54]. Consequently, it has already been approved by the FDA and is in use for the production of prostheses (contact lenses, parodontal tissues, surgical sutures) [55]. Chitosan suspensions or microparticles have also been shown to

- possess immune stimulating capacity, such as the accumulation (via chemotaxis) and specific activation (NO production) of macrophages [56],
- suppress tumor growth [57],
- induce non-specific resistance to infections [58,59],
- induce cytokines like IL-2, IL-10, TNF- α , IL-12, IFN- γ [60,61],
- enhance antibody responses and delayed type hypersensitivity [54,62] and
- activate cytotoxic T-cells as well as natural killer cells [63].

In an in vitro Caco-2 cell monolayer system, chitosan suspensions were able to penetrate the tight junctions and, therefore, allowed the enhanced paracellular transport of co-administered drugs across the epithelium [64–66]. Depending on the chitosan preparation, the model drugs atenolol or ^{14} C-mannitol were transported in 10–40 times higher amounts across the Caco-2 cell layers compared to baseline permeability.

In contrast to the soluble formulation, chitosan particles were endocytosed by the M-cells of PP and biodegraded by macrophages [52,67,68]. By derivatization with different coupling reagents and thiol groups, respectively (thiolated chitosans, thiomers), the mucoadhesiveness, the paracellular transport, the in situ gelling features, the cohesion and the stability could be increased, guaranteeing a prolonged release of embedded substances [69–71].

Chitin is also a major component of fungal spores. As the innate immune system of the lung is well adapted for the phagocytic clearance of airborne spores [72], it initiates Th1 responses to spores and to chitin. Actually, the uptake of chitin particles in the range of 1–10 μ m occurs via mannose receptor-mediated phagocytosis by macrophages [73]. Subsequently, the production of IL-12, TNF- α and IFN- γ is stimulated. In this respect, chitin particles provide an alternative to microbial products (e.g., heat-killed

bacteria or CpG-motifs), which also promote Th1 responses, but were criticized for possible overstimulation of Th1-cytokines, probably leading to autoimmunity [72].

In a ragweed-allergic mouse model of airway inflammation, Shibata et al. studied the effects of chitin as a substance on the allergic response [74]. Oral administration of empty chitin particles as prophylactic and therapeutic treatment resulted in down-regulation of allergen-induced IgE production in ragweed allergic mice by approximately 50% compared to the saline-treated group. Moreover, lung eosinophilia was reduced by over 50% in the prophylactic as well as in the therapeutic approach. These findings were paralleled by increasing ragweed-specific IgG2a/IgE ratios in serum, as well as increasing INF- γ and reduced IL-4 in supernatants of splenocytes. However, these changes in cytokine levels could not be observed in the therapeutic setting. The authors suggested that p.o. administered chitin particles are, at least in part, taken up by M-cells of PP, as has also been shown for poly(D,L-lactic-co-glycolic) acid (PLGA) and latex particles [75,76].

In an approach to cure OVA sensitized mice, animals were treated intranasally (i.n.) with plasmid DNA coding for IFN- γ in chitosan nanoparticles (CIN) [77]. Indeed, airway hyperresponsiveness and lung eosinophilia could be reduced two times and six times, respectively, in CIN-treated mice compared to the untreated or chitosan-treated control group. Th2-cytokines IL-4 and IL-5 were reduced fourfold in a therapeutic and also in a prophylactic approach. In addition, induction of IgE-antibodies was prevented in CIN-treated mice (10 ng/ml vs. 61 ng/ml in PBS-treated animals).

Roy et al. showed the potency of chitosan in targeting, transfection and immunologic protection against peanut allergy in oral gene delivery studies [78]. Mice were fed with a gene coding for the dominant peanut allergen Ara h 2 entrapped in chitosan nanospheres with a diameter of 150–300 nm. These animals were protected from a consecutive allergic reaction upon peanut challenge. This could be due to a three times higher IgG2a concentration in serum compared to the untreated group and four times higher mucosal IgA levels in feces compared to the group treated with naked Ara h 2-plasmid. The increase in Ara h 2-specific IgE was suppressed to

25%. A further allergen-DNA approach in chitosan nanoparticles proved successful for the treatment of house dust mite sensitized mice by priming of a Th1-immune response [79]. Mice were first fed and thereafter injected intramuscularly with chitosan–DNA nanoparticles with a size of about 506 nm. They showed sixfold higher levels of antigen-specific IgG2a than the group fed with empty vector or injected i.m. with naked DNA. Low levels of IgA and only basal levels of IgG1 specific for mite allergens were induced.

In another model, mice were sensitized to house dust mite (*D. pteronyssinus*) or fungi (*Aspergillus fumigatus*) allergens [80]. After daily i.n. immunization with the respective allergen extract, they were treated with PBS alone or with empty chitin microparticles. In this setting, i.n. treatment with the chitin microparticle suspension (<20 μ m) turned out to be effective in reducing total serum IgE by 45% of the house dust mite sensitized mice in comparison to PBS treatment. Impressingly, in the other model *Aspergillus*-specific IgE dropped by 80% and IgG1 by 79%. Peripheral blood eosinophils were reduced by 36% in the dust mite model and by 58% in the *Aspergillus* model. In parallel, intracellular IL-4 levels decreased by 34% and 27%, respectively. Airway hyperresponses could be diminished 1.6- to 3.3-fold, and peribronchial inflammation showed a 60% reduction in the *Aspergillus* model. The Th1-cytokines IL-12, IFN- γ and TNF- α increased 1.7 to 4 times in both models. These changes also remained after constant re-challenge with the allergens, which mimics the situation for individuals exposed to seasonal aeroallergens. The authors state that the Th1-mediated immune response evoked by chitin microparticles may also have a beneficial effect on symptom reduction of human diseases like allergic asthma.

Not only the application of allergens or genes for allergens, but also a peptide representing an immunodominant T-cell epitope (Der p 1 p111–139) of the house dust mite allergen Der p 1 adsorbed to chitosan was effective in inducing tolerance by sustained production of IL-10 [81]. This treatment unexpectedly led to enhanced IgE-titers after immunization with intact Der p 1, possibly due to an inhibitory effect of chitosan on IFN- γ synthesis. These results implicate that chitosan plays an ambivalent role and might not

be the ideal carrier for the treatment of allergic diseases.

2.7. Collagen

The collagen molecule is a polymer of three polypeptide chains that form a triple helix. On both ends of this tropocollagen, there are loose telopeptides, the main antigenic sites of collagen, which can be cut by proteolytic enzymes and result in the so-called atelocollagen (Fig. 3) [82]. Collagen is present as insoluble fibers in the living body, and prepared by pepsin digestion of bovine dermis, purification and sterilization. After this process, the telopeptides are removed, and atelocollagen forms fibers. These fibers do not immediately dissolve when administered to the body, but exist for about 35 days after s.c. administration [83]. Atelocollagen is degraded and absorbed similar to endogenous collagen [82].

Collagen has already been used in several medical applications such as resorbable surgical sutures, in cosmetic surgery, and as haemostatic and wound healing agent [84–86]. Although this material is biocompatible, the optimization for sustained delivery was difficult, because the release is not easy to control. Finally, Sano et al. were able to develop a novel system by creating minipellets of atelocollagen (reviewed in Ref. [82]). These minipellets proved successful as carriers for release of various proteins (HSA, human growth hormone, tetanus toxoid, diphtheria toxoid) [83,87–89]. Although they harbor low antigenicity, they elicited antibody responses similar or superior to conventional s.c. vaccination

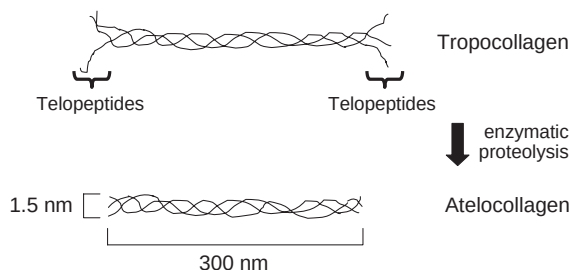


Fig. 3. Schematic presentation of a collagen molecule. The molecule consists of three polypeptide chains intertwined into a triple helix. The loose ends of this tropocollagen, the telopeptides, are the antigenic sites. Removing them by enzymatic proteolysis results in atelocollagen (adapted from Ref. [82]).

with alum. The predominant isotype was IgG1 in both, the alum and the minipellet group [90]. In immunizations of BALB/c mice with tetanus toxoid, the ratio of IgG1/IgG2a antibody titers was 14 in the alum group and 4.5 in the minipellet group. The intramuscular implantation of minipellets with plasmid DNA led to increased levels of gene-encoded protein on the local site as well as in serum [82,91,92].

Another approach was to combine the polysaccharide chitosan with type II collagen, (i) to promote mucosal uptake of native antigens and (ii) to protect the collagen from enzymatic breakdown [61]. In this combination, two times higher IL-10 levels were found in splenocytes of Wistar rats fed with the combined materials. High amounts of mRNA for transforming growth factor (TGF)- β , IL-4 and IL-10 were seen in PP. These results are interpreted by the authors as an oral tolerance induction, which may be beneficial in applications with antigens or allergens. However, evaluation of collagen particles for allergy therapy has not been done so far.

2.8. Gelatin microspheres

Gelatin consists of collagen, which was denatured by heat and hydrolyzed enzymatically, either under acidic or alkaline conditions. Typically, it is derived from bovine or porcine skin or bone by acid or basic extraction [93]. Gelatin preparations comprise polypeptide fragments of different sizes, isoelectric points and gelling properties. There is a high variability from lot to lot with regards to physicochemical features of the material [94]. This is due to the dependence on extraction method, thermal denaturation procedure and electrolyte content. Therefore, attempts have been made to produce collagen as well as gelatin recombinantly in yeast in a standardized fashion [95].

As gelatin can dissolve rather rapidly in aqueous solutions, the usage of the polymer for long-term delivery systems requires cross-linking by formaldehyde, glutaraldehyde, or, according to recent studies, genipin, a compound isolated from the plant *Gardenia jasminoides* (Fig. 4) [96]. These preparations have been shown to be biocompatible and biodegradable in intramuscular immunizations of Wistar rats. The inflammatory reactions and the degradation rate were determined as the density of inflammatory cells in the skeletal muscle tissues. It was significantly greater for

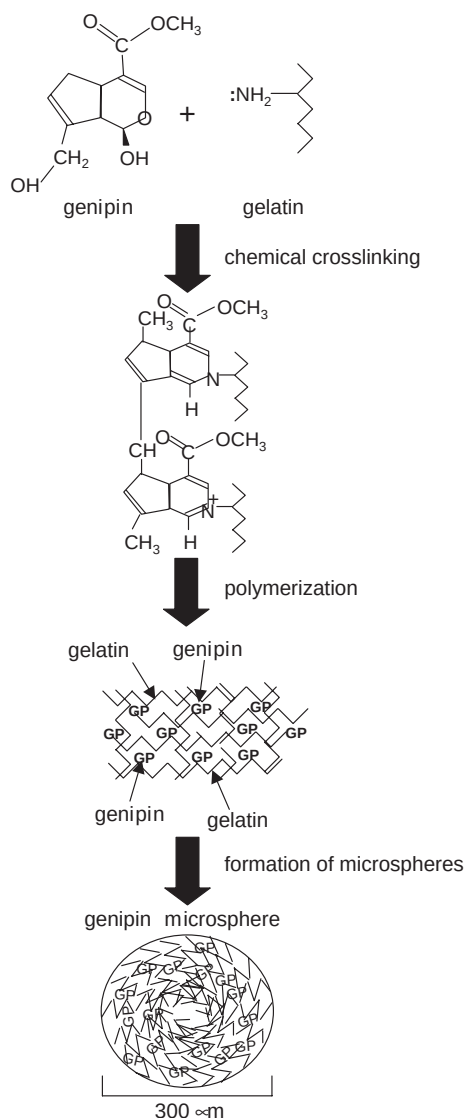


Fig. 4. Cross-linking of gelatin with genipin makes it stable in solution. As gelatin can dissolve rather rapidly in aqueous solutions, the usage of the gelatin polymer for long-term delivery systems requires cross-linking. This can be performed by different chemicals, e.g., as illustrated here by genipin, a compound isolated from the plant *Gardenia jasminoides*. Several resulting polymers form the microsphere (adapted from Ref. [96]).

glutaraldehyde particles (1300 cells/field) than for genipin particles (900 cells/field). This implies that the biocompatibility of genipin-cross-linked microspheres may be superior to the glutaraldehyde-cross-linked formulation.

The model antigen human gamma globulin (HGG) was incorporated into gelatin microspheres and the immune response examined after s.c. injection in mice [97]. The antigen-specific IgG levels induced by this preparation were 10 times higher compared to soluble antigen and even 3–4 times compared to antigen in Freund's incomplete adjuvant (FIA). These findings were paralleled by enhanced delayed type hypersensitivity, as the footpad thickness was about 10 times higher after HGG in gelatin microspheres than after free HGG.

For drug delivery, in vitro and in vivo release kinetics have major implications on the success of therapy. For gelatin particles, several studies point out that the release strongly depends on the rate of cross-linking [98], the particle size, the content of bioadhesive polymer (e.g., polyacrylic acid), the pH of the medium and the temperature [99]. In a rat model, the i.g. and i.n. emptying time of oxprenolol hydrochloride as model drug turned out to be retarded with increasing poly(acrylic acid) content of gelatin particles, suggesting the potential of this delivery system for controlled release of drugs for either oral or nasal application. This was further supported by in vitro characterization and biodistribution studies of bovine serum albumin (BSA) loaded into gelatin microspheres in BALB/c mice [100–103]. Microparticles with radiolabeled BSA were given orally to BALB/c mice and the radioactivity traced in different organs. The released BSA was predominantly taken up by PP and passed on through the mesenteric lymph pathways to the liver and spleen. As two differently sized particle solutions were used (1.2 μm and 7.0 μm, respectively), the researchers were able to show that the uptake was dependent on particle size. In the groups fed with the smaller particles, 35% of the total radioactivity was detected on day 1 in the stomach, in the small intestine with PP and in the mesenteric lymph nodes, whereas the percentage for the bigger particles was about 47% in these regions. This higher percentage implies that larger particles are taken up more slowly.

Disadvantageously, anti-gelatin IgE-antibodies leading to anaphylaxis were observed in some people after immunization with measles–mumps–rubella vaccines, where gelatin is used as heat stabilizer [104]. This may limit the usage of this material for the treatment of allergic diseases.

2.9. Immune stimulating complexes

Immune stimulating complexes (ISCOMS) were first described in 1984 by Morein et al. [105]. These are spherical complexes of about 40 nm, composed of saponin, cholesterol and phospholipids. They are derived from the bark of the soapbark tree *Quillaja saponaria*. The detergent saponin, a structural part of the vehicle, serves as build-in adjuvant [106]. The ring-shaped micellar molecules have a strong negative charge. They differ from liposomes (described in the following section) in respect of their polarity: in contrast to the bilayer of liposomes, ISCOMS consist of three layers, namely, hydrophilic–hydrophobic–hydrophilic. The hydrophilic parts are built up by carbohydrates and the hydrophobic regions by quillaic acid, a poisonous crystalline triterpenoid sapogenin obtained by hydrolysis of saponin from soapbark, which loses its toxicity if incorporated in these lipid particles [107]. Antigens can only be attached to the structure if either hydrophobic or electrostatic interactions occur. These limitations have been overcome by covalent coupling of fatty acid and subsequent incorporation of soluble antigens into ISCOMS [108]. ISCOMS prepared in this way with entrapped OVA presented highly immunogenic carriers, capable of generating a Th1-humoral immune response and delayed type hypersensitivity. However, in priming the immune system with OVA entrapped in ISCOMS by application in the mouse foot pad, both humoral and cellular Th1 and Th2 responses were evoked 1 week after re-challenge with OVA at the same site [109]. This phenomenon was seen in parenteral as well as in oral immunizations [110]. Interestingly, upon immunizations with ISCOMS, IL-4- and IFN- γ -receptor knockout C57Bl/6 mice were able to induce delayed type hypersensitivity, T-cell proliferation, cytotoxic T-lymphocyte activity and production of IL-5, IFN- γ , serum IgG and secretory IgA [111]. As the responses for oral as well as for parenteral administered OVA-ISCOMS were of a similar magnitude as those in wild-type mice, the authors suggested that the immunogenicity of ISCOMS is independent of IL-4 or IFN- γ . In general, ISCOMS have a wide range of immune activity, such as

- uptake by APC [112],
- activation of APC [112],

- induction of enhanced MHC II expression [113],
- production of cytokines, especially IL-2, IL-5, IL-10, IL-12 and IFN- γ [114–116],
- priming of antibody responses (IgG1, IgG2a, sIgA),
- CD4⁺ T-cell responses [109],
- CD8⁺ cytotoxic T-lymphocyte (CTL) responses [117].

ISCOMS made of purified low-toxicity *Quillaja* saponin (ISCOPREP 703) could induce high antibody responses already at doses as low as 1–5 μ g in s.c. immunizations of NMRI mice [115]. The OVA-specific antibody titers were similar to immunizations with alum, but OVA-ISCOMS did only induce IgG, whereas alum as adjuvant also induced IgE-antibodies besides predominant IgG1-titers.

Summarizing several studies, ISCOMS are potent activators of (murine) Th1 cells, and, therefore, may be applied in type I allergy treatment. In a human safety study with an influenza vaccine combined with ISCOMS, mild pain was observed at the injection site. Nonetheless, phase I clinical studies have been completed (reviewed in Ref. [107]). To date, one ISCOM vaccine is licensed in veterinary medicine [118].

2.10. Liposome group

In the liposome group, various particles in the submicron size are included. Most of them are based on a bilayer structure of phospholipids, creating an internal space, which allows the incorporation of hydrophilic antigens [119]. On the other hand, a number of variations is possible within this group, like vesosomes or cochleates.

2.10.1. Archaeosomes

Archaeobacteria show a unique polar lipid structure, which is characterized by fully saturated phytanyl chains attached via ether bonds to the glycerol backbone carbons in certain positions (*sn*-2,3). Ether glycerolipids can be extracted from various archaeobacteria and formulated into archaeosomes [120,121].

There are several advantages of archaeosomes. First, they are able to provide an antigenic depot (like microspheres and conventional liposomes), and they

have immune stimulating capacities (like cholera toxin lipid A and ISCOMS). Those vesicles are stable to oxidative stress, high temperature and alkaline pH, and resist attacks of phospholipases, bile salts and serum proteins [122,123]. They possess strong adjuvant activity and were used for the entrapment of different antigens (e.g., BSA, hen egg lysozyme, OVA, cholera toxin subunit B) [120]. The extent of anti-BSA antibody responses in BALB/c, C3H/HeJ and C57Bl/6 mice were comparable to those upon injections with Freund's adjuvant and substantially higher than those achieved with alum or conventional liposomes. The predominant subclass in murine serum of BALB/c mice was IgG1 after parenteral immunizations (i.p., i.m., s.c.), although also higher IgG2a and IgG2b titers were observed than with the other vaccine formulations. It has been shown that encapsulation of the antigens into archaeosomes was a precondition to achieve high antigen-specific antibody titers, because immunizations with antigen only mixed with archaeosomes elicited approximately 10 times lower antibody titers. Antigen-stimulated spleen cells of BALB/c mice immunized i.p. with antigen entrapped in archaeosomes induced IFN- γ and IL-4 production, whereas alum mainly induced IL-4 [120]. This delivery system evoked long-lasting memory: after primary immunizations on day 0 and 14 with BSA in archaeosomes, the anti-BSA antibody titer could be effectively boosted by gavages on day 210 and sustained until day 300. This was especially true for archaeosomes composed of *Thermoplasma acidophilum* lipids, suggesting that the type of archaeosome may have a great influence on the outcome of the immune response to the associated antigen.

It is suggested that these archaeosomes are sufficiently degraded in vivo, because internalized as well as surface bound BSA was equally able to evoke an antibody response. This vaccine delivery system was also efficient in oral application, and no apparent toxicological effects were observed in the mouse models [124,125]. Furthermore, s.c. immunizations with OVA entrapped in archaeosomes induced CD8⁺ T-cell responses in C57Bl/6 mice [126]. For evaluating the induced CTL activity, lung metastasis and melanoma cells with the gene encoding for OVA were used as targets. Cytotoxic effects were observed for these cells, but not towards control cells without OVA expression.

The potential of archaeosomes to evoke long-lasting antibody responses especially of the Th1 type [127] could lead to a reduction in the number of necessary applications and makes them interesting as a tool to balance the immune response in type I allergy.

2.10.2. Cochleates

The structure and properties of these lipid matrix-based formulations are very different from liposomes [128]. Cochleates are non-vesicular, rolled-up bilayer sheets, which consist of phosphatidylethanolamine, negatively charged phosphatidylserine and cholesterol. Calcium ions stabilize the formed spirals, which have no internal volume, by ionic interaction (Fig. 5). The administration of proteins (surface glycoproteins from influenza virus), peptides or DNA (coding for HIV envelope surface glycoprotein) formulated into cochleates was previously effective via various routes (i.n., oral, i.m. or s.c.) in mice [128–130]. Thereby, the activation of CD4⁺ and CD8⁺ T-cells was achieved, resulting in the induction of influenza-specific IgA in serum and saliva immediately after oral treatment, whereas in mice immunized with phosphatidylglycerol liposomes, no IgA was induced. In addition, high levels of serum IgG1 and IgG2a were achieved after oral and i.m. immunizations with cochleates. The efficacy after oral administration was ascribed to the solid rolled-up structure of the cochleates, which may

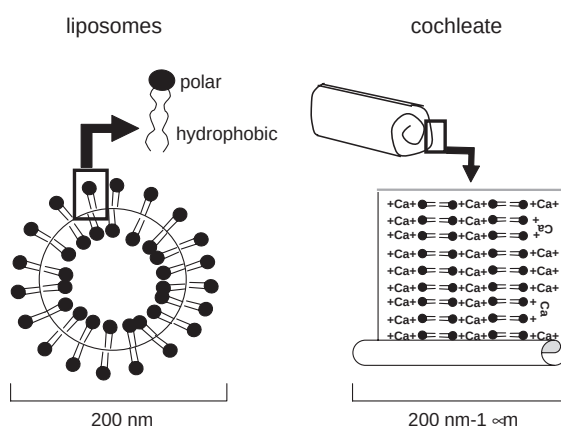


Fig. 5. Schematic comparison of liposomes and cochleates. Liposomes consist of a bilayer (hydrophobic–hydrophilic) and form particles. In contrast, cochleates are bilayer sheets, which form rolled-up structures without an internal space. They are stabilized by Ca²⁺ ions (adapted from Ref. [128]).

contribute to their survival in the stomach and to the protection of the proteins inside. Therefore, cochleates proved suitable as a vaccine delivery system for mucosal as well as for parenteral treatment. This even led to the initiation of clinical trials with HIV therapeutic vaccines [131], but so far, no experiences were collected in the field of IgE-mediated allergy.

2.10.3. Liposomes

Liposomes are non-toxic, biodegradable and biocompatible phospholipid vesicles, which have been checked for adjuvant activity as well as for delivery of antigens. In addition to phospholipids, those preparations often contain cholesterol to increase the stability. Although the production of empty liposomes is easy, the incorporation of antigens demands extensive optimization [132]. Oral applications are difficult as liposomes have a poor stability at low pH and are susceptible to attack from bile salts and gastrointestinal enzymes such as lipases [133]. Liposomes with encapsulated allergen extracts of house dust mite, cat hair and dander, and cocksfoot could be readily freeze-dried and reconstituted without changing their properties [134].

Liposomes have also been used as carrier for OVA. Unfortunately, in OVA-sensitized mice, feedings with OVA entrapped in liposomes had no additional effect regarding the suppression of IgE compared to soluble OVA [135]. Intraperitoneal immunization of BALB/c mice with OVA coupled to the surface of liposomes after primary immunization with OVA in alum enhanced specific IgG1 8-fold and IgG2a titers 12-fold, but no IgE was induced. In contrast, in the alum group IgE increased nearly twofold after booster injections [136]. This was also true the other way round, where mice were primed with OVA-liposomes and challenged with OVA-liposomes or OVA in alum. It was interesting to see that the humoral response did not correlate with the cytokine pattern in a further study [137]. Although all groups showed IgG1/IgG2a ratios of about 100–500, IL-4 was only detected in mice immunized with OVA in alum. This was also the group with the highest anti-OVA IgE titers. In contrast, IFN- γ , IL-5 and IL-10 were detected in all groups.

Distribution studies in mice revealed that liposome-entrapped radiolabelled *Artemisia* pollen allergens were retained for a longer time period in the

tissues after i.p. injection compared to free allergen [138,139]. Regarding immune responses of these animals, the IgG/IgE ratio was four to eight times higher in the groups treated with liposome-entrapped pollen allergens than after immunization with free allergen.

Mite allergens incorporated into liposomes have already been tested in clinical studies [140,141]. Asthmatic patients treated s.c. for 1 year had fewer bronchial, nasal and ocular symptoms as well as reduced drug consumption. In addition, the reactions to allergens in skin tests and bronchial challenges were reduced twofold and sixfold, respectively, in the actively treated group. This was accompanied by a significant decrease of serum eosinophilic cationic protein of 10 $\mu\text{g/L}$, whereas no significant change was observed in the placebo group. Furthermore, liposome treatment resulted in a transient increase in IgE by 47% and a stable increase of serum IgG1 and IgG4 by 289% and 793%, respectively. These immunological changes resemble the results seen in classical allergen immunotherapy, although further studies have to be performed to clarify the long-term effects of liposome immunization. As a disadvantage, it has been observed that non-IgE mediated pseudoallergy against liposomal formulations can be induced [142].

2.10.4. Non-ionic surfactant vesicles (NISV; niosomes, novasomes)

Niosomes are small unilamellar vesicles made of non-ionic surfactants, i.e., non-phospholipid liposomes composed of dioxyethylene cetyl ether, cholesterol and oleic acid. They were able to induce IgG antibody responses in mice and rabbits to encapsulated or admixed tetanus or diphtheria toxoid comparable to immunization with Freund's adjuvant or aluminum phosphate (260–370 $\mu\text{g/ml}$ in mice and 640–1900 $\mu\text{g/ml}$ in rabbits) [143,144]. The main IgG subclass was IgG1, but niosomes also induced around 10-fold higher IgG2a and IgG2b levels than the conventional adjuvants. Their immunogenicity for BSA after s.c. injection was shown to be as high as with Freund's complete adjuvant [145]. After oral administration in mice, OVA-filled niosomes increased the specific IgG and IgA titers, which were 10 times higher than after feedings with soluble OVA [146–148]. More hydrophilic niosomes were not capable of eliciting humoral responses. Other

researcher groups further examined the influence of modifications of niosomes with varying proportions of surfactant, cholesterol, dicetyl phosphate or external oil emulsions on in vitro release, antigen stability, morphology, particle size and entrapment efficiency [149]. They intended to optimize these particles also for i.n. and percutaneous applications. Niosomes proved to be more stable and more effective than liposomes. So far, no applications in humans or in allergy treatment were reported.

2.10.5. Transfersomes

These interesting molecules are highly deformable liposomes, based on phosphatidylcholine (e.g., from soybean) and edge-active substances (e.g., sodium cholate and sodium dodecyl sulfate) [150]. Transfersomes can pass spontaneously through the skin. They mimic the behavior of parasites, such as helminths, by first creating a passage and then creeping through the skin barrier. They use the transepidermal water activity difference as natural energy gradient (hydrotaxis). Transfersomes are much more deformable than other lipid particles. They are easily pushed through pores (approximately 20 nm), which are much smaller than their own size (about 190 nm). This is achieved by judiciously combining at least two lipophilic/amphipatic components (phospholipid plus biosurfactant). With this mechanism, they are able to transport even macromolecules of more than 500 Da through the intact epidermal layer. These particles thus provide a means for the non-invasive, needle-free permeation of antigens through the intact skin barrier.

When these transfersomes were applied epicutaneously on mice, they elicited immune responses to the model antigens HSA and gap junction protein that were comparable or even higher than those after s.c. injections. About twice as much serum IgA could be induced via the epicutaneous route than the s.c. route [151]. Therefore, it is tempting to speculate that transfersomes may be suitable for prevention of allergy or for the induction of mucosal immunity.

2.10.6. Vesosomes

A modification of the cochleates described earlier is achieved by molecular-recognition processes [152]. This self-assembly method is mediated by lipophilic receptors and substrates (e.g., biotin–streptavidin

complex) and is used to achieve a multi-compartmental aggregate of vesicles encapsulated within a large bilayer vesicle.

Another technique uses ethanol–lipid sheets, which close on themselves and the surrounding solution to form closed compartments [153]. Through this efficient and spontaneous encapsulation, several biological macromolecules, colloidal particles, condensed DNA or even other vesicles can be entrapped in a stable and efficient way (Fig. 6). The advantages of vesosomes over liposomes are manifold: (i) the bilayer of the interior vesicles can be composed differently than the outer membrane; (ii) interior vesicles can incorporate charged lipids that might lead to aggregation if exposed to serum; (iii) different drugs can be delivered simultaneously; (iv) spikes in drug release can be minimized, as was shown using carboxyfluorescein as a marker substance; and (v) the internalized substances are protected from lipolytic enzymes. In this respect, vesosomes might provide an interesting carrier system, also suitable for oral allergen delivery.

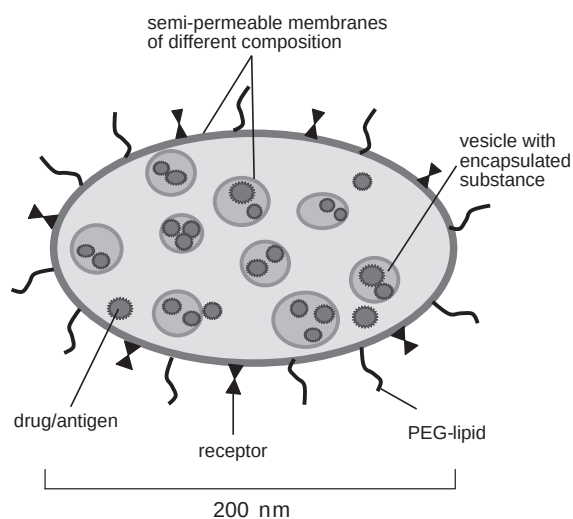


Fig. 6. Schematic presentation of a vesosome. Vesicles, efficiently loaded with different drugs by pH gradient, are trapped through self-assembly (e.g., by increasing the temperature) within further vesicles. They may consist of different membrane types. The resulting vesosome is stabilized by polyethyleneglycol lipids to extend the time of circulation. Receptors for targeting of different molecules can be included to enhance the binding capacity (adapted from Ref. [153]).

2.10.7. Virosomes

Virosomes are small unilamellar vesicles, based on viral membrane proteins, a subgroup of virus-like particles (see section 2.14). They contain hemagglutinin embedded into the membrane for the targeting of sialic acid residues on APCs [154]. For instance, immunopotentiating reconstituted influenza virosomes (IRIVs) are prepared by detergent removal from influenza surface glycoproteins, hemagglutinin and neuraminidase, and natural phospholipids [155]. These virosomes with a diameter of approximately 150 nm are taken up by endocytosis. Inside the endosomes, the acidic milieu is responsible for a conformational change of the hemagglutinin, which results in fusion of the virosome with the endocytic membrane. After this process, MHC class II presentation occurs [156,157]. On the other hand, the usage of viral membrane proteins in virosomes combines the targeting and fusogenic features of the native viral membrane proteins and possibly results in the efficient delivery of entrapped antigens into the cytosol for CTL induction [158]. In Europe, these vehicles are already approved in humans for vaccination with hepatitis A (Epaxal®) and influenza antigens (Inflexal V®) [155,159]. A novel formulation including an additional adjuvant was tested in a clinical phase II trial. Eighty healthy adults were treated with inactivated influenza vaccine adjuvanted with heat-labile toxin (HLT) from enterotoxigenic *E. coli* [155]. The immunization via the i.n. route revealed an excellent mucosal immunogenicity for virosomes. After i.n. applications, secretory IgA antibodies in nasal fluid increased 2.8-fold in the group with HLT, whereas the group without adjuvant showed only a 1.6-fold increase. In this trial, the vaccines were generally well tolerated, although adverse reactions, like headache, shivering, malaise, sneezing or stuffed nose were observed in all groups. The induction of mucosal antibody responses suggests that this application system could be used in the field of allergy prevention or tolerance induction.

2.11. Poly-(D,L-lactic-co-glycolic) acid particles

Poly-(D,L-lactic-co-glycolic) acid particles (PLGA) particles possess a well-established biocompatibility and therefore are already used in human surgery as resorbable sutures and implants [160,161].

These particles can be prepared by different techniques, either by spray freeze drying [162] or by solvent evaporation (emulsion) technique [163]. PLGA degrades through bulk erosion [164]. The resulting substances, lactic and glycolic acid, are innocuous to the human organism [165–175]. During recent years, PLGA particles were applied as carriers for several protein antigens [176] or plasmid DNA [177]. They deposit the entrapped antigen and release it in a delayed continuous or pulsatile manner [178], thereby mimicking booster injections [179,180]. The effects of PLGA-entrapped antigen on the immune response have been shown to be superior to application of soluble, free antigen [179,181–185]. In BALB/c mice immunized i.p. with BSA in PLGA microspheres, anti-BSA IgG antibody titers in serum were twice as high as in mice, which were treated with BSA alone or BSA only mixed to microspheres. This humoral response was comparable to that supported by Freund's adjuvant, but advantageously PLGA particles did not induce any adverse reactions at the injection sites. They were capable of stimulating both, the systemic and mucosal immunity through different application routes [186], not only on the humoral, but also on the cellular level.

In recent studies, these polymers were used for allergen delivery [187–192]. We demonstrated recently the capacity of PLGA particles in therapy [193,194] of type I allergy. During our studies, the Th2-specific humoral and cellular immune response to Bet v 1, the major birch pollen (BP) allergen, could be redirected to a Th1-biased antibody profile by a single s.c. shot in a BALB/c mouse model. In this study, IgG1 antibody titers dropped between 30% and 50% in an already established allergic response, whereas IgE levels were not affected. In addition, cytokine levels could be balanced in PLGA treated mice, as IFN- γ (40 pg/ml) and IL-10 (20 μ g/ml), but only low levels of IL-4 (<1 pg/ml) were induced in spleen cells upon stimulation with Bet v 1 [193]. This led us to the assumption that vaccination with PLGA particles can counterbalance an ongoing Th2 response. Furthermore, the oral administration of BP extract in PLGA microspheres (Fig. 7) with the enterocyte-targeting molecule wheat germ agglutinin primed the immune system towards a Th1 response [194]. The immune modulating effect was even more pronounced when coupling *Aleuria aurantia* lectin to

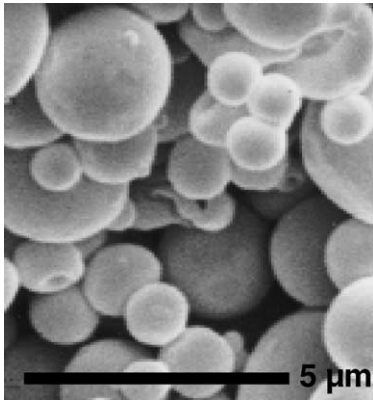


Fig. 7. Scanning electron micrograph of PLGA microspheres with heterogeneous size distribution. Particles were prepared and filled with birch pollen extract as described earlier [194].

the particles for targeting M-cells overlying PP. Feedings of naive mice with AAL-coated PLGA-BP particles evoked three times higher levels of IgG2a than uncoated PLGA-BP, but similar levels of IgG1 [195]. In a subsequent study, even a modulation of an ongoing allergic response towards Th1 and IgG2a production could be achieved, whereas other isotype levels did not change [196].

PLGA represents a controlled release system, which protects the entrapped antigen from destruction during the gastrointestinal transit [196,197], and allows the reduction of necessary booster shots. Nanospheres at the parenteral route, as well as microspheres targeted to M-cells in oral applications are capable of counter-balancing an allergic response. Therefore, we conclude that biodegradable PLGA particles are suitable vehicles for allergens and their derivatives in the few-shot treatment of type I allergy, and that they are superior to the conventional formulation of allergen extract with alum.

2.12. Proteosomes

Proteosomes originate from detergent-solubilized outer membrane of meningococci and mainly consist of highly hydrophobic proteins. They form multi-molecular liposome-like vesicular structures. Antigens, like proteins and peptides, with native or covalently added hydrophobic moieties can be anchored to the proteosomes and thereby become highly immunogenic [198,199]. This formulation is capable

of eliciting a humoral IgG immune response without an additional adjuvant. It was also efficient in BALB/c mice for i.n. application of recombinant influenza hemagglutinin (HA) [200] or for three different synthetic peptides representing B-cell, T-cell and CTL epitopes thereof [201]. These i.n. immunizations evoked humoral and cellular immune responses in mice. Secretory IgA levels induced in lung fluid were about three times higher when peptides were anchored to proteosomes than in controls immunized with PBS, or with peptides coupled to lauric acid alone. The same was true for anti-virus titers, which protected proteosome-treated mice during challenge with virus. The serum IgG1/IgG2a ratio was 60-fold lower for the i.n. proteosome immunization with influenza HA compared to recombinant HA applied i.m. The incorporation of a tumor antigen (ganglioside GD3 of melanoma and other neoplastic cells) into proteosomes was used for the induction of IgM and IgG antibodies, showing that the immune potentiation is not only possible for lipopeptides, but also for amphipathic carbohydrate epitopes (e.g., surface gangliosides) [202]. Hydrophobic or modified allergens should be used for trials with these proteosomes, which may support tolerance induction and mucosal immunity by the nasal route.

2.13. Sepharose particles

The production of carbohydrate-based Sepharose® particles (Pharmacia Diagnostics, Uppsala, Sweden) with a diameter of 2 μm was used for the application of the major timothy grass pollen allergen Phl p 5 [203]. The s.c. immunization of BALB/c mice rendered a high IgG response, with IgG1 as the predominant subclass. Although this isotype was equally high in the different groups, twice as high titers of IgG2a were found in the particle–allergen group compared to alum-adsorbed allergen. The cytokine pattern was comparable between the groups regarding IL-4 and IL-5, but IFN- γ was about 3.5-fold higher in the group immunized with carbohydrate particles. After s.c. administration of these formulations, no granuloma formation in histopathological sections was detected. The sera of carbohydrate particle-treated mice were similarly effective in inhibiting the binding of human IgE of allergic patients to Phl p 5 as were sera of aluminum treated animals. Sepharose

particles may be candidates for the treatment of allergy, although their immune modulatory potency has not been shown in an allergy model yet.

2.14. Virus-like particles

Some viral proteins spontaneously assemble into highly repetitive virus-like particles (VLPs). The main carrier used for these approaches is the hepatitis B core antigen (HBcAg). These particles induce strong B-cell responses even in the absence of adjuvants [204–207]. Into these particles, also foreign epitopes can be inserted. To enhance the presentation capacity for longer amino acid sequences, the assembly system has been modified to possess free cysteines, which enables the chemical cross-linking of antigens onto the VLPs [207,208]. These constructs were able to induce long-lasting immune responses up to 100 days after i.v. application in BALB/c mice, as was shown with the short peptide CGGDYKDDK or a 66 amino acid domain from *Toxoplasma gondii*, or with the M2 protein of influenza virus [205]. The same working group also used phospholipase A2, the major bee venom allergen, for s.c. immunizations of mice in this study. In all these approaches, mice immunized i.v. produced strong and comparable IgG-responses to both the above listed peptides and allergen and the VLP. In accordance with other particle preparations, the coupling of the peptides to VLP produced a superior specific immune response than the simple mixing of the compounds or the application of soluble antigens (about five times higher IgG-responses).

On the level of CTL responses, VLPs in combination with the model antigen p33 from lymphocytic choriomeningitis virus glycoprotein were tested [206]. Surprisingly, the construct p33-VLP did only evoke weak CTL responses in C57Bl/6 mice and was therefore combined with an agonistic anti-CD40 antibody or with CpG motifs to activate APCs. In this context, APC were triggered and the CTL response was as strong as those observed after infection with viruses. After intradermal injection of this construct, presentation of peptide p33 in draining lymph nodes was mainly restricted to CD8⁺ skin-derived dendritic cells and macrophages, although both cell types revealed different mechanisms of antigen presentation [209].

3. Conclusions

Aluminium salts are widely used adjuvants for human vaccines. Over the past 20 years, multiple attempts have been made to find novel adjuvants or even replace them by vehicles which have the ability to induce cellular and humoral immune responses. The particles used constitute a wide range of materials or polymers, such as protein/peptide/amino acids, carbohydrates, polyesters of lactic and glycolic acid, lipids or virus antigens. Most of them proved to be superior for the induction of antigen or allergen specific immune responses compared to antigens alone. They were efficient in the therapy and prevention of diseases, or for the delivery of drugs, at least in animal models. The consecutive immune responses have been analyzed in numerous studies and help to decide for a specific particle dependent on the intended application. Some of the particles have already been tested in human clinical trials and it is reasonable to expect that conventional immunization methods will be replaced by more convenient, biocompatible, few-shot delivery systems with little side effects. Moreover, the oral application of many antigens has been studied and is going to be optimized. This would be a further step towards an enhanced compliance and a great benefit for patients, especially for children.

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