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Recent Advances in the Use of Nanoparticles for Allergen-specific Immunotherapy

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Abstract

The number of patients suffering from allergic asthma and rhino-conjunctivitis has increased dramatically within the last decades. Allergen-specific immunotherapy (AIT) is the only available cause-oriented therapy so far. AIT reduces symptoms, but has also a disease modifying effect. Disadvantages are a long-lasting procedure, and in a few cases potential systemic adverse reactions. Encapsulation of allergens or DNA vaccines into nanostructures may provide advantages compared to the conventional AIT with non-capsulated allergen extracts: The protein/DNA molecule can be protected from degradation, higher local concentrations and targeted delivery to the site of action appears possible and most importantly, recognition of encapsulated allergen by the immune system, especially by IgE antibodies is prevented. AIT with nanoparticles (NPs) may offer a safer and potentially more efficient way of treatment for allergic diseases. In this review we summarize the use of biodegradable NPs consisting of synthetic or natural polymers, liposomes and virus-like particles as well as non-biodegradable NPs like dendrimers, carbon- or metal-based NPs for AIT. More or less successful applications of these NPs in prophylactic as well as therapeutic vaccination approaches in rodents or other animals as well as first human clinical trials are discussed in detail.

Abbreviations

AIT, allergen-specific immunotherapy

BAL, bronchoalveolar lavage

DCs, dendritic cells

DOTAP, *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride

DOTIM, octadecenolyoxy[ethyl-2-heptadecenyl-3-hydroxyethyl] chloride

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DPPC, dipalmitoylphosphatidylcholine
DPPE, dipalmitoylphosphatidylethanolamine
DSPC, distearoylphosphatidylcholine
LPS, lipopolysaccharide
NPs, nanoparticles
ODN, oligodeoxynucleotide
OVA, ovalbumin
PBMCs, peripheral blood mononuclear cell
PC, phosphatidylcholine
PCL, poly(caprolactone)
PEG, poly(ethylene glycol)
PGA, poly(glutamic acid)
PHEA, Polyhydroxyethylaspartamide
PLG, poly(lactide-co-glycolide)
PLGA, poly(lactic-co-glycolic acid)
TGF, transforming growth factor
Th, T helper cell
Treg, regulatory T cell
VLP, virus-like particle

Introduction

The prevalence of IgE-mediated allergic diseases like asthma and rhinitis has increased dramatically during the last century. To date, allergen-specific immunotherapy (AIT) is the only cause-oriented therapy for these hypersensitivity reactions. Following the first controlled human trials published by Noon and Freeman in 1911, AIT consists of repeated subcutaneous or sublingual applications of increasing doses of the specific allergen, resulting in restored tolerance towards this allergen. The exact mechanisms underlying AIT are mainly but not fully understood and have been reviewed elsewhere. Briefly, successful AIT is characterized by a shift from a Th2/Th17 immune response towards a Th1 response together with the induction of regulatory T cells leading to a decrease of allergen-specific IgE and an increase of protective IgG antibodies. Furthermore, infiltration and activation of eosinophils, mast cells, and basophilic leucocytes is reduced. Although effectiveness of AIT was confirmed in the majority of clinical trials, it still suffers from potential systemic side-effects, mainly occurring after s.c. injection, long-term duration and sometimes limited treatment effect, which all affect therapy safety and treatment adherence/patient compliance (1, 2).

Encapsulation of allergens into nanoparticles (NPs) might be one strategy for improvement of AIT due to the following reasons: NPs may serve as a carrier system for proteins, peptides, or DNA molecules and may also have adjuvant effects (3-5). There are two possible ways of transportation: encapsulation or surface coating of the cargo. Encapsulation offers protection from enzymatic or acidic degradation, delivery and co-delivery with other molecules to the targeted site of action enabling high local concentrations and preventing detection of the cargo molecule by the immune system. During AIT treatment, shielding of the allergen prevents recognition by IgE bound on the surface of mast cells or basophils, which in turn should mitigate or even prevent side-effects (Fig. 1). This property may also be useful for the realization of AIT against food allergies, where it is not yet routinely available (2). An oral administration route for food AIT seems natural, but it brings along additional requirements for the nanocarrier; as the cargo needs to be delivered to the intestine, NPs have to be resistant to gastric acid and need to cross the epithelial barrier and the mucosa to reach the lamina propria (3). Another important feature of NPs depending on the design (size, charge, particle shape) is improved and accelerated cellular uptake, which in turn may allow fewer treatments and may increase convenience. Allergen-coated NPs, on the other hand, facilitate cross-linking of IgE on the surface and thereby activation of the immune cells and are usually used for investigation of prophylactic allergen vaccinations in mice. Empty NPs without allergen encapsulation or coating have also been tested for allergy treatment, mostly in a prophylactic approach and show promising results enabling the restoration of a balanced Th1 and Th2 immune response due to their adjuvant activity (3, 5, 6).

There are thousands of publications using particles (mainly NPs, but also microparticles/microspheres depending on their size measured in nanometer or micrometer) as carrier systems for drug delivery with focus on vaccination strategies against cancer, infectious diseases or autoimmune diseases (5, 7-9). Considerable less research has been undertaken for the use of NPs in AIT (3, 6, 10) which will be discussed below separated into biodegradable and non-biodegradable NPs.

Biodegradable Nanostructures

Biodegradable nanostructures can be divided into synthetic polymers (polyesters), natural polymers (polypeptides, polysaccharides), liposomes and virus-like particles (Fig. 2). An overview for the use of biodegradable NPs in AIT including their composition and manufacturing method is shown in Tables 1a and 1b.

Synthetic polymers

The most popular polyesters used in drug delivery are poly(lactic acid) and poly(lactic-co-glycolic acid) (PLGA), also abbreviated PLG (poly(lactide-co-glycolide)), which degrade under biological conditions via enzymatic hydrolysis. However, a rather long degradation time of up to 24 months together with the acidic degradation products that may result in inflammation at higher concentrations, and the difficulty of derivatization are the main drawbacks of these materials. To overcome these limitations, copolymers of basic character to neutralize generated acidity or copolymers to allow derivatization can be utilized (11-13). Encapsulation of different allergens or allergen-DNA in PLGA-NPs or -microparticles was investigated by several groups. In general, intranasal or oral immunization of mice with these particles led to a Th1 immune response (reduced allergen-specific IgE and increased allergen-specific IgG2a levels) and expression of IL-10 preventing mice against sensitization (14-19). *In vitro* experiments showed that dendritic cells (DCs) matured after particle-uptake and were more effective in T cell activation (19). There are also some reports showing a therapeutic effect in sensitized mice treated with PLGA-NPs or -microparticles which induced a conversion from Th2 to Th1 responses and an up-regulation of regulatory T cells leading to decreased lung inflammation and mucus secretion, reduced numbers of total cells and eosinophils in BAL fluid as well as decreased anaphylaxis (20-26). In either cases, co-encapsulation of Th1 activator CpG-ODN or the immunosuppressor rapamycin enhanced the therapeutic or prophylactic effects (18-20, 25, 26).

Poly(ϵ -caprolactone) (PCL) is another popular polymer for biodegradable drug delivery systems because of the biocompatibility of this aliphatic polyester, its high permeability, and low glass transition temperature. The latter may enable fast disassembly and therefore release of the cargo molecules upon temperature rise to body temperature (27). In ovalbumin (OVA)-sensitized mice, treatment with OVA-PCL elicited similar OVA-specific IgG1 levels as treatment with OVA-alum but lower levels of IgE together with lower production of serum histamine, leading to a higher survival rate after allergen challenge due to reduced anaphylaxis (28).

Poly(anhydrides) degrade within days to several months. This property in concert with biocompatibility, low melting points and solubility in organic solvents renders them interesting candidates for drug delivery (29). Immunization of naïve mice with peanut protein-loaded lyophilized poly(anhydride) NPs induced a balanced Th1 and Th2 antibody and cytokine response, while spray-dried NPs led to an enhancement of Th1 and Treg cytokines (30).

Natural polymers

Natural biodegradable polymers are usually obtained from plants or – in the case of chitin – from marine organisms, such as crustaceans. Polysaccharide examples are chitosan, dextran, alginate, starch, or cellulose derivatives. Biodegradable natural polymers also encompass proteins (e.g. collagen, albumin, globulin, gelatin) (11). For drug delivery purposes, these natural poly(peptides) or synthetic polyamides like polyhydroxyethylaspartamide (PHEA) are used due to their aqueous solubility and biodegradation. Natural water-soluble polymers have not only been used to encapsulate allergens but also DNA or CpG-ODN.

Protamines are arginine-rich proteins derived from the sperm of different fish species. In nature, protamine complexes DNA by electrostatic interaction and can bind nucleotides or peptides/proteins. It could be shown that protamine-based NPs (proticles) complexed with Ara h 2 from peanuts together with CpG can counteract Th2-dominated allergic immune responses (increased specific IgG2a antibodies, no specific IgE, low ratio of IL-5/IFN- γ , lower type I skin test reactivity and no granuloma formation after s.c. injection into mice). Furthermore, stimulation of bone marrow-derived DCs with Ara h 2-loaded proticles resulted in an upregulation of CD11c, CD80 and IL-6 (31).

Poly(γ -glutamic acid) (γ -PGA) is formed by bacterial fermentation and can be used as a carrier and adjuvant simultaneously. *In vitro* stimulation of DCs from allergic donors with a mixture of a γ -PGA graft copolymer and grass pollen extract enhanced proliferation and IL-10 production of autologous memory T cells (32). Furthermore, γ -PGA copolymer NPs within L-leucine microcarriers for dry powder inhalation have been shown as promising vehicle for pulmonary vaccine delivery due to excellent bovine serum albumin adsorption and aerosolization behavior (33). Another group investigated the biodistribution of s.c. injected fluorescent labelled γ -PGA-phenylalanine NPs in mice. The authors observed complete disappearance of the NPs at the injection site and major organs within one month. When the NPs were administered systemically via the i.v. and i.p. route, they mainly accumulated in the liver and were more rapidly cleared compared to non-biodegradable NPs which is important to avoid unexpected and undesired side-effects to the human body (34).

Licciardi *et al.* suggested an allergen-copolymer nanoaggregate consisting of *Parietaria* pollen allergens and PHEA as potential vaccine against *Parietaria*-induced allergies. However, the authors only show that this formulation was not toxic and did not alter IgE cross-linking and basophil activation *in vitro* without providing further immunologic tests (35).

Neoglycocomplexes of mannan-conjugates with OVA or papain generated by carbohydrate oxidation were efficiently taken up by DCs *in vivo*. Both proteins induced elevated specific humoral immune responses and a shift from IgE to IgG with a predominance of IgG1 in mice. Additionally, papain glycocomplexes combine the DC targeting and simultaneously mask B-cell epitopes revealing hypoallergenicity (36). Proteins can also be coupled to the surface of polysaccharides by cyanogen halides (37). Grass pollen or cat allergens coupled to sepharose microbeads by this method induced a mixed allergen-specific Th1/Th2 immune response when injected into mice and did not lead to granulomatous tissue reactions compared to alum-adsorbed allergens. Furthermore, prophylactic and therapeutic immunotherapy with these carbohydrate-based particles prevented/reduced airway hyperreactivity and eosinophils in bronchoalveolar lavage (BAL) fluid, and induced a shift from IgE towards IgG2a production. *In vitro*, the particles were internalized by human DCs leading to an upregulation of CD86, IL-8 and TNF- α (38-41).

Chitin was reported to up-regulate Th1 immune responses. Oral or intranasal administration of chitin particles during sensitization resulted in a decrease of IgE, lung eosinophil numbers and airway hyperreactivity. *In vitro* re-stimulation of spleen cells showed a decrease of Th2 cytokines along with an increased production of IFN- γ (42, 43). Similarly, immunization of mice with grass pollen extract conjugated to the adjuvant alginate isolated from the cell walls of brown algae led to higher allergen-specific IgG (no specific subclasses are shown) and lower IgE production compared to alum-adsorbed allergens (44). In clinical trials, immunotherapy with alginate-conjugated allergens increased IgG1 and IgG4 levels while allergen-specific IgE was reduced (45, 46). Gelatin consists of denatured and hydrated collagen derived from animals. Treatment of allergic horses with gelatin NP-based CpG increased IL-10 expression and caused a significant decrease of allergic symptoms (respiratory effort, nasal discharge, tracheal secretion, and viscosity) (47). Chitosan is a linear amino-functional polysaccharide derived from chitin by deacetylation and is known from different drug delivery approaches (48, 49). Due to its cationic character after protonation it has been used to complex DNA or proteins in several murine studies, and successful prophylactic and therapeutic protection against allergic diseases was demonstrated with OVA, peanut, and house dust mite protein or DNA. These NPs as well as allergen-free chitosan NPs loaded with IFN- γ or TGF- β expressing DNA induce Th1 or Treg responses (50-58). *In vitro*, allergen-loaded chitosan NPs led to an enhanced uptake, processing and antigen presentation by DCs, a reduction of cellular inflammation in human keratinocytes, and a reduced IFN- γ and IL-10 secretion by human PBMC from allergic donors (58, 59).

Liposomes

Liposomes consist of lipids that self-assemble to a vesicular system, in which a lipid double layer separates an aqueous compartment from the aqueous environment. Liposomes have been investigated as drug delivery platforms or as adjuvants for several decades and are extensively reviewed elsewhere (60, 61). The first report of liposomes as delivery vehicles for allergens demonstrated that allergen-loaded liposomes were able to avoid anaphylactic reactions in pre-sensitized guinea-pigs (62). *Arora et al.* published several studies using *Artemisia scoparia* pollen extract-loaded liposomes made from phosphatidylcholine (PC), cholesterol, and phosphatidic acid by ultra-sonication. Intraperitoneal treatment of sensitized BALB/c mice with these liposomes resulted in a Th1-immune response and increase of Treg compared to free allergen or allergen adsorbed to alum. The authors further found an extended retention in all organs except kidneys in liposome-treated mice and higher survival rates after allergen challenge due to lower plasma histamine levels. They conclude that liposome entrapped allergens could be used as a safe and effective tool in immunotherapy (63, 64). *Genin et al.* introduced negatively charged multilamellar liposomes, where one third of the allergen was associated at the surface and two third were encapsulated. Immunogenicity of these liposomes was measured by basophil activation tests and skin tests in allergic patients resulting in similar reactions compared to allergens in solution (65). Immunization of mice with the same liposomal formulation increased Th1 and decreased Th2 responses as well as eosinophilia compared to free allergen (66). Similarly, liposomes which were generated from dipalmitoylphosphatidylcholine (DPPC) and loaded with purified mite allergens induced a higher specific IgG2a/IgG1 subclass ratio and a reduced cellular infiltration with eosinophils compared to allergen adsorbed on alum, while IgE levels were not elevated (67). Testing differently charged liposomes for induction of immune responses in mice revealed the highest IgG titers (no specific subclasses are shown) and lowest IgE levels with positively charged liposomes compared to the neutral and negative ones (68). A very recent report demonstrated reduced serum IgE and intranasal mucus production together with a shift in the cytokine profile from Th2 towards Th1 and Treg after AIT of sensitized mice with liposome-entrapped cat allergens (69). A decreased IgE level could also be observed after intragastrical administration of OVA-loaded liposomes into pre-sensitized mice, but compared to application of free OVA there was no additional benefit for the liposomal preparations (70).

Application of allergen-loaded liposomes in human clinical trials of AIT revealed that liposomal formulations were slightly more effective than adsorbed extracts, but less favorable systemic safety and complex manufacturing process led to the conclusion that liposomes are not suited for AIT (71). However, another study with fifty-five asthmatic patients reported no side-effects and reduced symptom and medication scores by at least 60% in nearly half of the patients vaccinated with a liposome-encapsulated house dust mite allergen compared to 12% in the placebo group and a decreased organ sensitivity measured by skin prick test, intradermal testing, and bronchial challenge. Accordingly, specific IgG, IgG1 and especially IgG4 production was augmented during the therapy, while no change in specific IgE production was observed (72).

Preventive as well as therapeutic vaccination of mice with oligomannose-coated liposomes led to decreased IL-4 and IL-5 production and decreased specific IgE and IgG1 levels, while specific IgG2a and IFN- γ was enhanced (73). The same modified liposomes were also able to induce CD8⁺-Treg leading to the suppression of allergic diarrhea in a murine food allergy model. Additionally, allergen-specific IgE was reduced and IL-10, IgG1, IgG2a and soluble IgA was elevated (74). In another murine study two different liposome-adjuvanted cockroach allergy vaccines were compared. The authors could show that liposomes loaded with the refined protein Per a 9 were superior to alleviate airway inflammation than crude-extract loaded liposomes (75).

Immunostimulatory complexes of liposomes, CpG-DNA and allergen were used in AIT of dogs with atopic dermatitis which resulted in an improvement of the pruritus score and a decrease in IL-4 mRNA expression in PBMC compared to the pretreatment values while IFN- γ and IL-10 mRNA as well as medication score did not change (76). Treatment of chronic rhinitis in cats with liposome-IL-2 DNA complexes showed significant improvement in frequency of sneezing, but no differences in cytokine profile. The authors further demonstrated that chronic rhinitis in cats is not a disease with a Th2 cytokine bias (77). AIT of pre-sensitized mice with liposome-protamine-DNA NPs resulted in a reduction of IgE as well as induction of IgG2a, IFN- γ , and T-bet together with higher lymphoproliferative responses (78). In another approach OVA was covalently coupled to distearoylphosphatidylcholine (DSPC)-cholesterol liposomes. When pre-sensitized mice were treated with these liposomes, OVA-specific IgE was strongly suppressed whereas IgG1 levels were not affected, but also decreased using high-density OVA-liposomes. Additional

loading of these liposomes with doxorubicin further reduced OVA-specific IgE probably by eradication of OVA-specific B cells in the spleen (79). Finally, multilamellar, small unilamellar, and reversed phase vesicle liposomes were analyzed regarding encapsulation efficiency of house dust mite allergens, but no cellular tests were performed (80).

Virus-like particles

Virus-like particles (VLPs) have been applied as adjuvants or drug delivery systems (81, 82). In a placebo-controlled, double-blind, randomized trial house-dust mite allergic patients were treated with VLPs consisting of bacteriophage Q β coat protein associated with CpG-ODN resulting in lower rhinoconjunctivitis symptoms and better quality of life score. Additionally, a 10-fold increase in allergen tolerance was demonstrated by conjunctival provocation test (83). In another clinical trial, the same VLPs were used as an adjuvant for ATI with house dust mite allergen extract. Symptoms of rhinitis and allergic asthma were significantly reduced and humoral response was shifted towards Th1 (84). In mice, a single s.c. vaccination of Fel d 1 coupled to bacteriophage Q β -derived VLPs led to protection against type I allergic reactions without inducing local or systemic reactions. Additionally, the allergen-coupled VLPs did not lead to degranulation of human basophils *in vitro* (85). Immunization of mice with adeno-associated VLPs expressing an OVA-derived B-cell epitope also led to a reduction of allergen-specific IgE compared to treatment with soluble OVA, while OVA-specific IgG1 titers were elevated. No anaphylactic reaction was observed for VLP-treated mice upon OVA challenge (86).

Conclusion Biodegradable Nanostructures

PLGA is by far the most intensely investigated biodegradable polymer applied in AIT to date. Other polyester materials like polydioxanone, polycarbonates, or poly(ortho esters) as well as phosphorus-based polyesters such as poly(phosphate)s are well-known materials for biomedical applications and may be interesting candidates in AIT. Poly(alkyl cyanoacrylate), which is degradable under acidic conditions, is also known in drug delivery (13, 87). However, extensive studies regarding immune responses to these materials as well as their metabolism products need to be performed. Natural water-soluble polymers possess great potential as drug delivery systems in AIT. So far, chitosan has been extensively investigated as transport vehicle for DNA and protein delivery. Other materials like alginate or chitin are tested in the field just now, but there remains an elusive amount of possible polysaccharides like xanthan gum, pectins, dextran, carrageenan, cellulose ethers, hyaluronic acid, starch, or starch-based derivatives, to name only a few (11). The employment of liposomes as allergen delivery vehicles for AIT was successfully shown in many animal studies as well as in human

clinical trials but due to local and systemic reactions their safety needs to be reconsidered. Many structural varieties of liposomes tested in other applications are promising but have not yet been transferred to AIT, like stealth liposomes that are shielded by PEG chains, liposomes with targeting moieties, or liposomes with cross-linked core or shell to improve stability of liposomes until arrival at the targeted location. VLPs and especially CpG-loaded VLPs represent potent delivery systems with proven immunogenicity and clinical efficacy. Additionally, they are well tolerated and often combine transport and adjuvant function, and, importantly, they might even be employed for allergen-free immunotherapy (88).

Non-biodegradable Nanostructures

Non-biodegradable polymers find wide application in everyday life as bottles, packaging materials, plastic films, toys, and a myriad of other applications. Only a few materials used for AIT are described in the existing literature. Inorganic NPs have physical and structural properties that can be tailored for the desired interactions with biological systems. Surface coating of inorganic NPs inaugurates pathways for diagnosis and delivery systems (89). In AIT, most inorganic NPs do not have an exclusive influence on allergic inflammation, but generally act immunomodulatory (Fig. 3, Table 2).

Polymer NPs

Dendrimers, which are perfectly branched, tree-like polymer structures with a high number of end groups that can be used for conjugation, were investigated as drug delivery systems (90). Vaccination of mice with a dendrimer made from poly(propylene imine) loaded with Bet v 1a pDNA resulted in decreased specific IgE combined with an enhanced Th1 response (91). Furthermore, guanidine-terminated dendrimers containing an amyloid-promoting OVA-peptide effectively associated with RAW264 cells, but unfortunately no further immunological tests were shown. In both cases, it is likely that the cargo was transported by several molecules that assemble to form NPs, but not by one dendrimer molecule itself (92). Preventive and therapeutic application of CpG coupled with poly(propylene sulfide) NPs enhanced recruitment of activated DCs and led to a shift towards Th1 immunity which was superior compared to free CpG (93). *Garaczi et al.* assembled NPs by mixing poly(ethylene imine) with OVA pDNA. In a murine allergic rhinitis model, topical administration of these NPs reduced nasal symptoms of about 50% and induced a balanced Th1/Th2 response (94). *Madan et al.* encapsulated *A. fumigatus* in poly(vinylpyrrolidone) NPs and could demonstrate intact integrity and immunoreactivity of the allergens as well as a sustained allergen release in 9 weeks. Mice immunized with these NPs in one single dose showed

decreased specific IgE as well as persisting IgG levels (no specific subclasses are shown) (95). AIT with OVA conjugated to a copolymer of N-vinylpyrrolidone and maleic anhydride modified with epsilon-aminocaproic acid led to a decrease of specific IgE and enhanced IgG levels (no specific subclasses are shown). The authors further observed a reduced allergenicity in a leukocyte histamine release assay while the activation of the OVA-specific T-cell hybrid 3DO-548 was comparable to that of unconjugated OVA (96). Gantrez® AN NPs made from poly(methyl vinyl ether-co-maleic anhydride) were loaded with OVA or *L. perenne* and in some cases with LPS as immunomodulator. Immunization of pre-sensitized mice with these NPs protected them from anaphylactic shock and increased IgG2a levels. However, full protection against anaphylaxis and survival of the mice was only achieved by OVA plus LPS entrapped NPs (97, 98). Poloxamine (copolymers of ethylene oxide and propylene oxide) nanospheres loaded with Der f 1 plasmid were tested regarding therapeutic effect on asthmatic mice, and a reduction of airway hyperreactivity together with a decrease in inflammatory cytokines in the BAL could be observed (99).

An interesting method to prepare nano-sized peptide-surfactant complexes by solid-in-oil nanodispersions with proteins was reported by two groups demonstrating facilitated skin penetration of the allergen peptide and suppressed IgE levels in mice (100, 101). Oral treatment of pre-sensitized guinea pigs with a copolymer of methacrylic acid and methylmethacrylate (Eudragit L-100), which was used to give an enteric coating of grass pollen allergen extract in order to protect it against gastric degradation, induced increased secondary antibody responses compared to aqueous presentation (102). Oral treatment of ragweed-sensitized patients with ragweed pollen extract encapsulated in polymethacrylic acid co-polymer NPs led to the production of high allergen-specific IgG and IgG4 titers while specific IgE antibodies were not increased. The therapy was possible without side-effects and decreased symptomatic medication (103). The same NP system was applied for encapsulation of timothy grass pollen. In addition to decreased medication and symptom scores the authors discovered a reduced allergen-specific T cell proliferation and IL-5 mRNA production *in vitro* (104).

The most prominent synthetic water-soluble polymer is poly(ethylene glycol) (PEG), an aliphatic polyether, that is widely used in pharmaceutical applications. PEG is often described as the “gold standard” for biomedical applications and obtained this status because of its unique properties (105). As PEG is a non-biodegradable polymer, cleavage sites need to be introduced for controlled delivery of proteins by PEG-based NP in AIT. To this end, acid-labile PEG-macromonomers that degrade at pH 5 (physiological pH inside the

endolysosome) were synthesized by *Pohlitz et al.* Encapsulation of allergens into these NPs resulted in targeted delivery and induction of T cell proliferation concurrently with allergen shielding from recognition by IgE antibodies in human *in vitro* experiments (106).

Carbon-based NPs

Carbon nanotubes are extensively investigated in the field of drug delivery, especially in tumor-targeting and as biosensors. The toxicity and influence on the immune system is controversially discussed (107). Investigation of the immunomodulatory activity of multi-walled carbon nanotubes in PBMC from healthy and allergic donors revealed that they may either promote or suppress immune responses, and may therefore be suitable adjuvants (108). In a mouse model of asthma, however, allergen-induced systemic immune responses and airway inflammation were worsened by such nanotubes (109). For more than two decades, fullerenes, a carbon allotrope, have been investigated for possible application in biotechnology and medicine. *Ryan et al.* described a negative regulator function of C60 fullerene on mast cells and basophils, which showed an inhibition of IgE dependent mediator release. Due to blockade of signaling molecules and cytoplasmic reactive oxygen species levels, fullerenes prevented the *in vivo* release of histamine and anaphylaxis (110). In two other studies, treatment of OVA-sensitized mice with tetraglycolate fullerenes or a water-soluble form of C60 fullerene resulted in a shift in the cytokine production from Th2 to Th1 as well as reduced airway inflammation, eosinophilia, and bronchoconstriction triggered by the production of an anti-inflammatory P-450 eicosanoid metabolite or an increase in Foxp3 and filaggrin mRNA, respectively (111, 112).

Metal-based NPs

Gold NPs are known for their application as biosensors and gene delivery vehicles, and in general for their anti-inflammatory and anti-oxidant effects (113). Intranasal administration of gold NPs prior to allergen challenge inhibited accumulation of inflammatory cells, mucus production, airway hyperreactivity, and production of pro-inflammatory cytokines and reactive oxygen species in OVA-sensitized mice (114). Magnetic NPs combine the advantage of good detectability for imaging purposes and drug delivery and gained attention e.g. in hyperthermia-based therapy (115). *Marengo et al.* presented a possible delivery platform for AIT made of dextran-coated magnetic NPs conjugated with bovine β -lactoglobulin or ovomucoid. They observed higher uptake of the conjugated compared to

non-conjugated NPs by human monocytes and confirmed internalization by confocal laser scanning microscopy (116). Nanodecoys consist of a solid core made of hydroxyl apatite which provides structural stability and a carbohydrate coating to which peptides and proteins can adsorb. Pre-sensitized mice treated with ultrafine nanodecoys coated with trehalose and adsorbed by ovalbumin, also called aquasomes, showed lower levels of IgE and serum histamine, higher survival rate and reduced symptoms during induction of anaphylaxis (117).

Conclusion non-biodegradable polymers

Non-biodegradable polymers as drug delivery systems for allergen or allergen-DNA possess potential but the fate of the carrier in the body needs to be thoroughly investigated. Although these materials do not naturally degrade, they are usually biocompatible as no low molecular toxic degradation products are formed. This advantage paves the way for applications in e.g. implants and catheters. Due to repeated NP application during AIT, discharging of NP remnants from the body is inevitable. Degradation can be promoted by artificially enclosed predefined degradation points. Positively charged polymers such as polyethylenimine and poly(L-lysine) have a common draw back as they often show cell toxicity, which might explain the few reports of these materials in drug delivery (118). In the last decades, the variety of water-soluble non-degradable synthesized polymers has expanded incredibly. Some of them are currently explored regarding their potential as drug delivery systems, e.g. poly(vinyl alcohol), with the advantage of high stability and many hydroxyl groups available, poly(vinyl pyrrolidone), poly(acrylic acid), poly(acrylamides), *N*-(2-Hydroxypropyl)-methacrylamide, poly(divinyl ether-co-maleic anhydride), and poly(oxazoline)s (119). Only few systems of inorganic NPs have been described for AIT so far. Inorganic NPs may contribute with structural properties desirable during AIT like the intrinsic Th1 activating effect or the option for facile modification. However, the fate of inorganic nanostructure in the body is of particular importance as a lack of elimination from the organism may result in undesired side-effects. Furthermore, the impact of highly anisotropic NPs such as carbon nanotubes on cells needs to be investigated in more detail.

Summary and Outlook

Although it is the only disease modifying therapy for allergic rhino conjunctivitis and asthma at present, AIT suffers from potential systemic side-effects mainly occurring during s.c. treatment. Prevention of these side-effects resulting in safer therapy as well as a more efficient treatment - even though there is no reliable biomarker predictive of AIT efficacy so

far - is a main goal for improving strategies in AIT. Approaches to achieve this goal include a molecular-based diagnosis to refine the prescription, and a therapy with physically adsorbed allergens as (semi-) depot extracts, modified allergens like allergoids or hypoallergenic recombinant allergens or peptides or co-administration of anti-IgE-antibodies (120). Another concept as an alternative to AIT aims at shifting the imbalance of T cell subtypes towards a more balanced one by unspecific induction of Th1 cells with adjuvant moieties, e.g. immunostimulatory DNA, monophosphoryl lipid A, or lactic acid bacteria. NPs can provide delivery function preventing enzymatic degradation as well as IgE cross-linking of the cargo molecule, can co-deliver or act as adjuvants themselves, and may have an additional targeting function.

AIT with proteins and DNA molecules protected by a variety of polymer structures and nanomaterials has predominantly been analyzed in murine allergy models, but also in some human clinical trials. The most intensively investigated materials for NPs are chitosan and the rather apolar, water-insoluble PLGA. Degradation of these materials and thereby cargo release depends on hydrolytic/enzymatic degradation and may last several weeks. This timeframe may be suitable for a depot function with sustained release behavior, but due to unspecific degradation shelf-life in aqueous solutions is affected. NP engineering of structures that show extended shelf-life, targeted drug delivery, as well as desired degradation behavior can be achieved by designing materials susceptible to external stimuli like reducing conditions, enzymes, pH changes, or light. Stimuli-responsive materials, possibly with attached target functions like antibodies, ensure delivery of the cargo at the desired site of action with less cargo release in a non-specific way (121). Only a NP that shields the cargo from detection by the immune system and that is stable until it reaches its destination could prevent side-effects during AIT. Furthermore, sterile and endotoxin-free non-toxic formulations are required for safe clinical application. Sterilization of NPs after their synthesis may be problematic as this may alter the properties of the NP and/or the cargo. Storage of NP formulations needs also to be taken into account considering patient's applicability. Moreover, characterization and understanding of protein corona formation is required to accomplish the desired delivery and targeting.

Certainly, research of NPs in AIT will be intensified, motivated by the promising results obtained so far and the huge diversity of well-tolerated NP formulations already described for other drug delivery purposes. This is exemplified for the usage of self-assembly processes, nature-mimicking structures, polymer nanogels, or multilayer capsules, to name only a few possibilities (122). Improvements from other fields of NP engineering may be exploited for targeting cells or organs, and for predetermined degradation mechanisms. Findings

concerning the effects of NPs on the immune system made in other contexts might be transferred to application in AIT and knowledge regarding possible effects of long-term biocompatibility and immunogenicity upon repeated injections need to be broadened.

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Figure legends

Fig. 1: Comparison of allergen-specific immunotherapy with soluble allergen and with allergen encapsulated in NPs.

Fig. 2: Overview biodegradable nanostructures used in AIT.

Fig. 3: Overview non-biodegradable nanostructures used in AIT.

Table 1a: Overview of biodegradable NPs for AIT: synthetic and natural polymers

NP composition and manufacturing method	Cargo	Vaccination approach	Species/ Route	Ref.
PLGA microparticles; double emulsion solvent evaporation method	<i>Olea europaea</i> Ole e 1 peptide	prophylactic	BALB/c mice, i.n.	(14)
PLGA microspheres; double emulsion solvent evaporation/extraction method	<i>Olea europaea</i> pollen extract	possible delivery platform	BALB/c mice, s.c.	(15)
PLG microspheres	β lactoglobulin	prophylactic	BALB/c mice, orally	(16)
PLGA microspheres; spray-drying method	bee venom phospholipase A2 DNA	prophylactic	CBA/J mice, s.c.	(17)
PLGA NPs; double emulsion solvent evaporation method	Der p 2-coated, CpG motifs encapsulated	prophylactic	C3H/HeBFeJ mice, s.c.	(18)
PLGA microparticles; double emulsion solvent evaporation method	ovalbumin (OVA) and CpG-ODN	prophylactic	murine DC2.4 cell line and C57BL/6 mice	(19)
PLGA microparticles; solvent extraction method	phospholipase A2, CpG-ODN and protamine	therapeutic	CBA/J mice, s.c.	(20)
PLGA NPs; double emulsion solvent evaporation method	<i>Chenopodium album</i> pollen rChe a 3	therapeutic	BALB/c mice, s.l.	(21)
PLGA NPs; double emulsion solvent evaporation method	OVA-coated or encapsulated	prophylactic and therapeutic	BALB/c mice	(22)
PLGA NPs; double emulsion solvent evaporation method	recombinant <i>Caryota mitis</i> profilin	therapeutic	BALB/c mice, s.c.	(23)
PLGA NPs; double emulsion	Bet v 1	therapeutic	BALB/c mice,	(24)

solvent evaporation method			S.c.	
PLGA microparticles; double emulsion solvent evaporation method	OVA and CpG-ODN	therapeutic	BALB/c mice, i.d.	(25)
PLGA NPs; double emulsion solvent evaporation method	OVA $\pm$ rapamycin (immunosuppressor)	therapeutic and prophylactic	BALB/c mice, i.v.	(26)
PCL microparticles; solvent extraction/ evaporation method	OVA	prophylactic and therapeutic	BALB/c mice, i.d.	(28)
poly(anhydride) NPs; solvent displacement method	raw or roasted peanut proteins	prophylactic	C57BL/6 mice, orally	(30)
protamine-based NPs (proticles); self-assembly in aqueous solution	CpG-ODN complexed with Ara h 2 extract	prophylactic	BALB/c mice, s.c.	(31)
γ -PGA NPs; self-assembly in aqueous solutions	mixture of γ -PGA NPs and <i>P. pratense</i> extract	possible delivery platform	human DCs <i>in vitro</i>	(32)
PGA-co- ω -pentadecalactone NPs within L-leucine microcarriers	bovine serum albumin	possible delivery platform	human A549 cell line	(33)
γ -PGA-phenylalanine-633; self-assembly in aqueous solutions	OVA	possible delivery platform	mice s.c., i.v. or i.p.	(34)
poly(hydroxyethyl)-aspartamide (PHEA); allergen-copolymer nanoaggregate	Par j 1 and Par j 2	possible delivery platform	murine Raw 264.7 macrophage cell line and human basophils <i>in vitro</i>	(35)
neoglycocomplexes of oxidized mannan and proteins	OVA and papain	prophylactic	BALB/c mice, i.d.	(36)
carbohydrate-based particles (micro sepharose)	Phl p 5b	prophylactic	BALB/c mice, s.c.	(38)
carbohydrate-based particles (micro sepharose)	rFel d 1	therapeutic and prophylactic	BALB/c mice, i.n. or s.c.	(39, 40)
carbohydrate-based particles (micro sepharose)	rFel d 1	possible delivery platform	human DCs <i>in vitro</i>	(41)
chitin particles ultrasonication of chitin powder	none	prophylactic	BALB/c and C57BL/6 mice, orally and i.n.	(42, 43)
sodium alginate carrier (Conjuvac®) purchased	grass pollen or Der p extract	prophylactic and therapeutic	BALB/c mice and clinical trial, s.c.	(44-46)
gelatin NP-bound CpG-ODN	none	therapeutic	horses, inhalation therapy	(47)
chitosan-DNA NPs complex coacervation	peanut allergen DNA (pCMVArach2)	prophylactic	AKR/J mice, orally	(50)
chitosan NPs complex coacervation	pDer p 1 and pDer p 1 DNA	prophylactic	BALB/c mice, orally	(51)
chitosan NPs	DNA vaccine	prophylactic	BALB/c mice,	(52)

complex coacervation	coexpressing Der p 1 allergen and murine ubiquitin		i.n.	
chitosan NPs complex coacervation	OVA-encoding DNA	prophylactic	BALB/c mice, orally	(53)
chitosan NPs peptide adsorbed on chitosan	Der p 1 peptide	prophylactic	C57BL/6 mice, i.n.	(54)
chitosan-triphosphate NPs ionotropic gelation	Der f allergen	therapeutic	BALB/c mice i.n.	(55)
chitosan NPs complex coacervation	IFN- γ pDNA	prophylactic	STAT4-/- BALB/c mice, i.n.	(56)
chitosan NPs complex coacervation	TGF- β expressing DNA vector	therapeutic	BALB/c mice, orally	(57)
chitosan triphosphate microparticles ; ionotropic gelation	OVA	therapeutic	BALB/c mice, s.l. and murine DC <i>in vitro</i>	(58)
chitosan-phenylalanine-mPEG NPs, self-aggregation	house dust mite allergen extract	possible delivery platform	human HaCaT cell line and PBMC <i>in vitro</i>	(59)

Table 1b: Overview of biodegradable NPs for AIT: liposomes and virus-like particles

NP composition and manufacturing method	Cargo	Vaccination approach	Species/ Route	Ref.
not known	Ragweed allergen	therapeutic	guinea pigs, i.p.	(62)
PC, cholesterol, and phosphatidic acid; dehydration/rehydration + ultra-sonication	<i>A. scoparia</i> pollen extract	therapeutic	BALB/c mice, i.p.	(63, 64)
PC, cholesterol, and L- α -tocopherol; dehydration/rehydration method	<i>Dactylis glomerata</i> , <i>D. pteronyssinus</i> , and cat hair and dander	possible delivery platform	human basophils <i>in vitro</i> and skin prick test	(65)
PC, cholesterol, and phosphatidic acid; dehydration/rehydration method	<i>Aspergillus fumigatus</i> glycoprotein (Af-gp)	therapeutic	BALB/c mice, s.c.	(66)
DPPC; dehydration/rehydration method	<i>D. siboney</i> Der s 1 and Der s 2	therapeutic	BALB/c mice, i.p.	(67)
DPPC, cholesterol, and optional dicetylphosphate or stearylamine; dehydration/rehydration + extrusion	<i>D. pteronyssinus</i> , <i>L. perenne</i> , <i>P. pratense</i> , <i>P. judaica</i> , <i>A. vulgaris</i> , and cat dander extract	therapeutic	BALB/c mice, s.c.	(68)
didodecyldimethylammonium bromide, PC, and cholesterol; dehydration/rehydration method	native Fel d 1 or crude cat hair extract	therapeutic	BALB/c mice, i.n.	(69)
PC or DSPC, cholesterol, and dicetylphosphate;	OVA	therapeutic	BALB/c mice, orally	(70)

dehydration/rehydration method				
PC, cholesterol, diacetylphosphate, and L- α -tocopherol; dehydration/rehydration method	grass pollen or Der p extract	therapeutic	clinical trial skin prick test, s.c.	(71)
DPPC, cholesterol, and tocopheryl acid succinate; dehydration/rehydration + extrusion	Der p 1 and Der p 2 extract	therapeutic	clinical trial, s.c.	(72)
DPPC, mannotriose-DPPE and cholesterol; dehydration/rehydration + extrusion	<i>C. japonica</i> Cry j 1 or OVA	prophylactic and therapeutic	BALB/c mice, i.d. or i.n.	(73) (74)
PC, cholesterol, and didecyldioctadecylammonium bromide; dehydration/rehydration method	<i>P. americana</i> crude extract and Per a 9	therapeutic	BALB/c mice, i.n.	(75)
DOTIM and cholesterol; dehydration/rehydration + extrusion	CpG-DNA and several allergen extracts	therapeutic	dogs, i.d.	(76)
DOTIM and cholesterol; dehydration/ rehydration + extrusion	feline IL-2 cDNA	therapeutic	cats, i.p.	(77)
DOTAP, cholesterol, and protamine; dehydration/rehydration + sonication	CpG and recombinant hybrid molecule	therapeutic	BALB/c mice, s.c.	(78)
DSPC and cholesterol; dehydration/rehydration + extrusion	OVA $\pm$ doxorubicin	therapeutic	BALB/c mice, i.v.	(79)
DPPC, phosphatidyl glycerol and cholesterol; dehydration/rehydration method	<i>D. pteronyssinus</i> extract	possible delivery platform	no cellular tests	(80)
virus-like particles (bacteriophage Qb coat protein); spontaneous assembly	CpG	therapeutic	clinical trial, s.c.	(83)
virus-like particles (bacteriophage Qb coat protein); spontaneous assembly	CpG and Der p 1	prophylactic and therapeutic	clinical trial, i.m. or s.c.	(84)
virus-like particles (bacteriophage Qb coat protein); spontaneous assembly	Fel d 1	prophylactic	BALB/c mice, s.c.	(85)
Adeno-associated virus-like particles spontaneous assembly	OVA	prophylactic	BALB/c mice, s.c.	(86)

Table 2: Overview of non-biodegradable NPs for AIT

NP composition and manufacturing method	Cargo	Vaccination approach	Species/ Route	Ref.
dendrosome Den123 amphiphilic polymer; self-assembly	pBet v 1 DNA	prophylactic	BALB/c mice, s.c.	(91)
guanidine-terminated dendrimers	amyloid-promoting peptide derived from OVA	possible delivery platform	murine RAW264 cell line	(92)
pluronic-stabilized poly(propylene sulfide) NPs	CpG	prophylactic and therapeutic	C57BL/6 mice, injections i.n.	(93)
polyethylenimine containing 3% mannobiose NPs; self-assembly	synthetic plasmid OVA pDNA	prophylactic	BALB/c mice, patch treatment (topically)	(94)
poly(vinylpyrrolidone) NPs reverse micelles method	<i>A. fumigatus</i> allergen	prophylactic	BALB/c mice, s.c.	(95)
N-vinylpyrrolidone and maleic anhydride; self-assembly	OVA	therapeutic	(CBAXC57BL/6)F1 mice, i.p. or s.c. human leukocytes <i>in vitro</i>	(96)
poly(methyl vinyl ether-co-maleic anhydride) NPs (Gantrez® AN NPs); solvent displacement method	OVA and LPS	prophylactic and therapeutic	BALB/c mice, orally or i.d.	(97)
poly(methyl vinyl ether-co-maleic anhydride) NPs (Gantrez® AN NPs); solvent displacement method	<i>L. perenne</i> extract and LPS	therapeutic	BALB/c mice, i.d.	(98)
poly(ethyleneimine) block copolymer 704; self-assembly	Der f 1 plasmid	prophylactic and therapeutic	BALB/c mice, i.m.	(99)
solid-in-oil nanodispersion with sucrose erucate as surfactant	<i>C. japonica</i> peptide	therapeutic	B10.S mice, transcutaneous	(101)
solid-in-oil nanodispersion with methacrylic acid and methylmethacrylate copolymers as surfactant	grass pollen extract	therapeutic	guinea pigs, orally clinical trial	(102)
allergen in poly(vinyl pyrrolidone) on sugar starch core, enterocoated with polymethacrylic acid copolymers	ragweed or timothy grass pollen	therapeutic	clinical trial, orally	(103, 104)
PEG-acetal-dimethacrylate NPs liposomes as templates	OVA, grass pollen/ house dust mite allergen	possible delivery platform	human T cells, DCs and basophils <i>in vitro</i>	(106)
multi-walled carbon nanotubes	house dust mite allergen extract	possible immune-modulatory nanostructure	human PBMC <i>in vitro</i>	(108)

multi-walled carbon nanotubes	house dust mite allergen	prophylactic	BALB/cByJ mice, injection	(109)
C(60) fullerenes	none	negative regulator of mediator release	human mast cells and basophils <i>in vitro</i> , C57BL/6 mice	(110)
C(70)-tetraglycolate fullerenes	OVA	negative regulator of mediator release	C57BL/6 and BALB/c mice, i.n. and human mast cells <i>in vitro</i>	(111)
C(60) fullerenes	OVA	therapeutic	BALB/c mice, s.c. or e.c.	(112)
gold NPs	OVA	therapeutic	Swiss-Webster (outbred) and A/J (inbred) mice	(114)
dextran-coated magnetic NPs chemical coprecipitation method	bovine β -lactoglobulin and ovomucoid	possible delivery platform	human monocytes <i>in vitro</i>	(116)
hydroxy apatite NPs, carbohydrate modified (aquasomes) self-precipitation method	OVA	prophylactic and therapeutic	BALB/c mice, i.d.	(117)

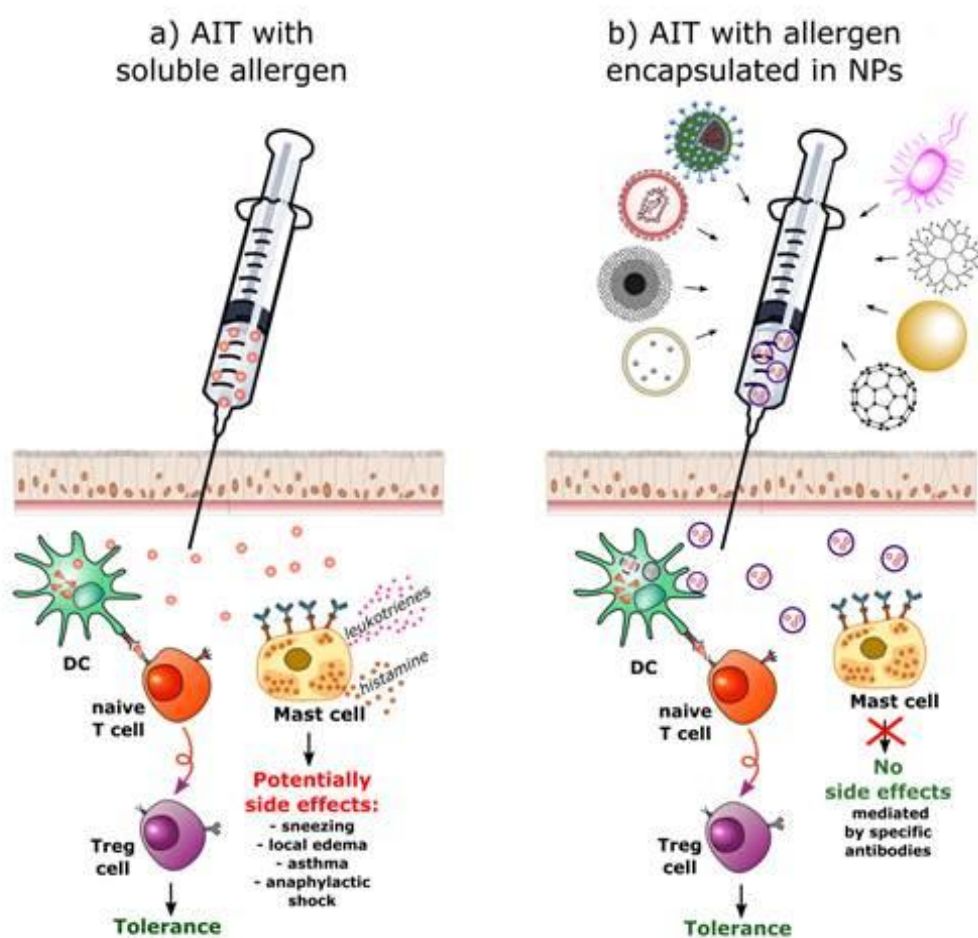


Fig. 1

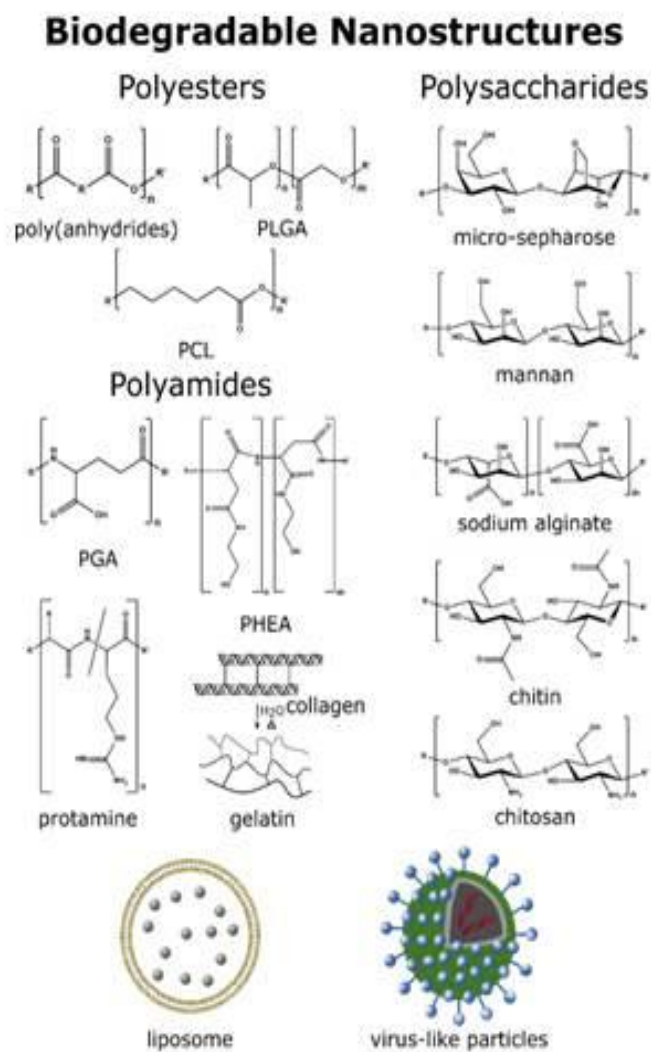


Fig. 2

Non-biodegradable Nanostructures

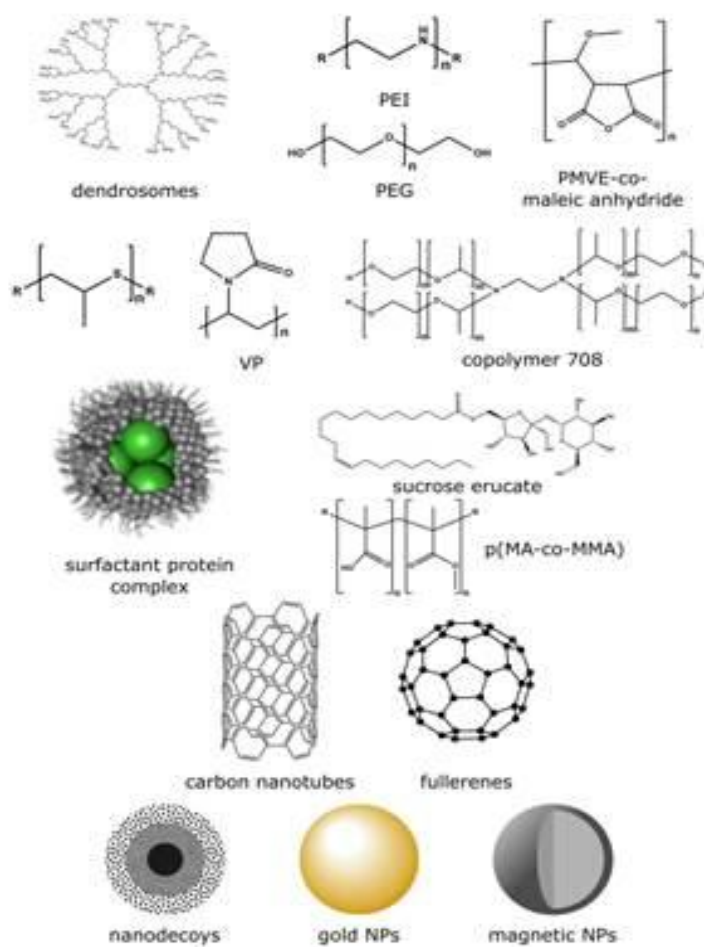


Fig. 3