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Biodegradable Nanoparticles as Vaccine Adjuvants and Delivery Systems: Regulation of Immune Responses by Nanoparticle-Based Vaccine

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Abstract Polymeric nano- and microparticles have recently been shown to possess significant potential as drug delivery systems. In particular, the use of biodegradable polymeric nanoparticles with entrapped antigens such as proteins, peptides, or DNA represents an exciting approach for controlling the release of vaccine antigens and optimizing the desired immune response via selective targeting of the antigen to antigen-presenting cells (APCs). The efficient delivery of antigens to APCs, especially in dendritic cells (DCs), and the activation of APCs are some of the most important issues in the development of effective vaccines. Using nanoparticle-based vaccine delivery systems, it is possible to target delivery to DCs, activate these APCs, and control release of the antigen. Nanoparticles prepared from biodegradable and biocompatible polymers such as poly(lactide-*co*-glycolide) (PLGA), poly(amino acid)s, and polysaccharides have been shown to be effective

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vaccine carriers for a number of antigens. This review mainly focuses on amphiphilic poly(amino acid) and PLGA nanoparticles as vaccine delivery systems and summarizes the investigations of our research group and others on the properties of these antigen-loaded naoparticles.

Keyword Adjuvant · Biodegradable nanoparticles · Poly(γ-glutamic acid) · Protein delivery · Vaccine

Contents

Abbreviations

APCs	Antigen-presenting cells
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CLSM	Confocal laser scanning microscopy
CT	Chitosan
CTL	Cytotoxic T lymphocyte
DCC	*N,N*-Dicyclohexyl carbodiimide
DCs	Dendritic cells
DDS	Drug delivery system
DLS	Dynamic light scattering
FCM	Flow cytometry

HBcAg	Hepatitis B core antigen
HIV	Human immunodeficiency virus
HOBt	1-Hydroxybenzotriazole
HTLV-I	Human T-cell leukemia virus type-I
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MPLA	Monophospholipid A
o/w	Oil-in-water
OVA	Ovalbumin
OVA-NPs	OVA encapsulating within γ-PGA-Phe nanoparticles
PCL	Poly(ε-caprolactone)
pDNA	Plasmid DNA
PEI	Polyethylenimine
PGA	Poly(glycolic acid)
PHB	Poly(hydroxybutyrate)
Phe	L-Phenylalanine
PIC	Polyion complex
PLA	Poly(lactic acid)
PLGA	Poly(lactide-*co*-glycolide)
SAXS	Small angle X-ray scattering
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
Trp	L-Tryptophan
w/o/w	Water-in-oil-in-water
γ-PGA	Poly(γ-glutamic acid)
γ-PGA-Phe	γ-PGA-*graft*-Phe copolymer
ε-PL	Poly(ε-lysine)
ε-PL-CHS	ε-PL-*graft*-cholesterol hydrogen succinate

1 Introduction

Vaccination to induce an adaptive immune response is expected for a broad range of infectious diseases and cancers. Traditional vaccines are mainly composed of live attenuated viruses, whole inactivated pathogens, or inactivated bacterial toxins. In general, these approaches have been successful for developing vaccines that can induce an immune response based on antigen-specific antibody and cytotoxic T lymphocyte (CTL) responses, which kill host cells infected with intracellular organisms (Fig. 1) [1, 2]. One of the most important current issues in vaccinology is the need for new adjuvants (immunostimulants) and delivery systems. Many of the vaccines currently in development are based on purified subunits, recombinant

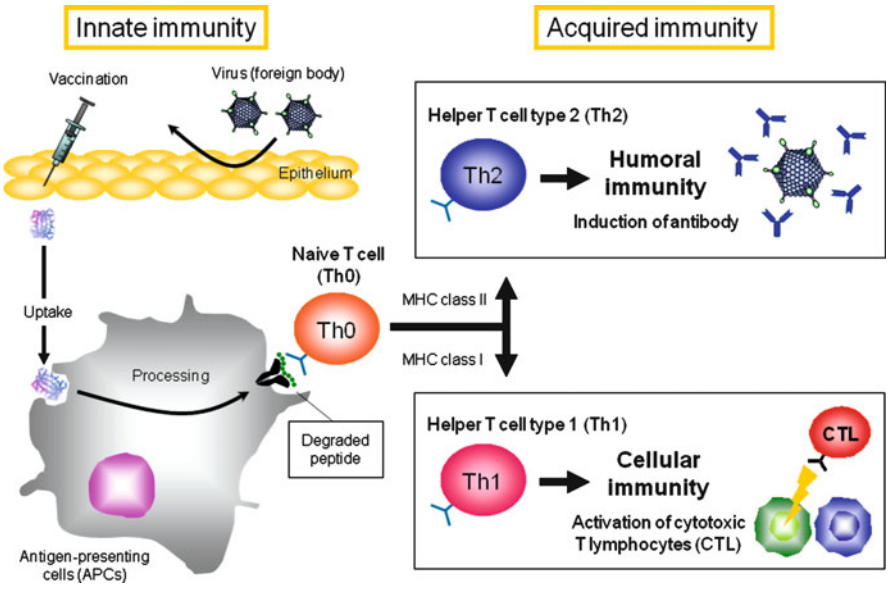

Fig. 1 Induction of immune responses by vaccination

proteins, or synthetic peptides. These new generation of vaccines are generally very safe, with well-defined components. However, these antigens are often poorly immunogenic, and thus require the use of adjuvants and delivery systems to induce optimal immune responses [3–5]. Immunological adjuvants were originally described by Ramon as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone" [6]. Until recently, the hydroxide and phosphate salts of aluminum and calcium were the only adjuvants licensed for human use. However, the use of alum-type adjuvants for vaccination has some disadvantages [7, 8]. They are not effective for all antigens, induce local reactions, induce IgE antibody responses, and generally fail to induce cell-mediated immunity, particular CTL responses. Therefore, the development of more efficient and safe adjuvants and vaccine delivery systems to obtain high and long-lasting immune responses is of primary importance.

Polymeric nanoparticles formulated from biodegradable polymers are being widely explored as carriers for controlled delivery of different agents including proteins, peptides, plasmid DNA (pDNA), and low molecular weight compounds [9–11]. Self-assembling polymer or block/graft copolymers that can form nanostructures have been extensively investigated in the field of biotechnology and pharmaceuticals. In general, hydrophobic interactions, electrostatic forces, hydrogen bonds, van der Waal forces, or combinations of these interactions are available as the driving forces for the formation of the polymer complexes [12–16]. Numerous investigators have shown that the biological distribution of drugs,

proteins, and DNA can be modified, both at the cellular and organ levels, using nano- or microparticle delivery systems [17–19]. For the development of effective vaccines, biodegradable nanoparticles show great promise as vaccine delivery systems. Controlled delivery systems consisting of nanoparticles can potentially delivery either the antigens or adjuvants to the desired location at predetermined rates and durations to generate an optimal immune response. The carrier may also protect the vaccine from degradation until it is released. Other potential advantages of the controlled delivery approach include reduced systemic side effects and the possibility of co-encapsulating multiple antigenic epitopes or both antigen and adjuvant in a single carrier. Biodegradable polymers provide sustained release of the encapsulated antigen and degrade in the body to nontoxic, low molecular weight products that are easily eliminated.

On the other hand, recent strategies for developing preventative and therapeutic vaccines have focused on the ability to deliver antigen to dendritic cells (DCs) in a targeted and prolonged manner. These strategies use nanoparticles because they can achieve longevity on intact antigen to increase the opportunity for DC uptake and processing. DCs are the most effective antigen-presenting cells (APCs), and have a crucial role in initiating T-cell-mediated immunity. DCs can control a substantial part of the adaptive immune response by internalizing and processing antigens through major histocompatibility complex (MHC) class I and class II pathways, and then presenting antigenic peptides to $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 1) [20]. Therefore, targeting DCs with an antigen delivery system provides tremendous potential in developing new vaccines [21]. Antigen uptake by DCs is enhanced by the association of the antigens with polymeric nanoparticles. The adjuvant effect of particulate materials appears to largely be a consequence of their uptake into DCs. More importantly, particulate antigens have been shown to be more efficient than soluble antigens for the induction of immune responses [22, 23]. Furthermore, the submicron size of nanoparticles offers a number of distinct advantages over microparticles, and nanoparticles generally have relatively higher intracellular uptake as compared to microparticles [24, 25]. There are several factors that can affect the immune response induced by immunization with particulate antigens. Among them are particle size, the chemical structure of particles, surface hydrophobicity, zeta potential, and adjuvants used within the formulations.

This review focuses on biodegradable polymeric nanoparticles as vaccine delivery systems and immunostimulants, and summarizes the preparation of antigen-conjugated particles and the mechanism of nanoparticle-based vaccines. Using these systems, it is possible to target antigen delivery to APCs, activate these APCs, and control intracellular release and distribution of the antigen. By understanding immune activation, we can rationally design particulate adjuvant to not only deliver antigen but also to directly activate innate immune cells providing the pro-inflammatory context for antigen recognition. The generation of more potent particulate adjuvants may allow the development of prophylactic and therapeutic vaccines against cancers and chronic infectious diseases.

2 Preparation of Biodegradable Polymeric Nanoparticles

2.1 PLGA Nanoparticles

Biodegradable polymeric nanoparticles have attracted much attention for their potential in biomedical applications, such as drug, gene, and vaccine delivery systems. The biodegradation rate and the release kinetics of loaded drugs can be controlled by the composition ratio and the molecular weight of the polymer and block/graft copolymers [26–28]. Furthermore, by modulating the polymer characteristics, one can control the release of a therapeutic agent from the nanoparticles to achieve a desired therapeutic level in a target tissue for the required duration for optimal therapeutic efficacy. The commonly used biodegradable polymers are aliphatic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), poly(hydroxybutyrate) (PHB) and their copolymers (Fig. 2) [29]. In particular, poly(lactide-*co*-glycolide) (PLGA) has been the most extensively investigated for developing nano- and microparticles encapsulating therapeutic drugs in controlled release applications [30–32] due to their inherent advantages. The copolymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several weeks. As polyesters in nature, these polymers undergo hydrolysis upon administration into the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle.

Several methods have been reported for the preparation of biodegradable nanoparticles from PLGA, PLA, and PCL by dispersing preformed polymers. Emulsion solvent evaporation techniques are frequently used to prepare nano- and microparticles [33, 34]. The polymer is dissolved in an organic solvent like dichloromethane, chloroform, or ethyl acetate and then emulsified into an aqueous solution to create an oil-in-water (o/w) emulsion by using a surfactant such as poly (vinyl alcohol). After the formation of a stable emulsion, the organic solvent is evaporated by increasing the temperature under pressure (Fig. 3). The effect of this process is variable, depending on the properties of the nanoparticles. Often, surfactants are used to stabilize the nanoparticles in aqueous solution in order to prevent the aggregation and/or precipitation of water-insoluble polymers. However, adequate removal of the surfactant remains a problem, and surfactant molecules are sometimes harmful in biomedical applications.

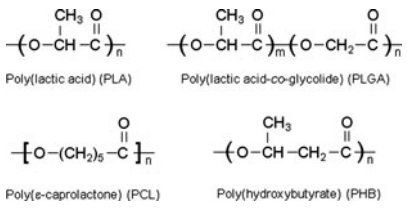

Fig. 2 Chemical structures of biodegradable polyesters used for preparation of nanoparticles

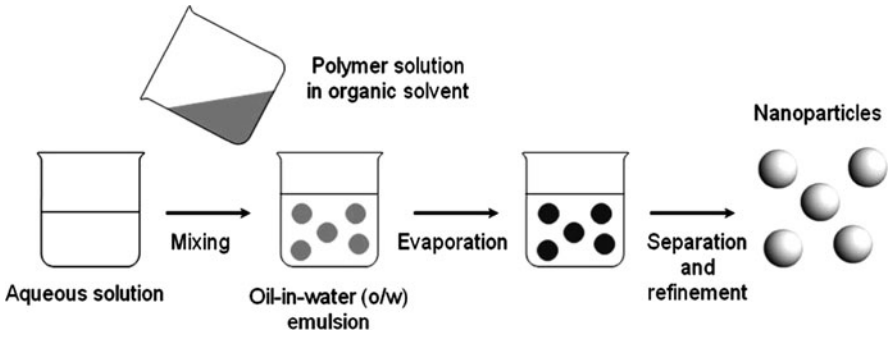

Fig. 3 Preparation of polymeric nanoparticles by emulsion solvent evaporation technique

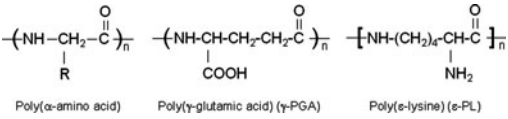

Fig. 4 Chemical structures of synthetic and naturally occurring poly(amino acid)s

2.2 *Amphiphilic Poly(amino acid) Nanoparticles*

Recently, many studies have focused on self-assembled biodegradable nanoparticles for biomedical and pharmaceutical applications. Nanoparticles fabricated by the self-assembly of amphiphilic block copolymers or hydrophobically modified polymers have been explored as drug carrier systems. In general, these amphiphilic copolymers consisting of hydrophilic and hydrophobic segments are capable of forming polymeric structures in aqueous solutions via hydrophobic interactions. These self-assembled nanoparticles are composed of an inner core of hydrophobic moieties and an outer shell of hydrophilic groups [35, 36].

In particular, poly(amino acid)s have received considerable attention for their medical applications as potential polymeric drug carriers. Several amphiphilic block and graft copolymers based on poly(amino acid)s have been employed, such as poly(α-L-glutamic acid) [37], poly(γ-glutamic acid) [38], poly(ε-lysine) [39] (Fig. 4), poly(L-aspartic acid) [40], poly(L-lysine) [41], poly(L-arginine) [42], and poly(L-asparagine) [43] as hydrophilic segments, and poly(β-benzyl-L-aspartate) [44], poly(γ-benzyl-L-glutamate) [45], and poy(L-histidine) [46] as hydrophobic segments. In general, amphiphilic copolymers based on poly(amino acid)s form micelles through self-association in water.

Poly(γ-glutamic acid) (γ-PGA) is a naturally occurring poly(amino acid) that is synthesized by certain strains of *Bacillus* [47]. The polymer is made of D- and L-glutamic acid units linked through the α-amino and the γ-carboxylic acid groups, and its α-carboxylate side chains can be chemically modified to introduce various bioactive ligands, or to modulate the overall function of the polymer [48–52]. Unlike general poly(amino acid)s, γ-PGA has unique characteristics of enzymatic degradation and immunogenicity. It has been reported that γ-PGA has resistance

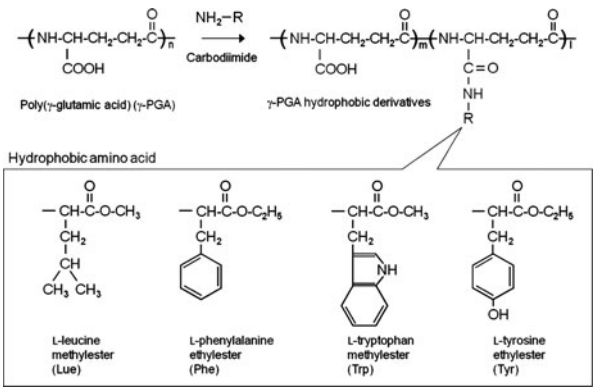

Fig. 5 Synthesis of γ-PGA hydrophobic derivatives

against many proteases because γ-linked glutamic acids are not easily recognized by common proteases [53, 54]. Moreover, several studies have shown that γ-PGA by itself is a poor immunogen and does not induce booster responses, probably because of its simple homopolymeric structure, similar to those of polysaccharides [55–59]. Therefore, the potential applications of γ-PGA and its derivatives have been of interest in a broad range of fields, including medicine, food, cosmetics, and water treatment [60].

Akashi et al. prepared nanoparticles composed of hydrophobically modified γ-PGA [38, 61, 62] (Fig. 5). γ-PGA (400 kDa) as the hydrophilic backbone and L-phenylalanine (Phe) as the hydrophobic segment were synthesized by grafting Phe to γ-PGA using water-soluble carbodiimide. The γ-PGA-*graft*-Phe copolymers (γ-PGA-Phe) with more than 50% grafting degree formed monodispersed nanoparticles in water due to their amphiphilic properties. To prepare nanoparticles, γ-PGA-Phe dissolved in dimethyl sulfoxide (DMSO) was added to various concentration of NaCl solution, and then the resulting solutions were dialyzed and freeze-dried. The γ-PGA-Phe formed monodispersed nanoparticles, and the particle size of the γ-PGA-Phe nanoparticles could be easily controlled (30–200 nm) by changing NaCl concentration [63]. Similarly, γ-PGA conjugated with L-tryptophan (γ-PGA-Trp) showed the same tendency (Fig. 6). The size of the nanoparticles increased with increasing NaCl concentration during formation of particles. The addition of NaCl leads to enhanced screening of the Coulomb interactions between the carboxyl groups of γ-PGA-Phe. Therefore, according to the increase in NaCl concentration, a larger number of graft copolymers was involved in the formation nanoparticles. The nanoparticles showed a highly negative zeta potential (−25 mV) due to the ionization of the carboxyl groups of γ-PGA located near the surfaces. The specific self-assembly behavior of γ-PGA-Phe in aqueous solution was due to multiple stacking of phenyl groups. Beside the particle formation of γ-PGA by using hydrophobic interaction, nanoparticles formed by complexation of γ-PGA with a bivalent metal ion complex [64] or by chemical crosslinking of carboxyl groups of γ-PGA [65] have been reported.

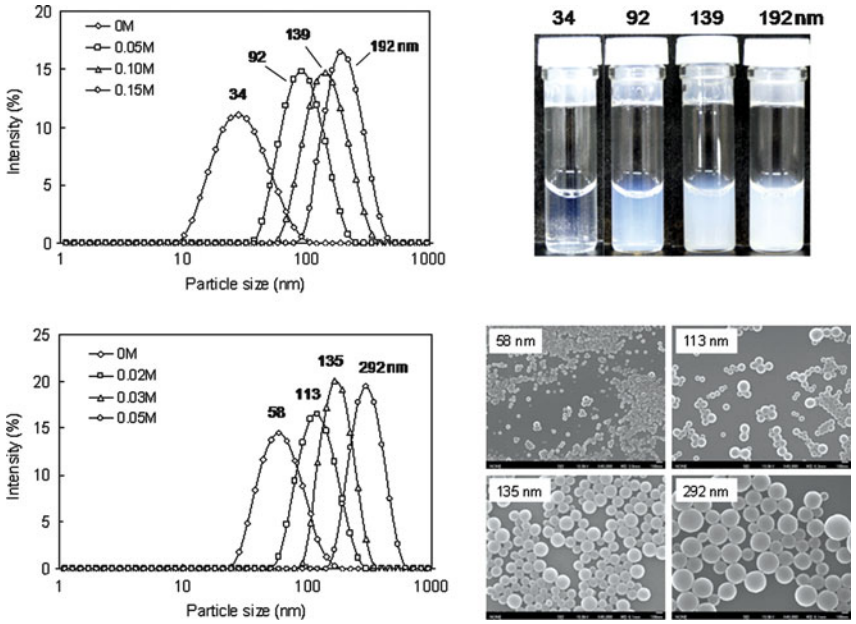

Fig. 6 Size changes of (**a**) γ-PGA-Phe and (**c**) γ-PGA-Trp nanoparticles prepared at various NaCl concentrations. The size of nanoparticles was measured by DLS. (**b**) Photographs of γ-PGA-Phe nanoparticles (2.5 mg/mL) dispersed in water. (**d**) Scanning electron microscope (SEM) images of γ-PGA-Trp nanoparticles prepared at various NaCl concentrations

Poly(ε-lysine) (ε-PL) is produced by a *Streptomyces albulus* strain, and has been used as a food additive due to its antimicrobial activities [66, 67]. ε-PL is water soluble and biodegradable and has a molecular weight of approximately 5,000. ε-PL is an L-lysine homopolymer (25–30 residues) with a linkage between the carboxyl group and the ε-amino group (Fig. 4). Matsusaki et al. reported the nanoparticle formation of amphiphilic ε-PL-*graft*-cholesterol hydrogen succinate (ε-PL-CHS) in water. ε-PL was hydrophobically modified by CHS in the presence of *N*,*N*-dicyclohexyl carbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) in *N*,*N*-dimethylformamide (DMF) (Fig. 7) [39]. ε-PL-CHS nanoparticles were prepared by the solvent (tetrahydrofuran) exchange method. ε-PL-CHS could form stable nanoparticles in water following the hydrophobic interactions of its CHS groups. The size of the ε-PL-CHS nanoparticles was approximately 150–200 nm. For the purposes of nonviral gene delivery, cationic polymers such as poly(L-lysine) and polyethylenimine (PEI) have been used as carriers for complexing gene vectors into polyplexes [68–70]. A polyplex can be easily formed when the oppositely charged DNA and polycation are mixed in aqueous solution and interact via electrostatic interactions. These polyplexes result in an increased net positive charge of the complexes, and promote cellular uptake and transfection efficiency. However, the in vivo applications of polyplexes are limited by low gene expression and toxicity due to their cationic nature [71–73]. ε-PL is

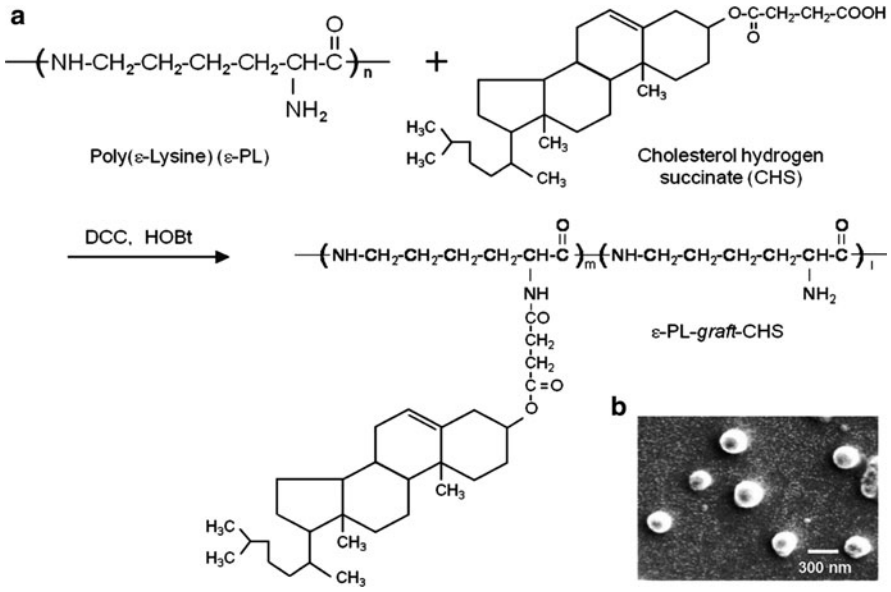

Fig. 7 (**a**) Synthesis of amphiphilic ε-PL-*graft*-cholesterol hydrogen succinate (ε-PL-CHS). (**b**) SEM image of nanoparticles prepared from ε-PL-CHS

a very safe material for use in humans. Therefore, the nanoparticles fabricated from ε-PL may be useful for DNA vaccine delivery and adjuvants.

2.3 Amphiphilic Polysaccharide Nanoparticles

Polysaccharidic hydrogel particles have been often used for designing protein-loaded systems for therapeutic applications. Polysaccharides are very hydrophilic polymers, and their hydrogels thus exhibit a good biocompatibility. Various type of hydrophobized polysaccharides, such as pullulan [74, 75], curdlan [76], dextran [77], alginic acid [78], and chitosan [79], have been used for preparation of nanoparticles. Akiyoshi et al. reported that self-aggregated hydrogel nanoparticles could be formed from cholesterol-bearing pullulan by an intra- and/or intermolecular association in diluted aqueous solutions [80]. Recently, much attention has been paid to chitosan as a drug or gene carrier because of its biocompatibility and biodegradability. Chitosan is a polysaccharide constituted of *N*-glucosamine and *N*-acetyl-glucosamine units, in which the number of *N*-glucosamine units exceeds 50%. Chitosan can be degraded into nontoxic products in vivo, and thus has been widely used in various biomedical applications [81, 82]. Chitosan has cationic characters even in neutral conditions to form complexes with negatively charged pDNA. Jeong et al. prepared nanosized self-aggregates composed of

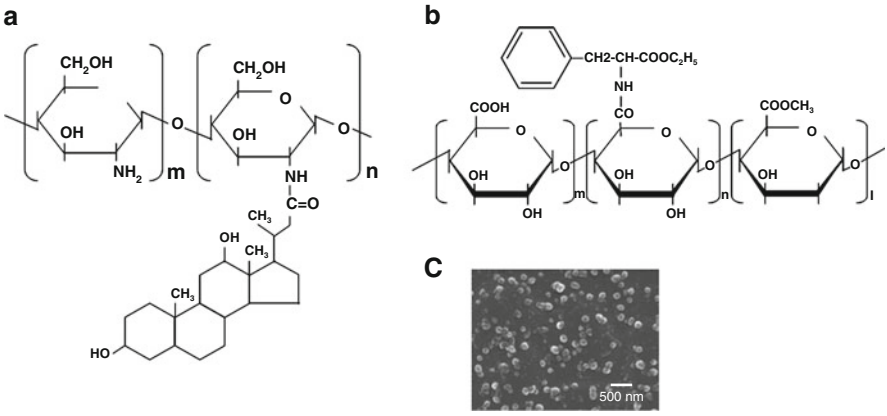

Fig. 8 Preparation of amphiphilic polysaccharide. Chemical structures of deoxycholic acid-modified chitosan (**a**) and Phe-modified pectin (pectin-*graft*-Phe) (**b**). SEM image of nanoparticles prepared from pectin-*graft*-Phe (**c**)

hydrophobically modified chitosans with deoxycholic acids (Fig. 8a) [83, 84]. The size of self-aggregates varied in the range of 130–300 nm in diameter, and their structures were found to depend strongly on the molecular weight of chitosan. To explore the potential applications of self-aggregates as a gene delivery carrier, complexes between chitosan self-aggregates and pDNA were prepared. The complex formation had a strong dependency on the size and structure of chitosan self-aggregates and significantly influenced the transfection efficiency of cells. It is expected that these approaches to control the size and structure of chitosan-derived self-aggregates will have a wide range of applications in gene delivery. Also, Kida et al. reported that novel polysaccharide-based nanoparticles were successfully prepared by the self-assembly of amphiphilic pectins, which were easily synthesized by the reaction of pectins with Phe as hydrophobic group (Fig. 8b) [85]. Pectin is a polymer of D-galacturonic acid. The galacturonic acid molecule has a carboxyl group on C5, some of which are esterified to form methyl esters. The pectin-*graft*-Phe could form about 200 nm-sized nanoparticles (Fig. 8c), and were able to retain entrapped protein in the nanoparticles for one week without any significant leakage.

2.4 Polyion Complex Nanoparticles

Polymer complexes associated with two or more complementary polymers are widely used in potential applications in the form of particles, hydrogels, films, and membranes. In particular, a polyion complex (PIC) can be easily formed when oppositely charged polyelectrolytes are mixed in aqueous solution and interact via

electrostatic (coulombic) interactions. Nanoscaled structural materials (e.g., nanoparticles, micelles, nanogels, and hollow nanospheres) composed of PIC are prepared by tuning the preparation conditions, such as the charge ratio of the anionic-to-cationic polymers, temperature, concentration, and type of polyelectrolyte [12, 86, 87].

PIC containing γ-PGA and chitosan (CT) as a cationic polymer has been used for preparation of nanoparticles, hydrogels, and films for biomedical applications. Sung et al. investigated the PIC particle formation of γ-PGA and CT by self-assembly in aqueous media [88]. Nanoparticles were obtained upon addition of a γ-PGA (160 kDa) aqueous solution (pH 7.4) into a low molecular weight CT (50 kDa) aqueous solution (pH 6.0). It was found that the particle size and the zeta potential of the prepared nanoparticles were mainly determined by the relative amount of the local concentration of γ-PGA in the added solution to the surrounding concentration of CT. The size (80–400 nm) and surface charge (from −35 to +25 mV) of γ-PGA-CT nanoparticles could be easily controlled by changing the mixing ratio of two polymers. Hajdu et al. also prepared γ-PGA (1,200 kDa)–CT (320 kDa) nanoparticles [89]. The size and size distribution of the nanoparticles depended on the concentrations of γ-PGA and CT solutions and their ratio as well as on the pH of the mixture and the order of addition. The particle size was in the range of 20–285 nm, as measured by transmission electron microscopy (TEM), and the average hydrodynamic diameters were between 150 and 330 nm.

The stability and characteristics of prepared PIC are influenced by various factors involving their chemical compositions and their surrounding environment. In particular, for PIC micelles or nanoparticles, the ionic strength and pH of the solution is a key parameter for stability because of the shielding effect of the ionic species on the electrostatic interactions [90]. Therefore, destabilization of PIC under physiological conditions limits their applications as a drug carrier. For the development of stable PIC nanoparticles under physiological conditions, Akagi et al. focused on a novel approach for the stabilization of PIC nanoparticles by hydrophobic interactions. Amphiphilic γ-PGA-Phe as the biodegradable anionic polymer, and ε-PL as the cationic polymer were used for preparation of PIC nanoparticles (Fig. 9) [91]. The PIC nanoparticles were prepared by mixing

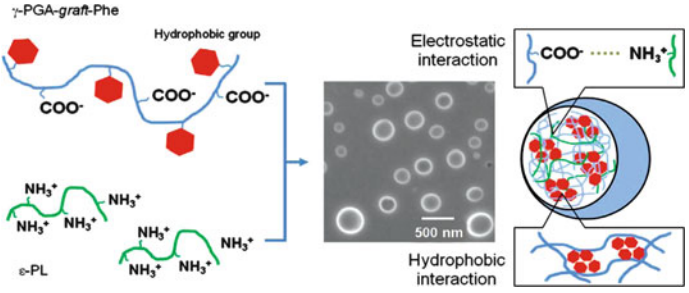

Fig. 9 Stabilization of polyion complex nanoparticles composed of poly(amino acid)s using hydrophobic interactions

γ-PGA-Phe (water soluble) with ε-PL in phosphate-buffered saline (PBS). The formation and stability of the PIC nanoparticles was investigated by dynamic light scattering (DLS) measurements. Monomodal anionic PIC nanoparticles were obtained using nonstoichiometric mixing ratios. When unmodified γ-PGA was mixed with ε-PL in PBS, the formation of PIC nanoparticles was observed. However, within a few hours after the preparation, the PIC nanoparticles dissolved in the PBS. In contrast, γ-PGA-Phe/ε-PL nanoparticles showed high stability for a prolonged period of time in PBS, and over a wide range of pH values. The stability and size of the PIC nanoparticles depended on the γ-PGA-Phe/ε-PL mixing ratio and the hydrophobicity of the γ-PGA. The improved stability of the PIC nanoparticles was attributed to the formation of hydrophobic domains in the core of the nanoparticles. The fabrication of PIC nanoparticles using hydrophobic interactions was very useful for the stabilization of the nanoparticles.

3 Polymeric Nanoparticles for Antigen Delivery and Adjuvant

3.1 Preparation of Antigen-Loaded Nanoparticles

Nanoparticles containing encapsulated, surface-immobilized or surface-adsorbed antigens are being investigated as vaccine delivery systems as alternatives to the currently used alum, with an objective to develop better vaccine systems and minimize the frequency of immunization. The encapsulation of antigenic proteins or peptides into PLGA nanoparticle carrier system can be carried out through mainly three methods: the water-in-oil-in-water (w/o/w) emulsion technique, the phase separation method, and spray drying. The w/o/w double emulsion process is popularly used to load proteins into nanoparticles (Fig. 10) [92, 93]. In this process, an antigen is first dissolved in an aqueous solution, which is then emulsified in an organic solvent to make a primary water-in-oil emulsion. This initial emulsion is further mixed in an emulsifier-containing aqueous solution to make a w/o/w double emulsion. The ensuing removal of the solvent leaves nano- and microparticles in the aqueous continuous phase, making it possible to collect them by filtration or centrifugation. However, the possible denaturation of the proteins at the oil–water interface limits the usage of this method. It has been reported that this interface causes conformational changes in bovine serum albumin (BSA) [94, 95]. Moreover, it has a disadvantage in that the entrapment efficiency is very low. The prevention of protein denaturation and degradation, as well as high entrapment efficiency, would be of particular importance in the preparation of nanoparticles containing water-soluble drugs such as a protein. Improved protein integrity has been achieved by the addition of stabilizers such as carrier proteins (e.g., albumin), surfactants during the primary emulsion phase, or molecules such as trehalose and mannitol to

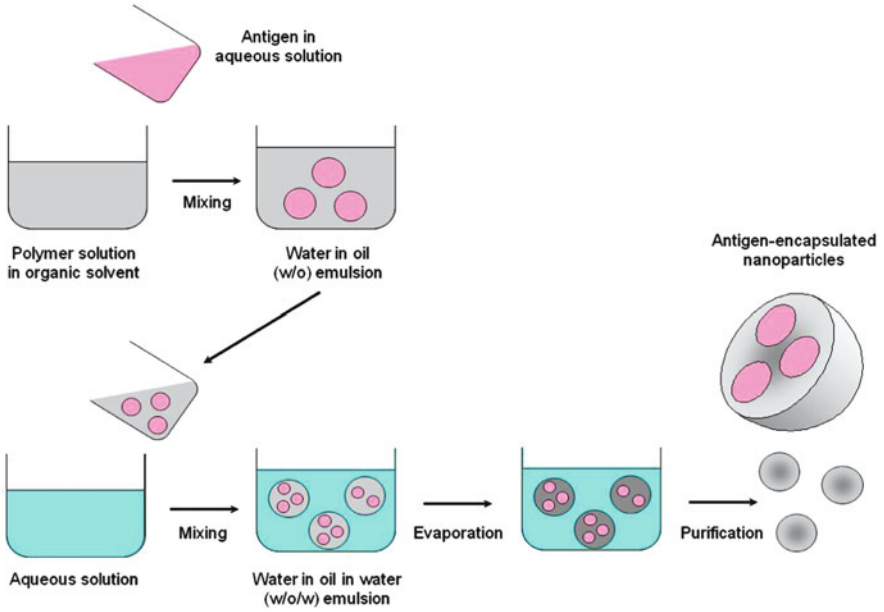

Fig. 10 Preparation of antigen-encapsulating nanoparticles by w/o/w emulsion method

the protein phase. Protein stability may also be enhanced if the protein is encapsulated as a solid rather than in solution.

We have recently found that nanoparticles consisting of amphiphilic poly(amino acid)s can efficiently and stably encapsulate various types of protein into the nanoparticles. Protein-loaded γ-PGA-Phe nanoparticles were prepared by encapsulation, covalent immobilization, or physical adsorption methods in order to study their potential applications as protein carriers [96, 97]. To prepare the protein-encapsulating γ-PGA-Phe nanoparticles, proteins with various molecular weights and isoelectric points were dissolved in saline, and the γ-PGA-Phe dissolved in DMSO was added to the protein solutions. The resulting solutions were then centrifuged and repeatedly rinsed (Fig. 11). The encapsulation of proteins into the nanoparticles was successfully achieved. All proteins used in this experiment were successfully encapsulated into the nanoparticles. The encapsulation efficiency was found to be in the range of 30–60% for most samples. For all samples tested, it was observed that the encapsulation efficiency for a given protein was not markedly influenced by the physical properties of that protein. Ovalbumin (OVA) encapsulated into the nanoparticles was not released (less than 10%) over the pH range of 4–8, even after 10 days. Moreover, it was found that the γ-PGA-Phe nanoparticles have some excellent properties. The enzyme-encapsulating nanoparticles showed high enzymatic activity. In the case of protein-encapsulating nanoparticles prepared by the self-assembly of γ-PGA-Phe, the encapsulated protein may be more stable than via the emulsion method. Proteins encapsulated into the nanoparticles appear to be adequate in terms

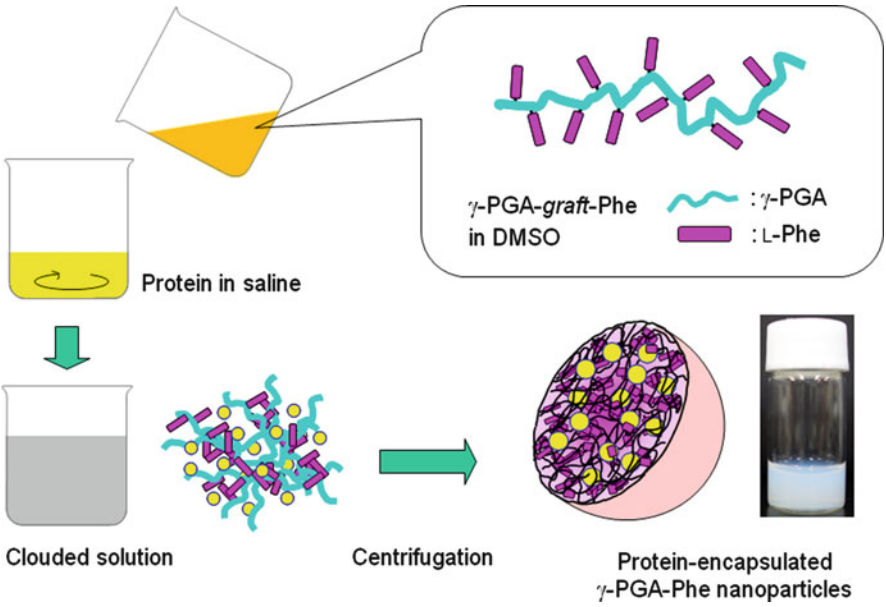

Fig. 11 Preparation of protein-encapsulating γ-PGA-Phe nanoparticles

of the preservation of the protein structure. The γ-PGA-Phe nanoparticles and protein-encapsulating nanoparticles could be preserved by freeze-drying. The results of cytotoxicity tests showed that the nanoparticles did not cause any relevant cell damage. Therefore, it is expected that the γ-PGA-Phe nanoparticles will have great potential as multifunctional carriers in pharmaceutical and biomedical applications, such as drug and vaccine delivery systems. Also, Portilla-Arias et al. reported preparation of nanoparticles made of alkyl esters of γ-PGA and described their potential application as drug and protein carriers [98].

3.2 *Delivery of Antigens Using Nanoparticles*

Antigen-loaded polymeric nanoparticles represent an exciting approach to the enhancement of antigen-specific humoral and cellular immune responses via selective targeting of the antigen to APCs [99, 100]. DCs are considered to be initiators and modulators of immune responses and are capable of processing antigens through both major histocompatibility complex (MHC) class I and II pathways. Immature DCs encounter pathogens (e.g., virus or bacteria), antigens, or particulate materials at the injection site and, after phagocytosis, the foreign bodies taken up into the DCs present antigens on MHC class II molecules or even on MHC class I molecules by cross-priming [101]. Therefore, the antigen delivery to DCs is of key importance in the development of effective vaccines (Fig. 12).

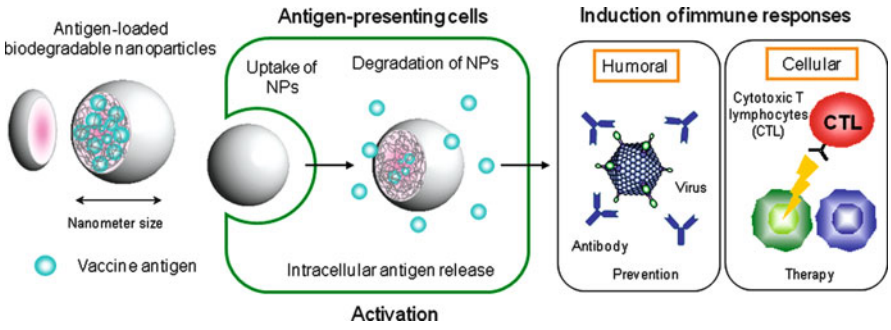

Fig. 12 Induction of immune responses by nanoparticle-based vaccine

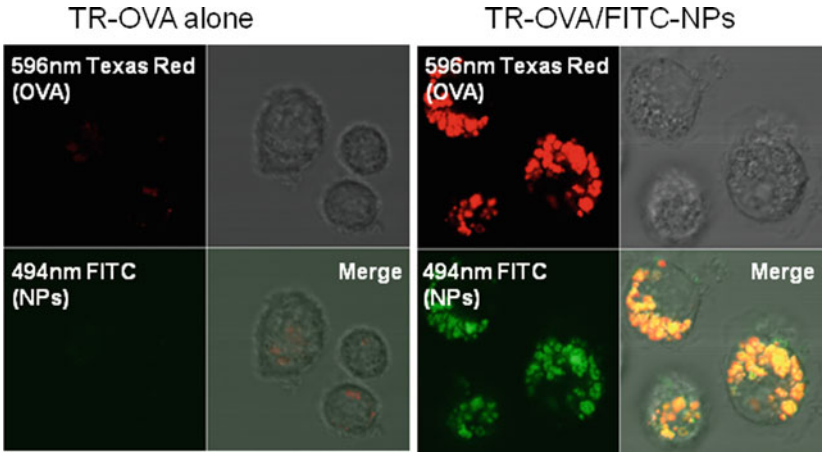

Fig. 13 Uptake of OVA-encapsulating γ-PGA-Phe nanoparticles by DCs. DCs were incubated with Texas Red-labeled OVA (*TR-OVA*) alone (**a**) or TR-OVA encapsulated within fluorescein-labeled nanoparticles (*TR-OVA/FITC-NPs*) (**b**). The intracellular localization of OVA (*red*) and NPs (*green*) was observed by confocal laser scanning microscopy

Akagi et al. demonstrated the use of nanoparticles composed of amphiphilic poly (amino acid) derivatives as vaccine delivery and adjuvants [62, 102–104]. To evaluate the uptake of OVA encapsulated within γ-PGA-Phe nanoparticles (OVA-NPs) by DCs, murine bone marrow-derived DCs were incubated with 250 nm-sized OVA-NPs for 30 min at 37 °C. The cells were then analyzed by flow cytometry (FCM) and confocal laser scanning microscopy (CLSM). OVA-NPs were efficiently taken up into DCs, whereas the uptake of OVA alone was barely detectable at the same concentration of OVA (Fig. 13). OVA-NPs were more efficiently taken up than OVA alone by DCs, and the uptake of OVA-NPs was inhibited at 4 °C. These results suggest that OVA-NPs were phagocytosed mainly via endocytosis by the DCs. In the case of OVA alone, an approximately 30-fold

higher concentration was required to elicit a similar amount of intracellular OVA as compared to OVA-NPs. Likewise, it has been reported that PLGA nanoparticles or liposomes are efficiently phagocytosed by the DCs in culture, resulting in their intracellular localization [105–107]. Foged et al. investigated DC uptake of model fluorescent polystyrene particles with a broad size range (0.04–15 μm). The results showed that DCs internalized particles in the tested size range with different efficiencies. The optimal particle size for DC uptake was 500 nm and below. In the smaller the particle size, a higher percentage of the DCs interacted with the polystyrene spheres [24]. Kanchan et al. also reported that PLA nanoparticles (200–600 nm) were efficiently taken up by macrophages in comparison to microparticles (2–8 μm) [25]. In contrast, in hydrogel particles composed of polyacrylamide, there was no difference in uptake by APCs of particles sized between 3.5 μm and 35 nm [108]. This disparity in uptake may be related to fundamental differences in the material properties of those carriers.

Particle shape and surface charge are equally important particulate physico-chemical factors and play crucial roles in the interaction between particles and APCs. In general, cationic particles induced high phagocytosis activity of APCs, because of the anionic nature of cell membranes [24]. Recently, particle shape has been identified as having a significant effect on the ability of macrophages to internalize particles via actin-driven movement of the macrophage membrane. Champion et al. observed that the cellular uptake of particles strongly depends on the shape of particles. The worm-like particles with very high aspect ratios exhibited negligible phagocytosis when compared to traditional spherical particles [109, 110]. These result suggest that uptake of particles by APCs strongly depends on the local geometry at the interface between particles and cells.

3.3 Activation of Dendritic Cells by Nanoparticles

Research on biomaterial adjuvant potential has been focused largely on determining the degree of DC maturation induced by exposure to polymeric nanoparticles or liposomes [111–113]. The maturation of DCs is associated with increased expression of several cell surface markers, including the co-stimulatory molecules CD40, CD80, CD83, CD86, MHC class I, and MHC class II. It is well known that DC maturation can be induced by inflammatory factors such as lipopolysaccharide (LPS), bacterial DNA, or inflammatory cytokines such as TNF-α, and the process is highly importance for the initiation of acquired and innate immune responses by these cells [114, 115]. Therefore, in addition to the antigen delivery to DCs, the control of DC maturation is deeply involved in the development of effective vaccines.

In vitro studies have shown that γ-PGA-Phe or PLGA nanoparticle-pulsed DCs result in DC maturation by upregulation of co-stimulatory molecule expression and cytokine production (Fig. 14). To determine whether the uptake of γ-PGA-Phe

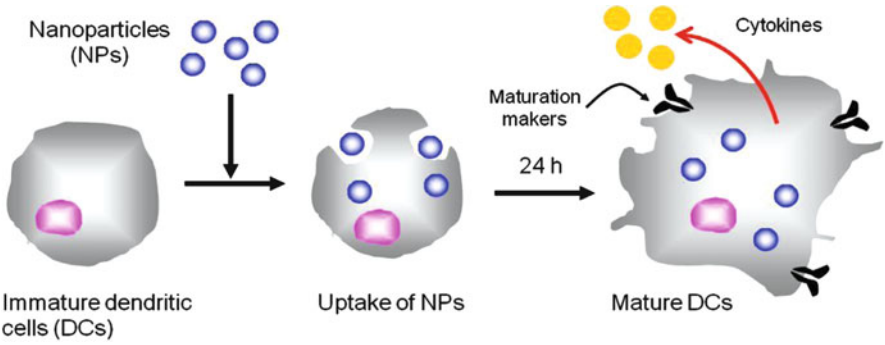

Fig. 14 Maturation and activation of DCs by nanoparticles

nanoparticles mediates the phenotypic maturation of DCs, the DCs were incubated with γ-PGA-Phe nanoparticles for 24 or 48 h, and the expression of surface molecules was measured by fluorescence confocal microscopy (FCM). Upon exposure of these DCs to the nanoparticles, the expression of co-stimulatory molecules (maturation markers) was increased in a dose-dependent manner. The expression levels of co-stimulatory molecules in nanoparticle-pulsed DCs were similar to those of LPS-pulsed DCs. These results suggest that γ-PGA-Phe nanoparticles have great potential as adjuvant for DC maturation [62, 102, 103]. The mechanisms responsible for DC maturation by γ-PGA-Phe nanoparticles are still unclear. However, it is hypothesized that not only the uptake of nanoparticles but also the characteristics of the polymers forming the nanoparticles are important for the induction of DC maturation. The DC uptake of 30 nm-sized nanoparticles was lower than for 200 nm-sized nanoparticles, but the effect of DC activation by the nanoparticles was high for the small sizes [116, 117]. Thus, it is considered that the surface interactions between the nanoparticles and DCs predominately affect DC maturation. In addition, soluble γ-PGA-induced innate immune responses in a Toll-like receptor 4 (TLR4)-dependent manner in DCs have been reported [118, 119]. TLRs are abundantly expressed on professional APCs. TLRs play a major role in pathogen recognition, and in the initiation of the inflammatory and immune responses. The stimulation of TLRs by TLR ligands induces the surface expression of co-stimulatory molecules, and this phenotypic modulation is a typical feature of DC maturation. Treatment with high molecular weight γ-PGA (2,000 kDa), but not low molecular weight γ-PGA (10 kDa) induced a significant upregulation of CD40, CD80, and CD86 expression in wild-type DCs. The stimulatory capacity of γ-PGA was not significantly affected by pretreatment with Polymyxin B (PmB). In contrast, DCs from TLR4-defective mice did not show an enhanced expression of maturation markers in response to the 2,000 kDa γ-PGA treatment. It is suggested that the γ-PGA-Phe nanoparticles also induce DC maturation in a TLR4-dependent manner using the same 2,000 kDa γ-PGA, because γ-PGA is located near the nanoparticle surface.

Tomayo et al. have also reported that poly(anhydride) nanoparticles act as agonists of various TLRs. The nanoparticles were useful as Th1 adjuvants in immunoprophylaxis and immunotherapy through TLR exploitation [120].

Similar results have been obtained with PLGA nano- and microparticles [121, 122], liposomes [107], cationic polystyrene microparticles [123], polystyrene nanoparticles [124], and acid-degradable cationic nanoparticles [125]. Elamanchili et al. examined DC maturation by PLGA nanoparticles. The results showed that after PLGA nanoparticle pulsing, DCs exhibited a modest increase in the expression of MHC class II and CD86 compared to untreated controls. In addition, DCs pulsed with PLGA nanoparticles containing an immunomodulator, monophosphoryl lipid A (MPLA), induced further DC maturation [106]. The PLGA-based nanoparticulate system offers the flexibility for incorporation of broad range of TLR ligands. Copland et al. investigated whether formulation of antigen in mannosylated liposomes enhanced uptake and DC maturation. Exposure to liposomes containing OVA resulted in enhanced expression of maturation markers when compared to exposure to antigen in solution. Expression was highest following exposure to mannosylated liposomes [107]. These particulate systems hold promise as a vaccine delivery system and immunostimulant. However, it has also been reported that PLGA particles failed to mature DCs in vitro [126, 127]. These differences may be attributed to particle size, particle concentration in DCs, presence or absence of antigen, and experimental conditions.

3.4 Gene Delivery by Polyion Complex Nanoparticles

Gene delivery has great potential for the treatment of many different diseases. The basic idea of gene therapy involves delivery of an exogenous gene into the cells to express the encoded protein, which may be insufficiently or aberrantly expressed naturally [128]. DNA delivery is, however, a difficult process and a suitable vector is required for efficient protection as well as release. Both viral and nonviral vectors have been used for gene delivery. Nonviral gene delivery relies on DNA condensation induced by cationic agents. Cationic polymers have been widely chosen to condense DNA through electrostatic interactions between negatively charged DNA and the positively charged cationic sites [129]. PIC nanoparticles composed of γ-PGA and chitosan (CT) have been used as a DNA delivery system. CT/DNA complex nanoparticles have been considered as a vector for gene delivery. Although advantageous for DNA packing and protection from enzymatic degradation, CT-based complexes may lead to difficulties in DNA release at the site of action. To improve the transfection efficiency of CT/DNA complexes, γ-PGA/CT/DNA conjugated nanoparticles were prepared by an ionic-gelation method for transdermal DNA delivery using a low-pressure gene gun [130]. pDNA was mixed with aqueous γ-PGA (20 kDa). Nanoparticles were obtained upon addition of the mixed solution to aqueous CT (80 kDa). The prepared γ-PGA/CT/DNA nanoparticles were pH-sensitive and had a more compact internal structure with a

greater density than the conventional CT/DNA. Analysis using small angle X-ray scattering (SAXS) indicated that incorporating γ-PGA caused the formation of compounded nanoparticles whose internal structure might facilitate the dissociation of CT and DNA. As compared with CT/DNA, γ-PGA/CT/DNA nanoparticles improved their penetration depth into mouse skin and enhanced gene expression. Moreover, in addition to improving the release of DNA intracellularly, the incorporation of γ-PGA in nanoparticles markedly increased their cellular internalization [131]. Taken together, the results show that γ-PGA significantly enhanced the transfection efficiency of this developed gene delivery system. The results indicated that γ-PGA played multiple important roles in enhancing the cellular uptake and transfection efficiency of γ-PGA/CT/DNA nanoparticles. This delivery system may be useful for DNA vaccine development.

Kurosaki et al. also developed a vector coated by γ-PGA for effective and safe gene delivery [132]. To develop a useful nonviral vector, PIC constructed with pDNA, PEI, and various polyanions, such as polyadenylic acid, polyinosinic–polycytidylic acid, α-polyaspartic acid, and γ-PGA were prepared. The pDNA/PEI complex had a strong cationic surface charge and showed extremely high transgene efficiency although it agglutinated with erythrocytes and had extremely high cytotoxicity. The γ-PGA could electrostatically coat the pDNA/PEI complex to form stable anionic particles. The coating of γ-PGA dramatically decreased the toxicities of pDNA/PEI complex. Moreover, the pDNA/PEI/γ-PGA complex was highly taken up by the cells via a γ-PGA-specific receptor-mediated pathway and showed extremely high transgene efficiencies. Further studies are necessary to examine the detailed uptake mechanism and clinical safety as gene delivery vector.

4 Control of Intracellular Distribution of Nanoparticles

4.1 pH-Responsive Nanoparticles

In general, particulate materials can be easily internalized into the cells via endocytosis, depending on their size, shape, and surface charge. However, the internalized materials are mostly trafficked from acidic endosomes to lysosomes, where degradation may occur. Thus, degraded exogenous antigens are presented by the MHC class II presentation pathway, and a part of the pathway involves antibody-mediated immune responses. In contrast, antigens within the cytosol are processed into proteasomes and presented by the MHC class I pathway, a pathway involved in the cytotoxic T-lymphocyte (CTL) response [99–101]. Therefore, the induction of antigen-specific cellular immunity by exogenous antigens is needed for the regulation of intracellular distribution of antigens. The escape of internalized

antigens from endosomes to the cytoplasm is an important subject relating to control of the antigen processing/presentation pathways.

The release of biomolecules from acidic endosomes requires a membrane-disruptive agent, which can release the internalized compounds into the cytoplasm. Approaches include the use of membrane-penetrating peptides, pathogen-derived pore-forming proteins, and "endosome escaping" polymers or lipids that disrupt the endosomal membrane in response to the pH reduction that occurs in these compartments. Thus, in recent years, there has been significant interest in developing pH-sensitive nanoparticles that can enhance the cytoplasmic delivery of various biomolecules [133–136]. Standley et al. reported an acid-degradable particle composed of acrylamide and acid-degradable crosslinker for protein-based vaccines. These particles released encapsulated protein in a pH-dependent manner. They were stable at the physiological pH of 7.4 but degraded quickly in the pH 5.0 environment of endosomes. The degradation of particles led to the endosome escape of encapsulated proteins. The colloid osmotic mechanism generates a quick degradation of the particles into many molecules, thus increasing the osmotic pressure within the endosomes, leading to a rapid influx of water across the membrane, resulting in its disruption. In fact, the MHC class I presentation levels achieved with these particles were vastly enhanced as a result of their ability to deliver more protein into the cytoplasm of APCs. In a mouse immunization study, these acid-sensitive particles could induce antigen-specific CTL responses and showed antitumor activity [137, 138]. Hu et al. also reported the endosome escape of pH-responsive core–shell nanoparticles. pH-sensitive poly (diethylamino ethyl methacrylate) (PDEAEMA)- core/poly(ethylene glycol) dimethacrylate (PAEMA)-shell nanoparticles were capable of efficient cytosolic delivery of membrane-impermeable molecules such as calcein and OVA to DCs. These particles effectively disrupted endosomes and delivered molecules to the cytosol of cells without cytotoxicity, and enhanced priming of $CD8^+$ T cells by DCs pulsed with OVA/PDEAEMA-core nanoparticles [139]. Polycations that absorb protons in response to the acidification of endosomes can disrupt these vesicles via the proton sponge effect. The proton sponge effect arises from a large number of weak conjugate bases with buffering capacities between 7.2 and 5.0, such as PEI, leading to proton absorption in acid organelles and an osmotic pressure buildup across the organelle membrane. This osmotic pressure causes swelling and/or rupture of the acidic endosomes and a release of the internalized molecules into the cytoplasm [140].

4.2 Amphiphilic Polymers for Cytosolic Delivery

Synthetic poly(alkylacrylic acid) [141, 142] and poly(alkylacrylic acid-*co*-alkyl acrylate) [143, 144] also have pH-dependent, membrane-disruptive properties.

These polymers contain a combination of carboxyl groups and hydrophobic alkyl groups, and are protonated at the endosomal pH range. Upon a decrease in pH, they increase their hydrophobicity, and penetrate into the endosomal membranes and disrupt them. The hydrophobicity of the polymers is important for disrupting lipid membranes. Foster et al. have applied these amphiphilic polymers to nanoparticle delivery systems [145]. pH-responsive nanoparticles (180 nm) incorporating OVA-conjugated poly(propylacrylic acid) (PPAA) (PPAA-OVA) were evaluated to test whether improved cytosolic delivery of a protein antigen could enhance $CD8^+$ CTL and prophylactic tumor vaccine responses. Nanoparticles containing PPAA-OVA were formed by ionic complexation of cationic poly(dimethylaminoethyl methacrylate) (PDMAEMA) with the anionic PPAA-OVA conjugate (PPAA-OVA/PDMAEMA). The PPAA-OVA/PDMAEMA nanoparticles were stably internalized and could access the MHC class I pathway in the cytosol by triggering endosome escape. In an EG.7-OVA mouse tumor protection model, PPAA-OVA/PDMAEMA-immunized mice delayed tumor growth for nearly 5 weeks, whereas control mice injected with PBS and free OVA developed tumors in less than 10 days. This response was attributed to the eightfold increase in production of OVA-specific $CD8^+$ T-lymphocytes and an 11-fold increase in production of anti-OVA IgG. However, these vinyl polymers are not biodegradable and, thus, their molecular weight presents a limitation for medical applications.

Recently, our group developed novel biodegradable nanoparticles composed of hydrophobically modified γ-PGA (γ-PGA-Phe). The nanoparticles showed a highly negative zeta potential (−25 mV) due to the ionization of the carboxyl groups of γ-PGA located near the surfaces. Protein-encapsulating γ-PGA-Phe nanoparticles efficiently delivered proteins from the endosomes to the cytoplasm in DCs [146]. To evaluate their potential applications as membrane disruptive nanoparticles, the nanoparticles were characterized with respect to their hemolytic activity against erythrocytes as a function of pH. The nanoparticles showed hemolytic activity with decreasing pH from 7 to 5.5, and were membrane-inactive at physiological pH. As the pH decreased, the hemolytic activity of the nanoparticles gradually increased, reaching a peak at pH 5.5. This activity was dependent on the hydrophobicity of γ-PGA. The mechanism responsible for the pH-dependent hemolysis by the nanoparticles involved a conformational change of γ-PGA-Phe and corresponding increase in the surface hydrophobicity. Increased polymer hydrophobicity resulted in increased membrane disruption. The γ-PGA-Phe has carboxyl side chain groups, so the p*K*a of the proton of the carboxyl groups is also a very important factor for the pH sensitivity of the γ-PGA-Phe [104].

It has also been reported that antigen delivery to DCs via PLGA particles increased the amount of protein that escaped from endosomes into the cytoplasm. How proteins or peptides encapsulated within PLGA particles become accessible to the cytoplasm is still not clear. It is suggested that the gradual acidification of endosomes leads to protonation of the PLGA polymer, resulting in enhanced hydrophobicity and attachment and rupture of the endosomal membrane [147].

5 Regulation of Immune Responses by Nanoparticle-Based Vaccines

5.1 Induction of Immune Responses Using Amphiphilic Poly(amino acid) Nanoparticles

Induction and regulation of an adaptive immune response by vaccination is possible for a broad range of infectious diseases or cancers. Vaccine delivery and adjuvant that can induce antigen-specific humoral and cellular immunity are useful for development of effective vaccine systems. Cellular immunity is required to remove intracellular pathogens, while humoral immunity plays a central role in neutralizing extracellular microorganisms. The efficacy of antigen-loaded γ-PGA-Phe nanoparticles on the induction of antigen-specific humoral and cellular immune responses was examined using OVA as a model antigen [102, 103, 148, 149]. The immune responses were investigated in mice after subcutaneous immunization with OVA-NPs. The OVA-specific CTL responses were not observed in the spleen cells obtained from the control (PBS) and OVA-alone-immunized mice. In contrast, the spleen cells obtained from the mice immunized with OVA-NPs showed a more potent CTL response than those obtained from mice immunized with OVA plus complete Freund's adjuvant (OVA + CFA) (Fig. 15). When anti-OVA antibody responses were examined and compared among the groups after immunization, both OVA-NP- and OVA + CFA-immunized mice showed significantly higher levels of OVA-specific total IgG, IgG1, and IgG2a antibodies than OVA-alone-immunized mice. These results indicate that the γ-PGA-Phe nanoparticles have the ability to prime cellular and humoral immunity by vaccination. It has been

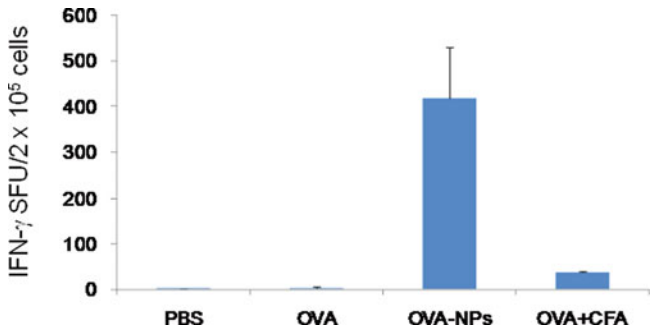

Fig. 15 Induction of cellular immunity by subcutaneous immunization with OVA-encapsulating γ-PGA-Phe nanoparticles. Mice were subcutaneously immunized one time with OVA alone (10 μg), 10 μg of OVA and 100 μg of NPs (*OVA-NPs*), 10 μg of OVA and 100 μL of complete Freund's adjuvant (*OVA + CFA*), or PBS (control). Splenocytes were obtained from the immunized mice on day 10 after the immunization and stimulated with the OVA peptide. The number of IFN-γ-producing cells was measured by enzyme-linked immunospot assay. *SFU* spot forming units

demonstrated that the γ-PGA-Phe nanoparticles are also effective for vaccines against human immunodeficiency virus (HIV) [150, 151], influenza virus [152, 153], Japanese encephalitis virus [154], human T-cell leukemia virus type-I (HTLV-I) [155], or cancers [146, 156, 157]. The antigen-loaded γ-PGA-Phe nanoparticles can provide a safe antigen delivery and adjuvant system for vaccination against viral infections or tumors because of their biocompatibility and biodegradability [158–161].

5.2 *Vaccination Using Antigen-Loaded PLGA Nanoparticles*

PLGA or PLA nano- and microparticles are suitable vehicles for the delivery of recombinant proteins, peptides, and pDNA to generate immune responses in vivo. Several studies have shown that PLGA nanoparticles can be used to modulate immune responses against encapsulated antigens due to their ability to efficiently target APCs and to facilitate appropriate processing and presenting of antigens to T cells [93, 162–170]. Gutierro et al. investigated the immune response to BSA-loaded PLGA nanoparticles after subcutaneous, oral, and intranasal administration to evaluate parameters that can affect the immune response [171]. These parameters include size, the internal structure of nanoparticles, surface hydrophobicity, zeta potential, and co-encapsulated surfactants, adjuvants or excipients during formulation, which are known to influence targeting strategies.

Many different vaccine antigens encapsulated into PLGA nanoparticles were shown to induce broad and potent immune responses. For example, hepatitis B therapeutic vaccines were designed and formulated by loading the hepatitis B core antigen (HBcAg) into PLGA nanoparticles (300 nm) with or without monophospholipid A (MPLA) adjuvant [172]. A single immunization with HBcAg-encapsulating PLGA nanoparticles containing MPLA induced a stronger cellular immune response than those induced by HBcAg alone or by HBcAg mixed with MPLA in a murine model. More importantly, the level of HBcAg-specific immune responses could be significantly increased further by a booster immunization with the PLGA nanoparticles. These results suggested that co-delivery of HBcAg and MPLA in PLGA nanoparticles promoted HBcAg-specific cellular immune responses. These findings suggest that appropriate design of the vaccine formulation and careful planning of the immunization schedule are important for the successful development of effective therapeutic vaccines for hepatitis B virus.

5.3 *Effect of Particle Size on Nanoparticle-Based Vaccines*

To design optimal drug carriers, polymeric nanoparticles have the advantage of being able to regulate their physicochemical properties, such as particle size, shape, surface charge, polymer composition, hydrophobicity, and biodegradability. In particular, a method for regulating the size of polymeric nanoparticles is essential for effective vaccine delivery, and to elicit a specific immune response.

Pluronic-stabilized polypropylene sulfide nanoparticles of 20, 45, and 100 nm diameter were prepared to compare the effective targeting of DCs in lymph nodes [173, 174]. Among the three different sizes of nanoparticles, 20 nm-sized nanoparticles were the most readily taken up into the lymphatics via interstitial flow, and activated lymph node-residing DCs more efficiently than the other sizes of nanoparticles. Different sized antigen-conjugated carboxylated polystyrene nanoparticles were also investigated for their size-dependent immunogenicity in vivo. The optimal size for an effective immune response was narrowly defined at 40–50 nm, in the viral range [175]. Furthermore, it has been reported that the size of antigen-loaded PLA particles modulated the immune response [25]. Immunization with PLA nanoparticles (200–600 nm) was associated with higher levels of IFN-γ production related to the T helper 1 (Th1)-type immune response. In contrast, immunization with PLA microparticles (2–8 μm) promoted IL-4 secretion related to the Th2-type immune response. Gutierro et al. also demonstrated that the vaccination of 1,000 nm-sized BSA-loaded PLGA particles generally elicited a higher serum IgG response than that obtained with the vaccination of 500 or 200 nm-sized particles, the immune response for 500 nm particles being similar than that obtained with 200 nm by the subcutaneous and the oral route, and higher by the intranasal route [171]. The vaccination of 1,000 nm particles generally elicited a higher serum IgG response than that obtained with the vaccination of 500 or 200 nm-sized particles, the immune response for 500 nm particles being similar than that obtained with 200 nm by the subcutaneous and the oral route, and higher by the intranasal route. These results suggest that the biodistribution of nano- and microparticles and the particle-related immune response can be regulated by controlling the size of the particles. Consequently, the size of the particulate delivery system is an important factor for modulating immune responses via differential interactions with APCs.

6 Concluding Remarks and Future Perspectives

Biodegradable nanoparticles with entrapped vaccine antigens, such as proteins, peptides and DNA, have recently been shown to possess significant potential as vaccine delivery systems. There are three primary mechanisms of adjuvant function: (1) stabilization of antigen, (2) delivery of antigen, and (3) activation of innate immunity. The duration of delivery is likely to affect immunity. Delivery of antigen is particularly important in cases where the vaccine is intended to act through DCs, as is the case for new vaccine applications requiring cell-mediated immunity. Nanoparticles are extremely flexible delivery systems capable of encapsulating a wide range of antigens. Improving delivery to DCs by nanoparticles will improve vaccine efficiency. Nanoparticle-based vaccine systems will also reduce the vaccine dosage frequency and will increase patient compliance. In the near future, these vaccine systems can be used for treating many infectious diseases or cancers.

γ-PGA is a very promising biodegradable polymer that is produced by various strains of *Bacillus*. Potential applications of γ-PGA as thickener, cytoprotectant, humectant, biological adhesive, fluoculant, or heavy metal absorbent, etc. have been reported. This review describes the preparation of polymeric nanoparticles composed of γ-PGA and their pharmaceutical and biomedical applications. The production of γ-PGA has already been established on the industrial scale because it can be produced easily and extracellularly in high yield by culture of bacteria in a fermenter. Moreover, various molecular weights of γ-PGA can be obtained commercially. γ-PGA by itself is shown to be weakly or non-immunogenic and safe. Amphiphilic γ-PGA nanoparticles have potential use as a new adjuvant instead of alum, and the nanoparticles are very suitable for use as vaccine delivery systems. These systems are expected to be introduced into clinical studies in the near future.

There is a growing interest in identifying the relationship between the size of nanoparticles and their adjuvant activities, but the results from recent studies remain controversial. Many investigators are in agreement that the size of the particles is crucial to their adjuvant activities. Some factors that may affect the conflicting findings include: (1) the polymeric materials used to form the nanoparticles, (2) the nature of the antigen used, (3) the methods of antigen conjugation, and (4) the immunization route. To clarify the influence of nanoparticles on adjuvant activity, there is a need to more comprehensively compare immune responses induced by precisely size-controlled nanoparticles prepared with the same materials and loaded with the same antigens by the proper method.

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