Development of Core-Corona Type Polymeric Nanoparticles as an Anti-HIV-1 Vaccine

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Abstract: We developed a novel technology for core-corona polymeric nanoparticles having hydrophilic polymer chains with functional groups. Polystyrene nanoparticles immobilized with the mannose-specific lectin concanavalin A could efficiently capture human immunodeficiency virus type 1 (HIV-1) particles and gp120 antigens on their surface. Since the antigen-capturing nanoparticles were capable of inducing humoral and cellular immune responses to HIV-1, they could be developed as a mucosal vaccine against HIV-1 infection.

Keywords: Polystyrene nanoparticles, concanavalin A, HIV-1, mucosal vaccine.

I. INTRODUCTION

Polymeric materials such as gels, films, beads, and particles are frequently used for the immobilization of biomolecules, such as antibodies and enzymes, to establish immuno assays [1, 2] and catalytic systems [3]. Among these, polystyrene beads and particles are quite useful because of their easy preparation. Since there is no functional group on their surfaces, the immobilization of biomolecules by means of covalent bonds is impossible. In addition, as the surfaces of the polystyrene beads and particles are highly hydrophobic, nonspecific adsorption of other biomolecules does not seem to be avoidable. To circumvent these problems, we recently developed technology for hydrophilic polymer chains with functional group-coated core-corona type polystyrene nanoparticles [4, 5], which was dispersed well in water and very useful for biomolecule immobilization. Using functional groups introduced on the coronas, proteins, peptides, saccharides, and metal nanoparticles could be easily conjugated on nanoparticle surfaces. Consequently, these core-corona nanoparticles have been utilized to various technological and biomedical applications, such as catalyst carriers [6], integrated materials [7], oral peptide drug carriers [8], diagnostic drugs, and virus captures.

In this review, we will summarize the preparation method of core-corona polymeric nanoparticles and their physicochemical properties. Furthermore, we will describe the application of lectin-immobilized polystyrene nanoparticles to capturing human immunodeficiency virus type 1 (HIV-1) particles and gp120 antigens and their potential for a prophylactic mucosal vaccine against HIV-1 infection.

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II. PREPARATION OF CORE-CORONA NANOPARTICLES

The preparation of nanoparticles for biomedical use can be roughly divided into two categories. One is based on physicochemical properties, such as phase separation and solvent evaporation [9]. The other is based on chemical reactions, such as polymerization and polycondensation. The first approach has been normally used for the preparation of nanoparticles made from hydrophobic or cross-linked water-insoluble hydrophilic polymers, such as polylactic acid and its derivatives, cellulose derivatives, and polyacrylate and polymethacrylate derivatives. In the other approach, emulsion polymerization has been extensively studied as a method for preparing nanoparticles with hydrophobic vinyl monomers [10].

Our group also developed a synthetic method for polymeric nanoparticles composed of novel graft copolymers having a hydrophobic backbone and hydrophilic branches [11]. A macromonomer method is frequently used to synthesize graft copolymers. The macromonomer is a chemical substance terminating in a highly-reactive group, such as vinyl group. Schulz and Milkovich reported that graft copolymers were obtained by copolymerizing macromonomers and other monomers [12]. When this method is used, it is easy to control configuration regularity, molecular weight, number, and site of graft chains. An example of synthesizing a hydrophilic macromonomer terminating in vinyl group is shown in Fig. (**1**). First, oligomers were prepared by the free radical copolymerization of hydrophilic monomers in the presence of a chain transfer agent, and the physical group, such as hydroxyl group, was added to the end of oligomers. Second, the physical group-terminated oligomers were reacted with highly polar vinyl monomers, such as *p*-chloromethylstyrene, to introduce a vinylbenzyl group into the oligomers; thereby the vinylbenzyl group-terminated macromonomers were synthesized.

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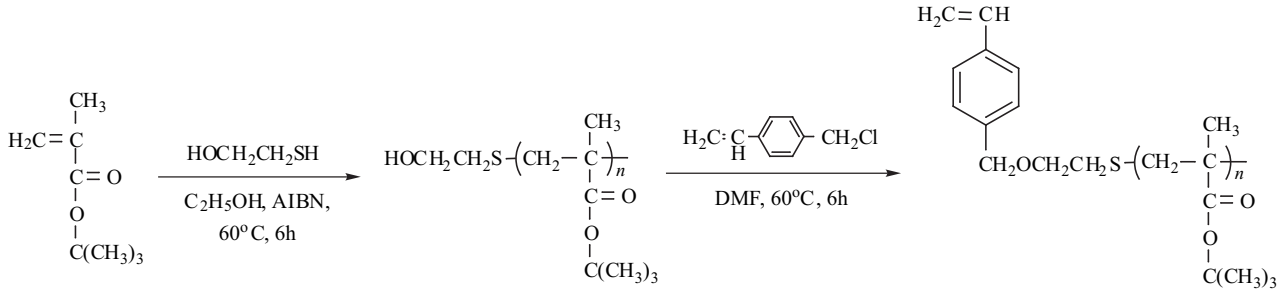

Fig. (1). Synthesis of a hydrophilic macromonomer terminating in vinyl group.

Monodisperse polymeric nanoparticles, which consist of hydrophobic core and hydrophilic corona on their surface, were prepared by the free radical dispersion copolymerization of hydrophobic monomers, such as styrene, and hydrophilic macromonomers in a polar solvent [13]. Instead of styrene, methyl methacrylate can also be used as a hydrophobic monomer. The mechanism of nanoparticle formation is shown in Fig. (**2**). Ethanol was added to dissolve the hydrophobic monomers, and dispersion polymerization processed in ethanol-water solution. To minimize an interfacial free energy, amphiphilic graft polymers with different surface free energies self-assembled in solution to form core-corona nanoparticles, which had hydrophobic cores and hydrophilic corona layers on their surfaces, resulting in excellent aqueous phase dispersion.

By designing and synthesizing different functional macromonomers, a variety of water-dispersible nanoparticles consisting of polystyrene cores and different functional polymeric branches on their surfaces were prepared by the macromonomer method. Surface charge of nanoparticles could be controlled by using cationic poly(vinylamine) and anionic poly(methacrylic acid) having a polymerizable end group as macromonomers [7, 14]. To prepare the ionic nanoparticles, macromonomer chains on the nanoparticle surface were hydrolyzed after dispersion copolymerization. However, it was confirmed that similar nanoparticles were obtained by dispersion copolymerization between hydrophobic monomers and ionic macromonomers. Thermosensitive nanoparticles could also be prepared by using the well-known thermosensitive polymer poly(*N*-isopropylacrylamide) as macromonomers [5, 15]. Furthermore, nanoparticles prepared by using poly(ethylene glycol) (PEG) macromonomers had potential to contact with biological systems including proteins and cells [16]. With respect to the synthesis of new functional particles, an appropriate choice of macromonomers may be able to provide the formation of particles with desired surface groups.

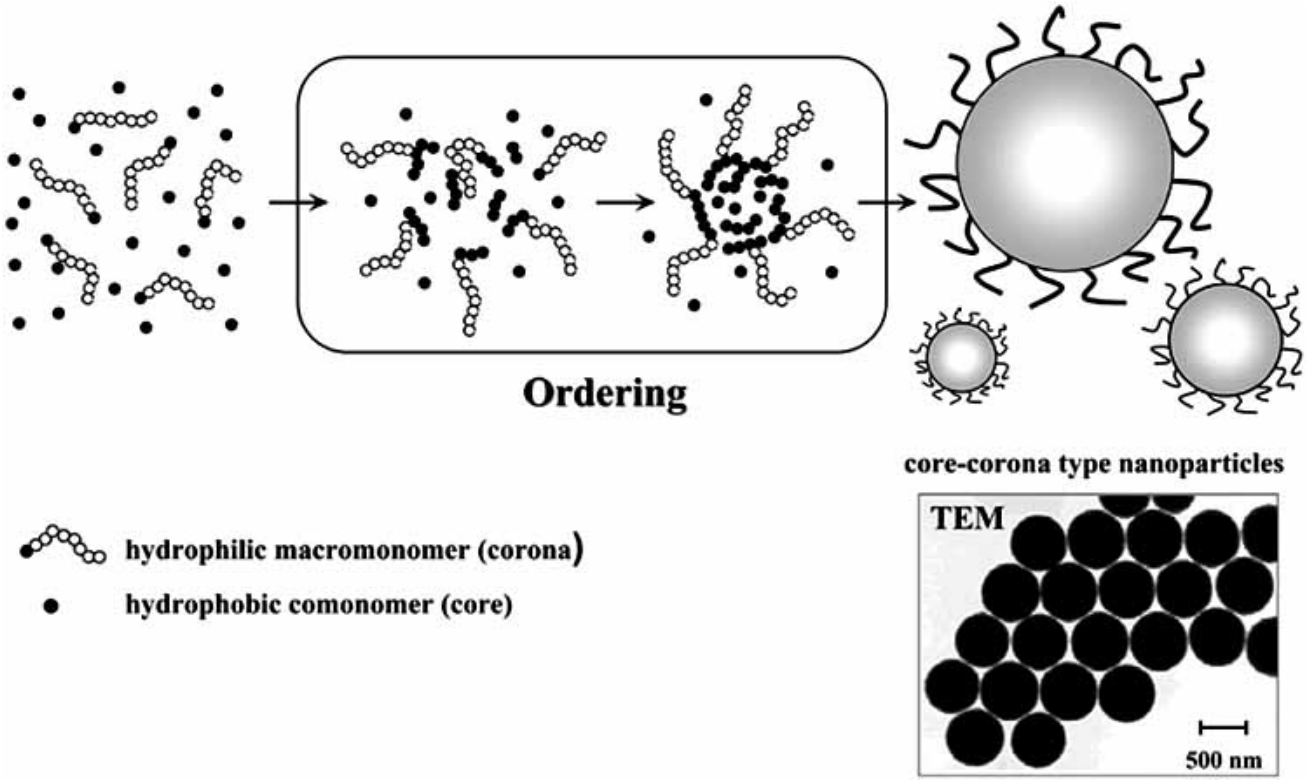

Fig. (2). Mechanism of nanoparticle formation.

Virus-like nanoparticles with many projections (Fig. (**3**)) were recently prepared by one-step polymerizations of styrene, acrylonitrile, and PEG macromonomers [17]. Although the mechanism of virus-like nanoparticle formation is still unknown, the nanoparticles with projections had a larger surface area than ordinary nanoparticles having a similar diameter and were expected to have excellent adsorption capacity similar to that of virus particles.

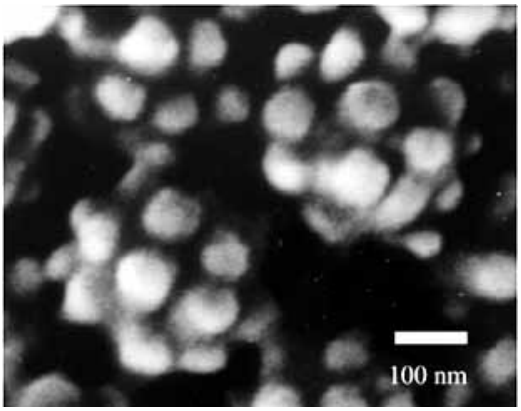

Fig. (3). SEM image of poly(styrene-co-acrylonitrile-co-PEG macromonomer) nanoparticles. Data from Ref. [17b].

III. PHYSICOCHEMICAL PROPERTIES OF CORE-CORONA TYPE NANOPARTICLES

The preparation of polymeric particles using macromonomers as a steric stabilizer was reported by other researchers [18]. Kobayashi *et al.* reported the synthesis of polystyrene particles by dispersion copolymerization of poly(2-oxazoline) macromonomers with styrene [18b]. Winnik *et al.* studied the dispersion copolymerization of styrene or *n*-butyl methacrylate with *p*-alkylstyrene terminated PEG macromonomers in methol/water mixed solvents [18c, d]. Distinct from the above methods, the macromonomer method developed by us has a unique characteristic in particle formation. The size of nanoparticles, the molecule weight of graft polymers, and the increased conversion of polymerization with increasing reaction time indicate the *in situ* self-assembling process for the nanoparticle formation (Fig. (**4**)) [13d].

Using various polymerization parameters, such as the concentration of macromonomers, initiators, initial styrene, solvent composition, and polymerization temperature, the size of core-corona nanoparticles can be readily changed ranging from 50 nm to 3000 nm with narrow size distribution [16]. Fig. (**5**) shows the range of nanoparticle size having PEG chains on the surface. The size could be controlled by varying each reaction factor. For instance, when high-molecular weight macromonomers were used, smaller particles were obtained. Furthermore, particle size can be controlled more extensively, when combined two or more reaction factors.

In terms of core-corona nanoparticle applications, the surface layer structures that correspond to corona have key roles in the functions of nanoparticles. The characteristics of nanoparticle surface are determined by the chemical structure of macromonomers located on the surface. An electron spectroscopy for chemical analysis (ESCA) and a dynamic light scattering (DLS) supported the accumulation of a hydrophilic macromonomer component on the surface of nanoparticles [5, 11, 19]. Recently, cross-sectional structures of nanoparticles with polystyrene cores and various hydrophilic macromonomer coronas were analyzed by transmission electron microscopy (TEM) [20]. The core and corona of nanoparticles were successfully visualized by exposure of their cross-sections in an osmium oxide atmosphere, resulting in TEM images with clear black and white contrasts for respective corona (increasing staining) and core (Fig. (**6a**)). Nanoparticles displayed a core-corona

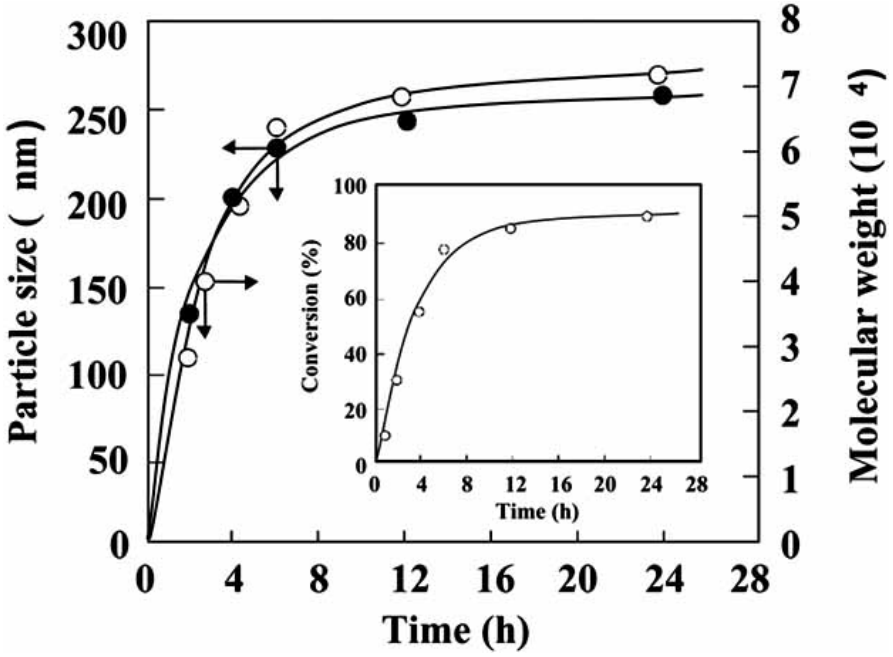

Fig. (4). Copolymerization of PEG macromonomer with styrene in ethanol/water. Data from Ref. [13d].

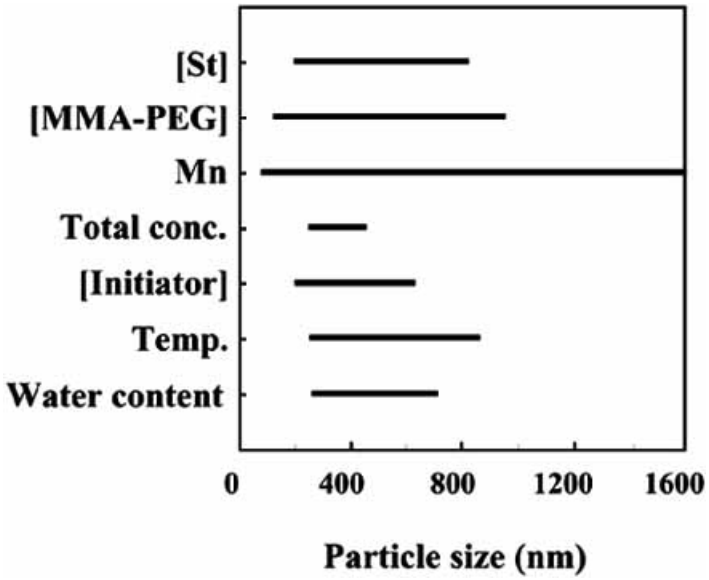

Fig. (5). The effect of reaction factors on the particle size. Data from Ref. [16a].

structure in all cases, indicating that the macromonomer method can generally generate nanoparticles with unique structure similar to the model deprived from DLS measurement (Fig. (**6b**)). Corona layers were basically comprised of polymer monolayers derived from macromonomers, although their structures seemed to be affected by macromonomer chemical species. In addition, hydrophilic branches of graft copolymers were partially incorporated in the cores during copolymerization. Interestingly, the corona layer thickness was always constant irrespective of nanoparticle sizes, when a macromonomer with the same molecular weight was used. The thickness increased with increasing the molecular weight of a macromonomer, although the particle size decreased (Fig. (**7**)). These findings appear to be very important for further utilization of core-corona nanoparticles and understanding their biomedical functions.

Taken together, our self-assembling core-corona polymeric nanoparticles have the following properties. (1) The diameter is monodispersed. (2) Particle diameters are controllable from nm to μm. (3) Long-term preservation is possible in a fine powder state obtained by freeze and dry. (4) Polymer chains (corona) densely accumulate on nanoparticles surfaces. (5) Various functions can be introduced easily by changing the hydrophilic macromonomer. Therefore, nanoparticles prepared by the macromonomer method are expected to have a lot of applications in various fields.

IV. LECTIN-IMMOBILIZED NANOPARTICLES AND CAPTURE OF HIV-1

HIV-1 is a spherical RNA virus with a diameter of 100 nm and possesses an envelope with glycoprotein gp120 and gp41 [21]. The gp120 is a positively charged mannose-rich glycoprotein, which strongly interacts with some high-mannose binding lectins, such as concanavalin A (Con A) [22]. It was reported that the HIV-1 envelope glycoprotein could be purified by Con A-agarose affinity chromatography [23]. Thus, it is possible that gp120 and HIV-1 virions are effectively captured by such lectins, if they are ideally immobilized to certain materials such as polystyrene

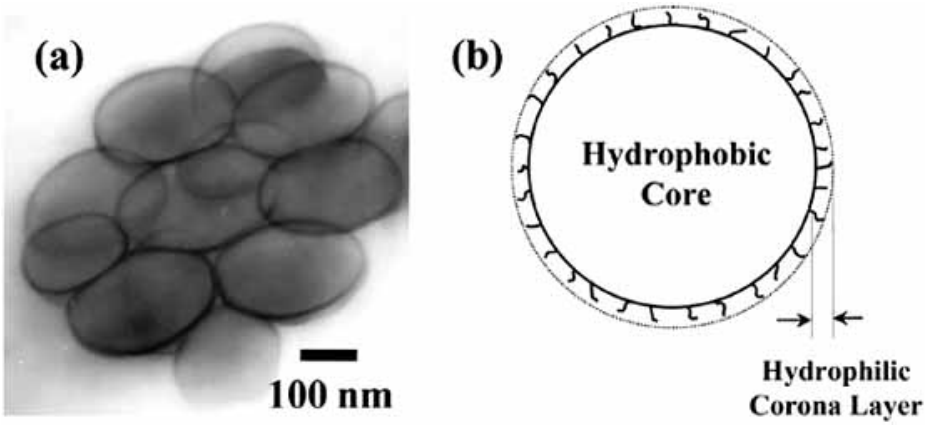

Fig. (6). (a) TEM image of ultrathin cross sections of core-corona nanoparticles stained with osmium tetraoxide. (b) Schematic presentation of a core-corona polymeric nanoparticle. Data from Ref. [20a].

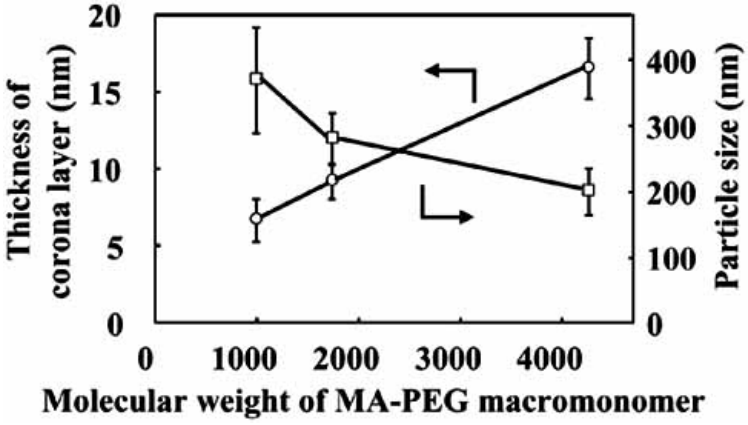

Fig. (7). Dependence of corona layer thickness and nanoparticle size on macromonomer molecular weight. Data from Ref. [20a].

nanoparticles. Actually, when lactose was conjugated to the surface of poly(vinylamine)-coated polystyrene nanoparticles by an amide linkage, the binding ability of the galactose-specific lectin RCA120 to the nanoparticle-associated lactose was at least 100 times higher than that to a monomeric lactose [24].

Poly(methacrylic acid)-covered polystyrene nanoparticles (approximately 400nm in diameter) were prepared by the copolymerization of styrene with the poly(*tert*-butyl methacrylate) macromonomer and the following acid hydrolysis according to the method described previously [4]. Using water-soluble carbodiimide, the condensation between the carboxyl group of poly(methacrylic acid) on the polystyrene surface and amino group of Con A gave Con A-immobilized polystyrene nanoparticles (Con A-NPs) (Fig. (**8**)) [25, 26]. Con A-NPs were mixed with HIV-1 suspension in medium. The amount of captured HIV-1 was determined by the residual gp120 antigen level and the viral infectivity in the supernatants after centrifugation of the mixed suspension (Fig. (**9**)). Con A-immobilized nanoparticles achieved a >3.3 log and 2.2 log reduction of viral infectivity at 2 and 0.5 mg/ml, respectively (Table **1**). In contrast, only a slight reduction of the viral infectivity was identified for the Con A-free nanoparticles, which was contributed by the static interaction between negatively charged nanoparticles and positively charged gp120. In addition to infectious HIV-1 particles, the virion-free gp120 antigen could also be captured by Con A-NPs [26]. The results of electron microscopy with immunostaining and microporous membrane filter experiments also demonstrated that virion-free gp120 could be captured by Con A-NPs (data not shown). Among the several mannose-specific lectins examined, Con A showed the highest capture activity. Furthermore, the capture activity of Con A-NPs was weakened by addition of excessive amounts of mannose but not galactose [25]. These results indicate that Con A-NPs primarily recognize the mannose molecules in HIV-1 gp120 and efficiently capture HIV-1 virions and virion-free gp120 antigens. Although heat inactivation (60°C, 30min) completely annihilated the infectivity of HIV-1 suspension, it did not affect the affinity of gp120 to Con A-NPs [27].

V. POTENTIAL OF INACTIVATED HIV-1-CAPTURING NANOPARTICLES AS MUCOSAL VACCINES

The development of highly active antiretroviral therapy (HAART) has achieved a reduced death rate of HIV-1

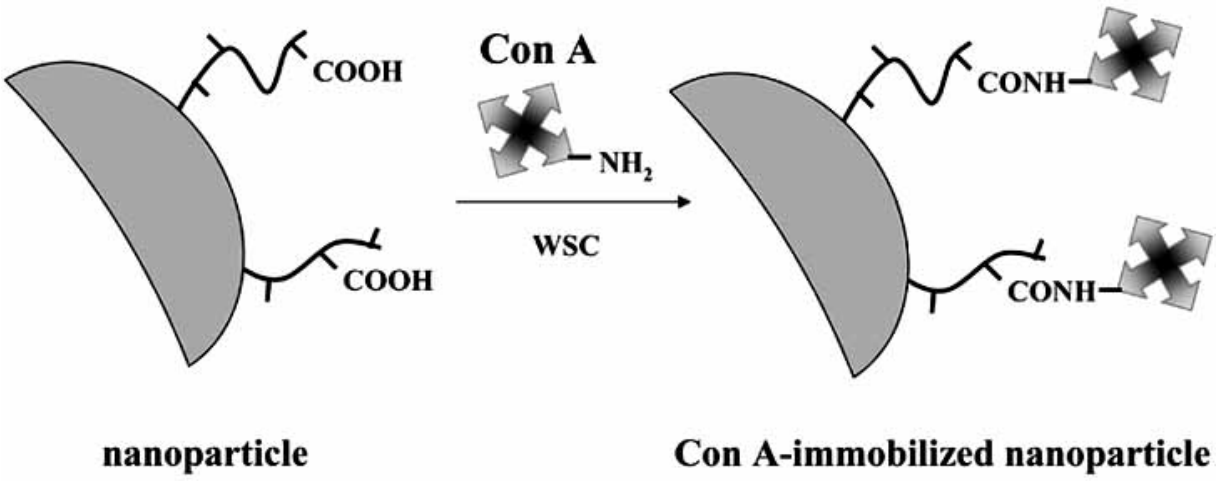

Fig. (8). Immobilization of lectin (Con A) onto the surface of nanoparticles.

Table 1. Effect of Con A-NP Treatment on HIV-1 Infectivity*

Nanoparticle	Concentration (mg/ml)	Infectivity[a] (x 10^4 $CCID_{50}$/ml)	%
Con A-immobilized	2	< 0.02	< 0.05
	0.5	0.22 ± 0.09	0.59
	0.13	5.6 ± 1.4	15
Con A-free[b]	0.5	19 ± 7	51
None	0	37 ± 14	100

*Data represent mean values ± SD for triplicate samples.

[a]The infectivity of HIV-1 (III_B strain) was determined by microscopic observation for virus-induced cytopathicity in MT-4 cells.

[b]Nanoparticles not immobilized with Con A.

Data from Ref. [26].

infection in developed countries. However, considering the high cost and low compliance of long-term HAART, it is obvious that the development of vaccines against HIV-1 is the most desirable for the prevention of viral transmission and disease progression [28]. The majority of prophylactic vaccine candidates consist of envelop proteins, peptides, and DNA, while researches for an inactivated HIV-1 vaccine have been minimal. Inactivated viruses seem to be advantageous over other component vaccines, because they contain a full set of viral proteins. Since the inactivated HIV-1-captured Con A-NPs (HIV-NPs) were covered with a number of HIV-1 particles and gp120 antigens on their surface, it was assumed to be a good immunogen, when administered *in vivo*. Furthermore, Con A-NPs may capture different HIV-1 subtypes and cellular tropism (R5 and X4), because the capture of HIV-1 particles fully depends on the interactions between the mannose of gp120 and the immobilized lectins.

Since a major route of HIV-1 infection is sexual intercourse, mucosal immune response must have critical effects on the establishment of infection. Some studies on

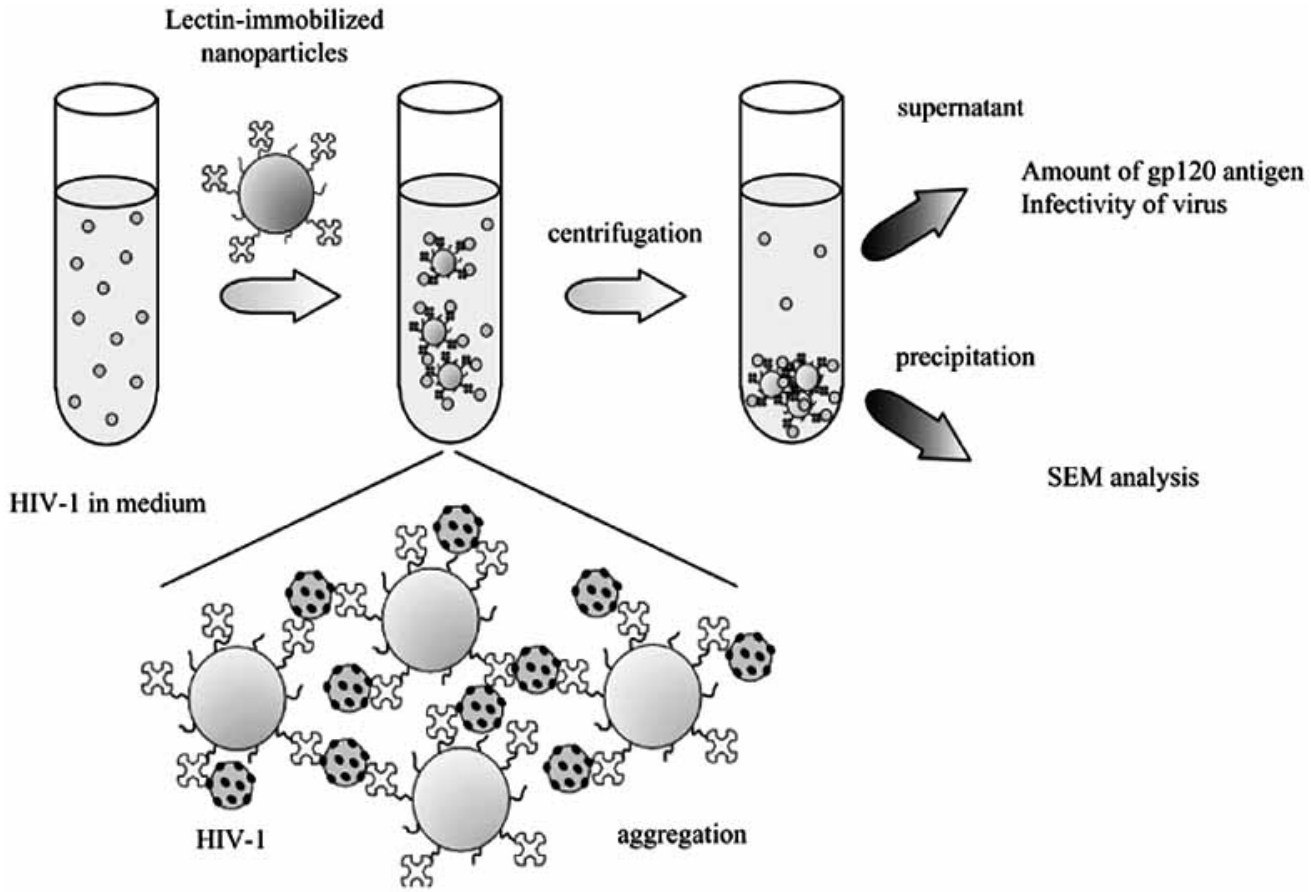

Fig. (9). Experimental methods to determine the capture of HIV-1 virions and gp120 antigen by Con A-NPs.

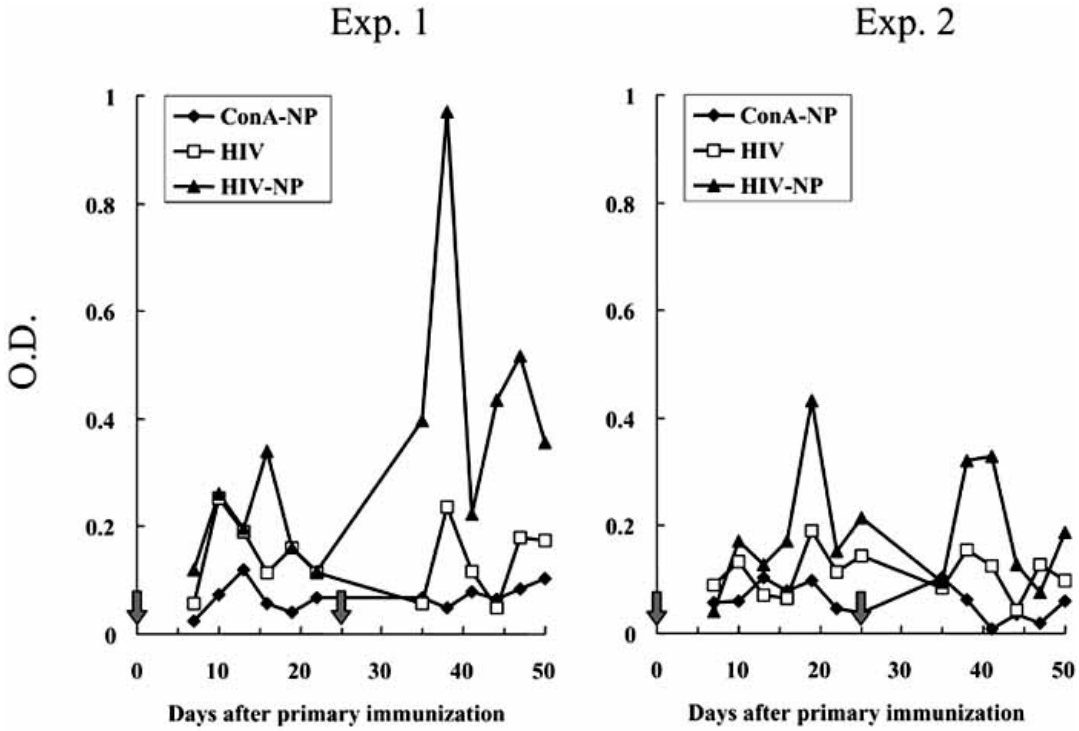

Fig. (10). Anti-gp120 IgA antibody levels in vaginal washes after intravaginal immunization (↓). Data from Ref. [27].

HIV-1 seronegative heterosexual partners of seropositive individuals and on uninfected commercial sex workers frequently exposed to HIV-1 suggest that virus-specific IgA in the genital tract may play an important role in protecting females against HIV-1 infection [29]. To determine whether HIV-NPs induce virus-specific IgA in the genital tract, HIV-NPs were administered intravaginally to female mice. After immunization, increased anti-gp120 IgA titers were identified in the vaginal fluids of the mice immunized with HIV-NPs, whereas such an IgA response was hardly detected in the vaginal fluids of immunized mice with HIV-1 alone or Con A-NPs alone (Fig. (**10**)) [27]. Furthermore, the vaginal fluids obtained from HIV-NP-administered mice could neutralize HIV-1 infectivity.

Nanoparticles possess considerable potential as a stable antigen carrier and an effective adjuvant through enhancing antigen stability and controlling antigen release. In fact, the immunohistochemical study revealed that the conjugation of inactivated HIV-1 with Con A-NPs could prevent the degradation of viral antigens and keep them in the pocket of vaginal lumen for a considerable period of time (data not shown). The colocalization of HIV-1 antigen and Con A-NP in the vaginal lumen also suggested that the interaction between HIV-1 gp120 with Con A had high affinity and that HIV-1 could not be easily exchanged by some natural ligands *in vivo* [27]. This may be a reason why Con A-NPs enhanced the immunogenicity of inactivated HIV-1 after intravaginal immunization. Interestingly, intranasal immunization with HIV-NPs could induce much higher IgA antibody titers in the genital tract than intravaginal or intraperitoneal immunizations [30]. Similar results were obtained for the IgG antibody titers in the genital tract. Furthermore, the vaginal fluids from intranasally immunized mice also displayed neutralizing activity against HIV-1 [30]. It has to be mentioned that only 10-30 ng of the antigen was sufficient for the induction of specific antibody response by intranasal immunization with HIV-NPs. However, more than 10 μg of the antigen was required to obtain a similar antibody response, when immunized HIV-1 alone. In addition, HIV-1-specific cytotoxicity T lymphocytes were detected in mice immunized with HIV-NPs [31].

The first immunological event after administration of antigens is their uptake by antigen-presenting cells, such as dendritic cells (DCs), monocytes/macrophages, and B cells [32]. Among these, DCs are the most potent professional antigen-presenting cells. The ability of DCs to prime naïve T cells with antigens and the presence in various peripheral tissues suggest their central role in mediating immune response to cancers and infectious diseases. Therefore, it is hypothesized that the interaction between DCs and Con A-NPs accounts for the enhancement of the immunogenicity of HIV-1 antigens captured on Con A-NPs. Indeed, Con A-NPs were taken up by pulmonary DCs after intranasal administration [31]. Furthermore, it is noteworthy that, as compared to gp120 alone, the amount of gp120 antigen taken up by murine DCs increased remarkably (approximately 100-fold), when it was captured onto the surface of Con A-NPs (Fig. **11a**). It was also demonstrated that the efficient uptake of Con A-NP-associated antigens by DCs was not due to the immobilized Con A but nanoparticles themselves, since blocking of the mannose-binding sites of Con A did not affect the uptake of Con A-NPs [31]. The efficient uptake and intracellular localization of nanoparticles in DCs were observed by confocal laser scanning microscopy with fluorescent nanoparticles (Fig. (**11b**)) [33]. To further characterize the effect of nanoparticles

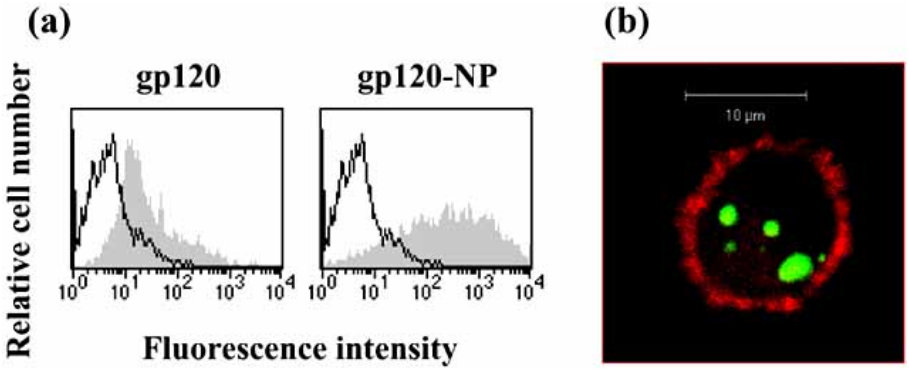

Fig. (11). (a) Effect of Con A-NPs on gp120 antigen uptake by DCs. Murine DCs were incubated with fluorescence-labeled gp120 or gp120-NPs (both 1 μg/ml for gp120) for 1 h at 37°C. After washing, the cells were examined for their fluorescence (hached histograms for gp120 and gp120-NPs, line histograms for unpulsed DCs). (b) Intracellular localization of nanoparticles (green) in DCs (red). Murine DCs were pulsed with fluorescence-labeled nanoparticles (green) for 1 h at 37°C. After washing, the cells were stained with a fluorescence-conjugated anti-mouse CD11c monoclonal antibody (red) and observed with confocal fluorescence microscopy. Data from Ref. [31 and 33].

on DCs, global transcripttional analysis using high-density DNA microarrays was performed. Nanoparticles upregulated the expression of several genes important for phagocytosis and immune responses in DCs [34]. The combination of antigens and nanoparticles specifically induced transcription of the genes related to protein transport, cell signaling, and immune response [34]. Those results indicated that the core-corona nanoparticles are excellent carriers to deliver viral antigens to DCs and strongly induce humoral and cellular immune responses after intranasal immunization.

Based on various advantages described above, intranasal administration of HIV-NPs may be predicted to be protective against HIV-1 infection in humans. To investigate this hypothesis, a macaque system infected with SHIV, a chimera virus of HIV-1 and simian immunodeficiency virus (SIV), was used for further experiments. Macaques were intranasally immunized with Con A-NPs alone or inactivated SHIV-capturing nanoparticles (SHIV-NPs) [35]. As expected, virus-specific IgA and IgG antibody responses were detected in SHIV-NP-immunized macaques but not in other control groups. Although the immunization with SHIV-NPs could not protect a macaque against intravaginal viral challenge, continuous suppression of plasma viral RNA was observed in this macaque but not ConA-NP-immunized and unimmunized macaques after intravenous challenge of SHIV (Fig. (**12**)).

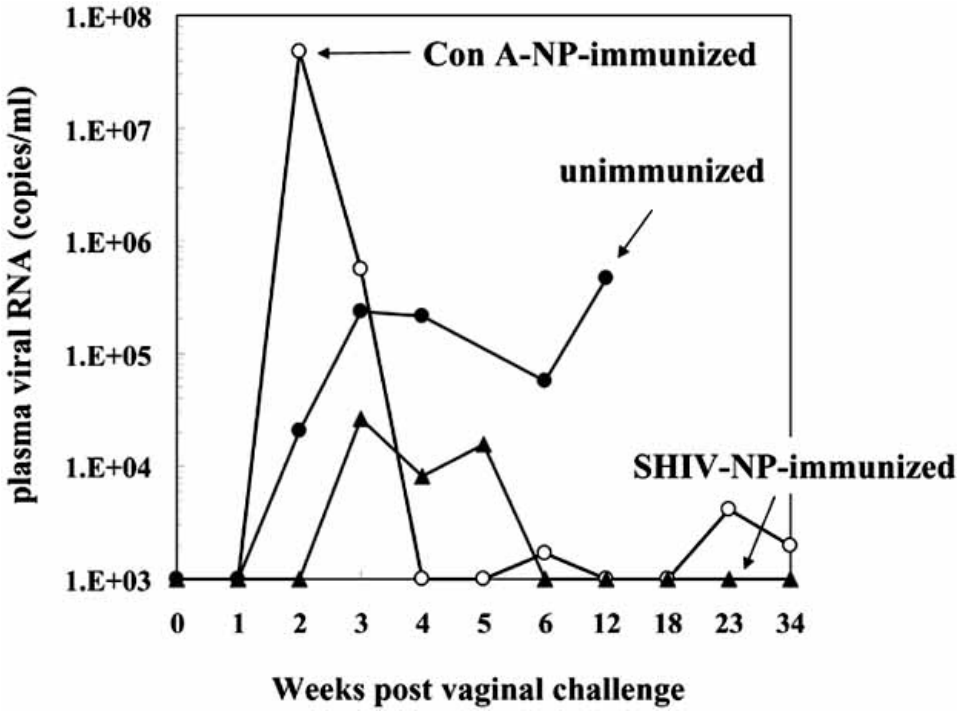

Fig. (12). Viral RNA loads in plasma of SHIV-infected macaques after intravaginal challenge. Macaques unimmunized and immunized with SHIV-NPs or Con A-NP were intravaginally inoculated with SHIV. Plasma viral RNA loads were measured by real-time polymerase chain reaction. Data from Ref. [35].

In conclusion, our core-corona type nanoparticles are an excellent antigen carrier and a vaccine candidate against HIV-1 infection. By introducing various functional groups onto the surface of nanoparticles, they may also be applicable to vaccine carriers against other infectious diseases. Further studies are ongoing to develop the second generation of core-corona nanoparticles, especially biodegradable nanoparticles.

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