Gold Nanoparticles Used as a Carrier Enhance Production of Anti-Hapten IgG in Rabbit: A Study with Azobenzene-Dye as a Hapten Presented on the Entire Surface of Gold Nanoparticles

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The azobenzene moiety, well-known not only for its reversible cis-to-trans photoisomerization but also as a hapten, does not induce antibodies on its own, but it reacts with antibodies raised against conjugates with protein carriers. Hence we selected azobenzene dye as an indicator to assess the possibility of having gold nanoparticles act as an immunological carrier instead of protein carriers. In rabbits, we confirmed an in vivo response against azobenzene dye presented on the entire surface of gold nanoparticles (azo-nanoparticles), where the gold nanoparticles appeared to play a role as a carrier for the hapten. A high yield of immunoglobulin G (IgG) against the azobenzene derivative took place in rabbits injected with azo-nanoparticles, whereas no increase in IgG was recognized in other rabbits treated solely with chemically equivalent azobenzene dye instead of azo-nanoparticles. Electron microscopy and surface plasmon resonance spectroscopy indicated that the IgG obtained specifically recognized the difference between the isomer conformations of the azobenzene moiety.

Key words: hapten; carrier; nanoparticles; electron microscopy; surface plasmon resonance

Size-quantized noble metal and semiconductor nanoparticles have attracted keen interest in various fields such as biotechnology, cell biochemistry, electronics, energy, and industrial products, where scientists and engineers can exploit the tools of nanotechnology to design materials at the molecular level.1,2) Their unique physical and chemical properties offer nanoscaled material engineering considerable potential applications in optical, electronic, and magnetic devices, in catalysis, as lubricants, and for many other uses.3–7) In the immunological field, reports are accumulating on development and applications using polymer-based nanoparticles and nanoparticles mainly consisting of biodegradable materials such as gelatin and chitosan.8–10) Indeed, many available vaccines, including protein antigens and DNA vaccines, are very unstable and need to be protected from degradation in the biological environment. In addition, their efficacy is limited by

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Abbreviations: azo-nanoparticle, a gold nanoparticle capped by unsymmetrical azobenzene disulfides; IgG, immunoglobulin G; BSA, bovine serum albumin; C6AzSSC12, unsymmetrical azobenzene disulfide [4-hexyl-4-(12-(dodecyldithio)dodecyl)oxy]azobenzene]; PBS, phosphate buffered saline; ELISA, enzyme-linked immunoorbent assay; SPR, surface plasmon resonance; SAM, self-assembled monolayer; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate; TEM, transmission electron microscopy
their poor capacity to overcome biological barriers and reach the target site. Therefore the design of appropriate antigen carriers that would help to overcome these problems has become a significance challenge. Metal-based nanoparticles are also being developed in clinical application for targeting and destruction of abnormal cells such as breast cancer cells. In this study, we confirmed for the first time that gold nanoparticles function as an immunological carrier in anti-hapten antibody production. They appear to assist in the display of small well-defined chemicals to immune system of mammals in vivo. In our study, there are two main distinct differences: a hapten, azobenzene dye, which alone cannot be an antigen, was used to conjugate with a carrier (gold nanoparticles) in order to allow us to assess the quality, especially the antigenic specification, of the resulting IgG, and gold nanoparticles were used as a carrier because gold is chemically stable, biologically undegradable, and known not to do harm to living bodies. Gold nanoparticles under our treatment might stay long in the body even if some are sequestrated or secreted. The size of the nanoparticles used was in the range commonly used in capture experiments using macrophages such as dendritic cells. Once the coating is removed from the gold nanoparticles in such immunologic cells, the cells should continue to present small chemicals (haptens) to the immune system. In order to examine the possibility of having gold nanoparticles act as immunological carriers in the production of anti-hapten antibodies as an alternative to the common protein carriers, such as hemocyanin and bovine serum albumin (BSA), we chose an azobenzene derivative as a model hapten, because the azobenzene moiety is well-known for its reversible geometric isomers’ (cis- and trans-form) sensitivity to light. This allows for rotation or inversion around the double bond, called reversible cis-to-trans photoisomerization. This feature of the moiety can assist in assessing the quality of the IgG yielded by stimulation.

Materials and Methods

Preparation of gold nanoparticles capped by unsymmetrical azobenzene disulfides. The unsymmetrical azobenzene disulfide, 4-hexyl-4’(12-(dodecylthio)decyloxy)azobenzene (C6AzSSC12), was synthesized according to a method reported previously. The unsymmetrical azobenzene disulfide-passivated gold nanoparticles were prepared in a two-phase water/tetraoctylammonium bromide/toluene system, as reported previously.

Time course monitoring in vivo and preparation of anti-azobenzene-dye IgG. Japan white rabbits of the female sex weighting 2–3 kg were used throughout the study. An amount of 0.1 mg of azobenzene disulfide, C6AzSSC12, which was stoichiometrically equivalent to the amount of C6AzSSC12 that resided on the entire surface of 1 mg of azo-nanoparticles, was dissolved in 1 ml of phosphate buffered saline (PBS) alone, and was mixed at a 1:1 ratio with a solution of 50% Freund’s complete adjuvant until the mixture was in a colloidal state, then injected intradermally into three rabbits as a control experiment. On the other hand, 1 mg of gold nanoparticles capped by C6AzSSC12, that is, azo-nanoparticles, was injected into another group of three rabbits after being dissolved in PBS, and was then mixed at a 1:1 ratio with a solution of 50% Freund’s complete adjuvant. When the mixture achieved a colloidal state, intravenous booster injection into each group of rabbits of the stoichiometrically same amount as for the hapten molecule was repeated several times with a one-week interval. The injection was performed under light conditions. The azobenzene moiety in the trans-form should be predominantly presented, and hence the antibody, which is trans-form specific should arise. Antisera were drawn once a week from each group of rabbits, and the stimulated anti-azobenzene-dye IgG was examined by the commercially recommended enzyme-linked immunosorbent assay (ELISA) method using antisera diluted 10 times. Finally, antisera containing the IgG of interest were collected and the IgG was purified by affinity chromatography with recombinant protein A (HiTrap rProtein A FF column, GE Healthcare UK, Buckinghamshire, UK). Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulfate (SDS–PAGE) was performed with PhastGel of 4–15% gradient by the PhastSystem (GE Healthcare UK) The committees for life-scientific research at NIAS and AIST approved the experiments on live rabbits, and all the experiments were performed in accordance with the relevant guidelines and regulations.

Observation by electron microscopy of the complex formed between anti-azobenzene-dye IgG and gold nanoparticles capped by unsymmetrical azobenzene disulfides. The antigenic specificity of the purified anti-azobenzene-dye IgG was observed morphologically by electron microscopy. Aliquots of the suspension containing gold nanoparticles capped by azobenzene disulfides were applied onto electron microscopic specimen grids covered with a thin carbon support film. The excess solution was adsorbed with a piece of torn filter paper. The other grids without negative staining, once the distribution manner of the azo-nanoparticles in the electron microscope was examined and the solvent was completely be evacuated using a vacuum environment, were taken out, then reacted with anti-azobenzene disulfide IgG. A droplet of solution containing IgG (10 μg/ml) was applied to the specimen grid where azo-nanoparticles covered with trans-formed azomencene dye resided and was incubated for 3 h at room temperature for immuno-reaction. After rinsing 3 times with the same buffer without IgG followed by removal of the excess solution with filter paper, the grid was immediately negatively stained with 1% uranyl acetate for 30 s
to make the condition identical between the experiments using IgG, then examined under an electron microscope (Tecnai F20, FEI Company, Eindhoven, Netherlands) operated at 120 kV. In order to change the conformation of the azobenzene moiety from the trans-form to the cis-form on the surface of the gold nanoparticles for the experiment to examine whether the resulting IgG would show antigenic specificity on the chirality of the azobenzene moiety, irradiation with UV (λ = 365 nm) for 2 h at room temperature was performed, and then a droplet of solution containing IgG was applied to the specimen grid and incubated for 3 h in the dark at room temperature for immuno-reaction. Images were recorded on computer media using a Digital Micrograph (Gatan, Pleasanton, CA) making use of a slow scan CCD camera (Gatan Retractable Multiscan Camera, Gatan) under a low electron dose condition at a magnification of 50,000x. Optical density profiles along the sections of interest in each EM image were analyzed using the Digital Micrograph. The density score was normalized so that completely black corresponded to a value of zero, and completely white to a value of 250.

Observation of interaction between anti-azobenzene-dye IgG and the azobenzene-dye SAM formed on a flat gold surface by SPR spectroscopy. In surface plasmon resonance (SPR) spectroscopy measurement, an appolarized He-Ne laser beam (λ = 632.8 nm) was used as the light source. It was mechanically chopped in conjunction with a lock in the amplifier before entry into the prism. The intensity of the beam reflected at the gold surface was determined with a photodiode detector, and was recorded against time at a fixed angle of incidence for kinetic-scan measurement. Prior to the all SPR experiments, the liquid cell with the bare gold substrate was filled with absolute hexane for more than 30 min. Self-assembling monolayer (SAM) formation was initiated by injection of the azobenzene disulfide solution into the cell, and the adsorption process was monitored by observing the change in reflectivity by kinetics-scan measurement (incidence angle, 66°). Reflectivity increased monotonically during SAM formation, and it reached a constant value within 2 h. Measurement was continued for at least 12 h to monitor all possible events. Time course measurement of the interaction of anti-azobenzene-dye IgG with azobenzene disulfide SAM was initiated by injection of a buffer solution of 10 mM Tris–HCl (pH 7.5) containing the IgG (9.5 μg/ml) into the cell. The photoisomerization reaction of the azobenzene dye in SAM was controlled by photoinfraredation of UV and visible light through a glass window on side in back. An ultrahigh-pressure mercury lamp with color filters was used as the UV (364 nm, 2.44 mW/cm²) and visible (440 nm, 2.70 mW/cm²) light source. Real-time observation of the adsorption manner of proteins (IgG and BSA) corresponding to the photosresponse in azobenzene SAM was carried out in the kinetics-scan mode.

Results

A schematic drawing of the structure in cross-section of the gold nanoparticles capped by unsymmetrical azobenzene disulfide dye (designated C6AzSSC12, formula wt. 683.14) is shown with the electron micrograph in Fig. 1(a). The nanoparticles had similar sizes, with an average diameter of 5.2 ± 1.3 nm. Azobenzene disulfide dye appeared to bind to the entire surface of the gold nanoparticles in a manner like piliform distribution at about 3.6 nm (theoretical) length with a preferred direction of the normal to the surface as seen in the middle in Fig. 1(a). The chemical structure of C6AzSSC12 is shown to the right in Fig. 1(a). According to calculation, about 170 molecules of the azobenzene disulfides resided on the surface per gold nanoparticle. As to the chemical features of the azobenzene disulfide, it alters conformation from the trans-form to the cis-form by UV irradiation, and from the cis-form to the trans-form by irradiation by visual light, reversibly, as is schematically summarized in Fig. 1(b).

The time courses of IgG production against azobenzene disulfide dye in living rabbits were monitored, and are shown with standard deviations in Fig. 2(a). The rise in anti-azobenzene disulfide IgG expression in rabbits injected with gold nanoparticles capped with azobenzene disulfides, azo-nanoparticles, was remarkable from about 7 weeks after the beginning of the experiment. On the other hand, no conspicuous increase in IgG concentration was recognized in the other rabbits, which were immunized with chemically stoichiometrically equivalent amounts of azobenzene disulfide dye. Times at which each antigen was boosted are indicated with arrowheads on the upper outside of the panel. The graph suggests that gold nanoparticles are able to act as an immunological carrier for the hapten, accompanying a high density of azobenzene disulfide dye, so that they can be exposed to the rabbit immune system. The purified IgG after the collection of antisera from the rabbits was investigated by SDS–PAGE (Fig. 2(b)). The two components of IgG, H- and L-chains, are clearly seen with CBB R-250. The typical final yield was 20 mg of IgG from 30 ml of antisera after affinity column chromatography using protein A.

The gold nanoparticles modified with azobenzene disulfide dye in suspension were examined by transmission electron microscopy (TEM), as shown in Fig. 3(a). Although individual particles are not so clearly visible in comparison with those without stain, from arbitrarily selected TEM images, we confirmed that circular nanoparticles (seen in dark black) irradiated with UV light before incubation with IgG (and thus on which the azobenzene moiety should take the cis-form) were never found surrounded by proteinaceous substances (Fig. 3(b)). However, azo-nanoparticles on which the azobenzene moiety took the trans-form appear to be surrounded by proteinaceous substances that correspond to the interacted IgGs (Fig. 3(c)). The individual shapes
of IgG are hardly seen, probably due to many protein molecules stacked and piled up together on the azo-nanoparticle when the specimen grid was negatively stained and air-dried. After the incubation of azo-nanoparticles with IgG on the grid, and just before staining with uranyl acetate, we included a rinsing step with the same buffer without IgG. Hence proteinaceous matter was seldom seen on the background of the carbon gold surface.

Fig. 1. Characteristics of Azo-Nanoparticles.

a. The structure and size of gold nanoparticles capped with unsymmetrical azobenzene derivative, C6AzSSC12. b. A schematic drawing of reversible cis-to-trans photoisomerization at the azobenzene moiety.

Fig. 2. Production of Anti-Azobenzene-Dye IgG in Vivo.

a. Time course of production of anti-azobenzene disulfide IgG in rabbits. The case of rabbits boosted solely with azobenzene-dye is indicated with open circles, and that with azo-nanoparticles with filled circles, in both cases together with standard deviations. Times when each antigen was boosted are indicated with arrowheads on the upper outside of the panel. b. SDS–PAGE of the purified anti-azobenzene-dye IgG stimulated by azo-nanoparticles.
**Fig. 3.** Electron Microscopy of Interaction between Anti-Azobenzene-Dye IgG and Azo-Nanoparticles.

a. Electron micrographic image of negatively stained azo-nanoparticles. b. TEM image of azo-nanoparticles, on which surface azobenzene moieties were in the *cis*-form, incubated with the anti-azobenzene-dye IgG produced in the study. Note that azo-nanoparticles, seen as dots in dark black, were never surrounded by proteinaceous substances. c. TEM image of azo-nanoparticles, on which surface azobenzene moieties were in the *trans*-form, reacted with the anti-azobenzene-dye IgG. The circular azo-nanoparticles appear to be surrounded by proteinaceous substances, *viz.*, anti-azobenzene disulfide IgGs. d. Superposition of schematic drawing to help in the interpretation of (c). Azo-nanoparticles are colored in blue and the proteinaceous substances, the anti-azobenzene disulfide IgGs produced in the study, are in light yellow. Scale bars represent 50 nm.

e. Optical density profile along the dotted line in red indicated in (a). A possible region for an azo-nanoparticle is suggested in the upper part of the panel.
f. Optical density profile along the dotted line indicated in (b). A possible region for an azo-nanoparticle is suggested in the upper part of the panel.
g. Optical density profile along the dotted line indicated in (c). Possible regions for an azo-nanoparticle and for proteinaceous substances, *viz.*, the anti-azobenzene-dye IgGs, are suggested by a solid band and a dotted squared band respectively in the upper part of the panel.

**Fig. 4.** SPR Measurement of Interaction between Anti-Azobenzene-Dye IgG and the Azobenzene-Dye SAM.

a. Experimental set-up for SPR measurement. A Tris-buffered solution containing anti-azobenzene-dye IgG was channeled from inlet through outlet. Specific adsorption of IgG toward the azobenzene moiety of SAM on a flat gold surface was promoted. Reversible *cis*-to-*trans* photoisomerization of azobenzene moiety was realized by incident UV or visible light. b. Real-time photoresponse of the adsorption of the anti-azobenzene-dye IgG onto the azobenzene disulfide SAM formed on a flat gold surface. The Y-axis shows the reflectivity record of SPR measurement. The types of isomers of the azobenzene moiety, *cis*- or *trans*-, are indicated together with the events of triggered irradiation (UV or vis) light. c. Real-time photoresponse of the adsorption of BSA onto the azobenzene disulfide SAM formed on a flat gold surface. The Y-axis shows the reflectivity record of SPR measurement. The types of isomers of the azobenzene moiety, *cis*- or *trans*-, are indicated together with the events of triggered irradiation (UV or vis) light.
film area (the upper left area in Fig. 3(c)). It is said that azobenzene disulfide, C6AzSSC12, usually takes the energetically stable trans-form, and it is most likely that the same happens in vivo. It is reasonable to suppose that IgG binds specifically to the surface of the gold nanoparticles, where trans-formed azobenzene disulfides are predominant. To help in the interpretation of the electron micrograph of Fig. 3(c), a schematic drawing was superimposed, and this shown as Fig. 3(d). Optical density profiles along the section lines, indicated by a dotted line in red in each EM image of Fig. 3(a), (b) and (c) are presented as (e), (f), and (g) respectively. Typical two neighbored azo-nanoparticles having similar sizes were chosen to make each optical density profile. A possible region for an azo-nanoparticle is suggested with squared belts in the upper part of each panel. The manners of appearance in the profile of both (e) and (f) are similar, while that of (g) is different from those of (e) and (f). The region most likely corresponds to the reflection of a proteinaceous substance, viz., IgG, even those stacked and piled up, is proposed with the squared shape filled with a dotted pattern, which is located on both sides of the squared belt corresponding to the region for azo-nanoparticles (Fig. 3(g)).

We also examined the binding interaction of IgG to the SAM of C6AzSSC12 formed on a flat gold surface by SPR measurement. The set-up of the measurement is shown in Fig. 4(a). As shown in Fig. 4(b), anti-azobenzene-dye IgG selectively adsorbed onto the SAM with trans-formed azobenzene disulfides, and saturated within 250 min. On the contrary, IgG adsorbed slowly to the SAM constituted with cis-formed C6AzSSC12, even though this process may contain nonselective bindings. Interestingly, when the conformation of the elements of the SAM was shifted to the trans-form by irradiation with visual light, the binding rate of the IgG dramatically increased, as seen in Fig. 4(b). This illustrates that IgG bound to the SAM of azobenzene disulfides, recognizing its predominant conformational difference. It is important to consider the phenomenon with proteins other than the IgG, e.g., BSA. BSA is a non-specific adsorptive protein. The result shows that no difference was recognized between the manners of BSA adsorption to SAM composed of trans- or cis-formed azobenzene disulfides (Fig. 4(c)).

Discussion

Many antigens are thymus-dependent.23) Hapten, a small molecule that binds to preformed antibodies but fails to stimulate antibody production, becomes immunogenic when coupled to an appropriate carrier such as a protein with a large molecular mass.19) Building on the knowledge that both T- and B-cells are necessary for an antibody response to a thymus-dependent antigen, and that only T-cells are activated by proteins, including proteinaceous degradates, a proteinaceous carrier stimulates T-helper cells that cooperate with the B-cell to enable them to respond to the hapten by providing accessory signals.24) Antigens such as azo-nanoparticles are not proteinaceous and do not appear to degrade readily in the living body, but at an appropriate spatial size, at a high enough concentration of the small chemical, can be thymus-independent in their ability to stimulate B-cells directly without the need of T-cell involvement.23,25) They appear to persist for long periods on the surfaces of macrophages specialized to locate at the lymph nodes and the splenic marginal zone.23)

Autoimmune disease is an immune reaction in which the immune system acts against itself. Some auto-immune diseases are known also as allergies. Acute type allergy includes anaphylactic shock as well as atopy. An easy and rapid way of obtaining IgGs of interest against a certain small chemical can serve as an important tool in further investigation of the mechanisms of allergic responses. It is interesting to consider the following phenomena appearing in the study: A group of rabbits that were once immunized solely with azobenzene disulfide dye, and that showed a negligible rise in IgG production, never produced anti-azobenzene disulfide IgG in high amounts, even when azobenzene disulfide dye was boosted as azo-nanoparticles. This phenomenon appears to be consistent with events known as immunotolerance and hypo-sensitization.19,26) Although it is beyond the scope of the present study to analyze in detail the clarifying components that figure here, it might provide further insight into mechanisms having to do with the immune response triggered by a hapten such as the azo-nanoparticles presented here.27)

Our method can indeed be generalized, because there are known chemical synthetic ways to introduce the -SH group to a small molecule of interest, which allow the formation of many molecules of a certain small chemical conjugated with a gold nanoparticle so as to cover the entire surface of the gold, and hence non-toxic nanoparticles. It is possible to highlight a particular small chemical molecule (a desired hapten) on the surface of gold nanoparticles so that it will be recognized by the immune system of mammals if it is in co-existence with shorter spacers such as alkyl-chains with the -SH group (alkane thiol). As illustrated on C6AzSSC12 in Fig. 1, the molecule contains the -S-S- sequence around the middle of its structure, and can be bent at the point of the -S-S- when it binds to the gold surface. In consequence, two different length chains derived from C6AzSSC12 come from the gold surface, one of which has the azobenzene moiety, and the shorter one of which makes an appropriate space around the azobenzene moiety. The space is likely to be a favorable environment for the IgG to approach, and makes possible interaction with the azobenzene moiety (see the middle of Fig. 1(a)). This in turn might create favorable conditions for the exposure of the azobenzene moiety to the immune system of a mammal. When some proteinaceous carriers are used and injected into an animal, many kinds of undesired IgGs
that possess different antigenic selectivities and the same biochemical features should rise also, because there are many candidates for epitopes on the proteinaceous carriers employed.\(^{28}\) It is hard to separate a certain kind of IgG from the remaining IgGs that share similar biochemical characteristics. Although it is often said that autologous proteins such as IgG are promising representatives that function well as immunological carriers, it is not easy to produce a hapten conjugated with autologous IgG, and the number of bindable sites of IgG for hapten molecules is limited. Although the safety of using nanoparticles should be examined before concluding that they can be used as a medication in the future, we have designed a novel alternative promising method of creating anti-hapten IgG. We need further evaluation of the safety of gold nanoparticles as a medication for mammals and of the novel methodology for producing anti-hapten IgGs with high efficiency making use of nanoparticles as a carrier. These results shed light on the application of non-toxic precious metal nanoparticles such as non-proteinaceous carriers. It is apparent that continued research along these lines should further expand the clinical applications of non-toxic precious metal nanoparticles as immuno-conjugate, and chemo-therapeutic drugs.\(^{29–31}\)

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References

(2002).


