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BRIEF COMMUNICATION

Surface modification of gold nanorods using layer-by-layer technique for cellular uptake

Hironobu Takahashi · Takuro Niidome · Takahito Kawano · Sunao Yamada · Yasuro Niidome

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Abstract Gold nanorods (NRs), rod-shaped gold nanoparticles, were modified with bovine serum albumin (BSA) and polyethylenimine (PEI) using layer-by-layer technique. From absorption spectroscopy and zeta potential measurements, it was obvious that NRs were wrapped with these polymers without aggregation of NRs. Following BSA modification, the surface-modified NRs (BSA-NRs) were well-dispersed without aggregation in biochemical conditions, verified from absorption spectroscopy. Further modification with PEI provided positively charged NRs (PEI-NRs). A transmission electron microscopy image of PEI-NRs revealed that the surface modification did not affect changing the shape of the initial NRs. In addition, the PEI-NRs retained the colloidal stability of BSA-NRs in biochemical conditions. We have evaluated that transfection activity of PEI-NRs with HeLa cells. From results of gene expression experiments, it was obvious that the stabilization of NRs by BSA and further modification with PEI realized transfection using NRs into cultured cells. Moreover, the cellular uptake of NRs enabled cellular imaging using light scattering from the NRs.

Keywords Gold nanorods · Gold nanoparticles · Gene delivery · Layer-by-layer deposition · Light scattering · Colloids

H. Takahashi · T. Niidome · T. Kawano · S. Yamada · Y. Niidome
Department of Materials Physics and Chemistry, Graduate School of Engineering, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan

T. Niidome · S. Yamada · Y. Niidome (✉)
Department of Applied Chemistry, Faculty of Engineering, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan
e-mail: ynidotcm@mbox.nc.kyushu-u.ac.jp

T. Niidome
Center for Future Chemistry, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan

Introduction

The use of anisotropic materials for biological sensing and gene delivery has attracted great interest in the fields of materials science and bioscience (Martin and Kohli 2003; Bauer et al. 2004). Because of their elongated structure, anisotropic nanoparticles have chemical and optical anisotropy, and can interact with cells and biomolecules in fundamentally new ways. Anisotropic gold nanoparticles are also a topic of discussion at present (Sau and Murphy 2004). In particular, rod-shaped gold nanoparticles, gold nanorods (NRs), have attracted much attention as a new class of gold nanoparticles, due to their unique optical properties (Yu et al. 1997; Murphy et al. 2005; Pérez-Juste et al. 2005). NRs show

two absorption peaks that correspond to the transverse and longitudinal surface plasmon (SP) bands in the visible region (~520 nm) and the near-infrared (near-IR) region, respectively (Link et al. 1999; Brioude et al. 2005). The intense longitudinal SP band affords absorption (Gorelikov et al. 2004), fluorescence (Li et al. 2005), and light scattering (Lee and El-Sayed 2005; Alekseeva et al. 2005) in near-IR region.

Because near-IR light is a powerful tool for biochemical and biomedical applications (Loo et al. 2005; Hintersteiner et al. 2005), the unique near-IR properties of NRs are particularly expected to provide advantages for diagnostic and therapeutic fields. For example, the use of NRs and near-IR light enable in vitro and in vivo two-photon luminescence imaging (Wang et al. 2005). On the other hand, we demonstrated that near-IR laser irradiation realized controlled release of plasmid DNA from complexes of NRs and plasmid DNA (Takahashi et al. 2005). This is because pulsed near-IR laser irradiation provides reshaping of NRs into spherical nanoparticles (Link et al. 2000). This photoreaction could potentially be useful for a new type of gene delivery (Nishiyama et al. 2005). Thus, NRs are possible candidates for use as probes for cellular imaging and/or functional materials for gene delivery (Takahashi et al. 2005; Huang et al. 2006; Chen et al. 2006).

Various kinds of nanoparticles including NRs require biocompatibility and colloidal stability for biochemical and biomedical applications (Otsuka et al. 2003). In the case of NRs, a large amount of cationic detergent, hexadecyltrimethylammonium bromide (CTAB), is required as a stabilizing agent (Yu et al. 1997; Murphy et al. 2005; Pérez-Juste et al. 2005). On the other hand, the CTAB in NR solutions interferes with biological processes, and exhibits high cytotoxicity (Connor et al. 2005). To improve the biocompatibility of NRs, we previously succeeded in preparation of phosphatidylcoline (PC)-passivated NRs (PC-NRs) with very low cytotoxicity (Takahashi et al. 2006a, 2006b). Thus, PC-NR is expected to be a potential biomaterial; however, further modification is needed to obtain functional NRs for gene delivery because it is difficult to keep the NR's well-dispersed, without aggregation in biochemical conditions (Takahashi et al. 2006c). To improve colloidal stability of gold nanoparticles in biochemical conditions, for example, surface modification with poly(ethylene glycol) (PEG) is a common strategy (Otsuka et al. 2003). We also reported in vivo stability of PEG-modified gold nanoparticles (Kawano et al. 2006) and gold nanorods (Niidome et al. 2006). On the other hand, Tkachenko et al. (2004) reported that bovine serum albumin (BSA) is useful for stabilizing nanoparticles under biochemical conditions. BSA modification provides simply biochemical stability via electrostatic interactions.

Layer-by-layer technique described by Decher (1997) allows nanoscale control over deposition of a large variety of functional materials through electrostatic interactions. Previously, Gole and Murphy (2005) reported that this technique is useful for altering the surface properties of NRs. In this study, the layer-by-layer technique was employed to modify PC-NRs with BSA and polyethylenimine (PEI). The surface modification of PC-NRs providing the colloidal stability realized transfection into cultured cells and cellular imaging using light scattering from the NRs.

Experimental

NRs, prepared with a slight modification of our method (Niidome et al. 2003), were supplied by Mitsubishi Materials Co. Ltd. The average length and width of the as-prepared NRs was +65 ± 5 nm and +11 ± 1 nm, respectively (aspect ratio: 5.9). The initial NR solution (~1 mM (Au atoms)) contained 80 mM of CTAB. Some of the CTAB precipitated when the solution was kept in a refrigerator (~4°C). The precipitated CTAB was removed by using a membrane filter (pore size, 0.8 μm). The residual CTAB in the NR solution (20 ml) was extracted into PC-chloroform solution (10 mg/ml, 10 ml; PC from egg yolk was purchased from Nacalai Tesque). After performing two more extraction procedures, the aqueous solutions containing NRs were centrifuged once, and then dispersed again in pure water (2 ml) (Takahashi et al. 2006a). Absorption spectra of the PC-NR solutions were measured with JASCO V-570. The zeta potentials of NRs

were evaluated using a Zetasizer NanoZS (MALVERN, He–Ne laser). Zeta potential was calculated using a theoretical model with spherical particles. Thus, the value qualitatively indicates the sign and magnitude of the zeta potential of the NRs. This is sufficient for relative evaluation of the surface charge of NRs.

BSA aqueous solution (600 μl, 2 mg/ml) was added to 50 μl of PC-NR solution with stirring. After 15 min of incubation, the NR solution was centrifuged (6,500*g*, 10 min). The precipitated NRs were redispersed into 100 μl of water, and the resultant NR (BSA-NR) solution (50 μl) was added to 200 μl of PEI solution (25 kDa, 10 wt%) with stirring. The mixture was centrifuged after 15 min of incubation, and the precipitates were redispersed in 80 μl of water. The resultant NRs (PEI-NRs) were observed by transmission electron microscopy (TEM). To evaluate the colloidal stability of the modified NRs (BSA-NRs and PEI-NRs), absorption spectroscopic measurements were carried out. After the BSA addition to PC-NRs, the BSA-NRs were divided into two tubes, and were centrifuged. They were redispersed into water and PBS buffer, respectively. The BSA-NRs in water were mixed with the PEI solution, and were condensed by centrifugation. To compare the colloidal stability of the PEI-NRs in PBS buffer with that in water, the PEI-NRs were divided into two tubes, and were redispersed after further centrifugation in water and PBS buffer, respectively. Besides, the absorption spectrum of the PEI-NRs in PBS buffer was measured after 12 h.

For agarose gel electrophoresis, plasmid DNA containing the firefly luciferase reporter gene (Ohsaki et al. 2002) (7 kbp, 0.5 mg/ml, 10 μl) was mixed with various concentrations of the PEI-NR solutions (3.125–12.5 μM (Au atoms), 10 μl). After the addition of 10 μl of 5×TBE buffer and 20 μl of water, the mixed solutions were electrophoresed. The plasmid DNA was stained with SYBR® Green I (Molecular Probes).

HeLa cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) at 37°C, 5% CO_2. The cells were plated at 5×10^3 cells/well in a 96-well plate, and incubated for 24 h. Cell viabilities of HeLa cells with PEI-NRs were estimated by a slight modification of the MTT assay (Takahashi et al. 2006a). Briefly, 10 μl of PEI-NR solution was added to the 100 μl of DMEM in each well (final NR concentrations: 0.25–1 μM). Following 24 h of incubation, 10 μl of Cell Counting Kit-8 (Dojindo Laboratories) was added to the medium. The absorbance of lysates after 2 h was measured at 450 nm by a 96-well microplate reader (Model 680 Microplate Reader, Bio-Rad Laboratories). To evaluate transfection efficiency for PEI-NR/DNA complexes, various concentrations (3.125–12.5 μM (Au atoms)) of PEI-NR solution (20 μl) were mixed with plasmid DNA (0.5 mg/ml, 20 μl), 10×PBS buffer and DMEM (final PEI-NR concentrations: 0.25–1 μM), and then the mixed solutions (250 μl) were poured gently onto the cells seeded as described above. After 3 h of incubation, the mixed solution was removed, and 250 μl of DMEM containing FBS was added to the well. After 48 h of incubation, luciferase assays were performed as described in the protocol of the Luciferase Assay System using a luminometer (Maltibiolumat LB9505, Berthold, Germany) (Niidome et al. 2004). The protein concentrations of the cell lysates were assessed with the Bradford assay (Protein Assay Kit, Bio-Rad).

For microscopic observation of the cultured cells treated with NRs, the cells were cultured in a glass base dish (50 mm, glass diameter: 12 mm) for 24 h. A PEI-NR/DNA complex solution and a PC-NR solution (final concentration: 1 μM) containing PBS and DMEM were poured into the dish. After 3 h of incubation, the solutions were removed, and fresh medium was poured into the dish. The near-IR light scattering images were obtained using a microscope (TE 2000, Nikon) equipped with a dark field condenser (T-CHA, Nikon), IR filter (HOYA R72) and CCD camera (NEPTUNE 100, Watec Co. Ltd.). All images were recorded with identical CCD gain and light illumination intensity.

Results and discussion

The effect of the surface modification of NRs on their colloidal stability can be evaluated from

their absorption spectra, because SP bands of NRs depend sensitively on their colloidal stability. Absorption spectra of PC-NRs before and after the addition of BSA are shown in Fig. 1. An absorption spectrum of a PC-NR solution (0.5 mM) shows typical characteristics of a NR solution (Fig. 1A) (Link et al. 1999; Brioude et al. 2005). The zeta potential of the PC-NRs was +15 ± 1 mV (initial NRs: about +60 mV) (Takahashi et al. 2006a). After addition of BSA solution (600 µl, 2 mg/ml) to the PC-NR solution (50 µl), the absorption spectrum of the BSA-NRs (0.25 mM) (Fig. 1Ba) was the same as that of the PC-NR solution (Fig. 1A). This indicates that the resultant NRs formed no aggregates because an absorption spectrum of NR aggregates shows broader SP bands than that of well-dispersed NRs (Takahashi et al. 2005, 2006c). On the other hand, in the case of lower BSA/NRs ratio, the absorption spectrum of the PC-NRs showed remarkable change (not shown). This is due to formation of PC-NR aggregates by electrostatic interactions between PC-NR and BSA. Considering that the zeta potential of the well-dispersed NRs (Fig. 1Ba) was negative (–20 mV), it is obvious that BSA molecules wrapped the PC-NRs without inducing aggregation of PC-NRs. The spectrum of the BSA-NRs in PBS buffer (0.25 mM) (Fig. 1Bb) was the same as that in water (Fig. 1Ba), despite PC-NRs form small aggregates in PBS buffer (Takahashi et al. 2006c). These suggested that BSA modification provided the colloidal stability in biochemical conditions to PC-NRs.

Figure 2a shows absorption spectrum of the PEI-NRs (0.25 mM) in water. When 50 µl the BSA-NR solution was added to 200 µl PEI solution (25 kDa, 10 wt%), the zeta potential of the PEI-NRs changed to positive (+8 mV). While this indicated that PEI molecules interacted with BSA-NRs, the absorption spectrum in Fig. 2a was identical to that of BSA-NRs (Fig. 1B), indicating the absence of NR aggregates. These suggested that BSA-NRs were modified with PEI without aggregation. A TEM image of the PEI-NRs is shown in Fig. 2b. The average length and width of the PEI-NRs are the same as those of as-prepared NRs. From these results, it was confirmed that the surface modification using the layer-by-layer approach gave positively charged NRs without changing the shape of the initial NRs.

Figure 3 shows absorption spectra of PEI-NRs (0.125 mM) in PBS buffer. The two curves in Fig. 3 indicate that the PEI-NRs were well-dispersed in PBS buffer for at least 12 h. Considering that PC-NRs form small aggregates in PBS buffer (Takahashi et al. 2006c), it is obvious that the addition of PEI did not induce BSA dissociation from the PC-NR surface. Thus, the BSA-NR was wrapped with PEI molecules and retained the colloidal stability in PBS buffer after the PEI modification. Gole and Murphy (2005) reported that a delicate balance of several parameters (the polymer type, concentration, chain length, and so on) is essential for preventing aggregation of NRs and achieving coating with polymers. In our study, the use of larger PEI (for example, 750 kDa) or higher NR concentrations than described above induced aggregation of NRs. To obtain desirable NRs, the balance described above are essential in this study.

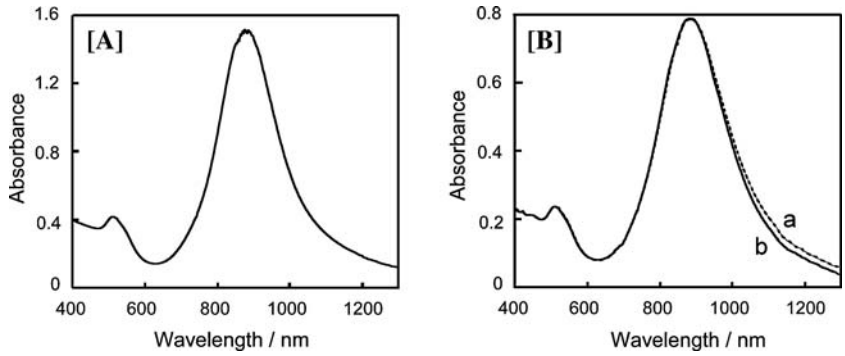

Fig. 1 Absorption spectra of PC-NRs (**A**) and BSA-NRs (**B**). (a, in water; b, PBS buffer)

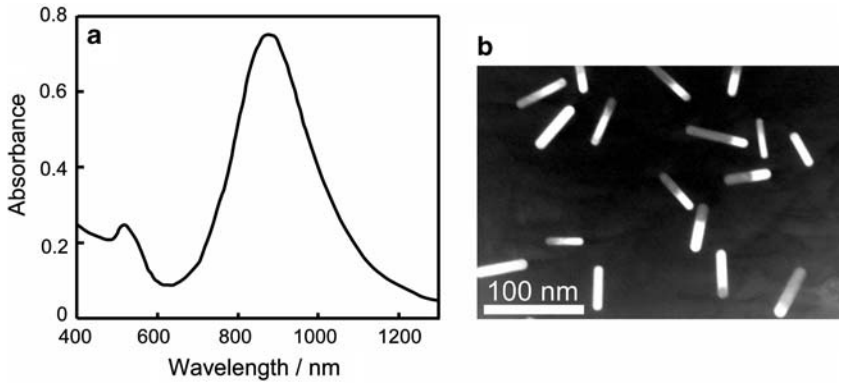

Fig. 2 Absorption spectrum (**a**) and TEM image (**b**) of PEI-NRs

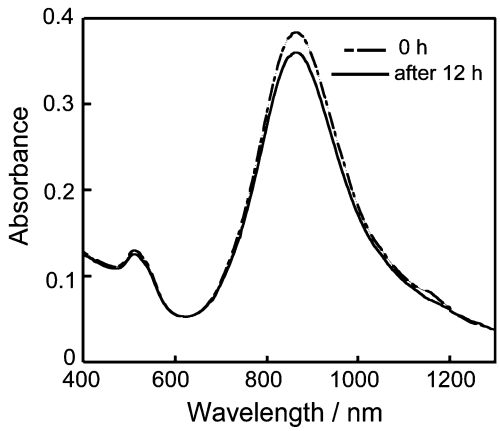

Fig. 3 Absorption spectra of PEI-NRs in PBS buffer just after mixing (dash-dotted line) and after 12 h (solid line)

In Fig. 4, the electrophoretic patterns of the plasmid DNA without (a) and with (b–d) PEI-NR solution are shown. The amount of migrating DNA decreased with increasing PEI-NR concentration. When 3.125 μM of PEI-NR solution was added to the DNA solution (b), a relatively small amount of the plasmid DNA migrated. Fluorescence around the well of lane (b) indicates that some of the plasmid DNA remains in the well even after electrophoresis. Addition of 6.25 μM (c) and 12.5 μM (d) of PEI-NR solutions completely suppressed the migration of the plasmid DNA. Thus, the electrophoretic patterns indicate the formation of PEI-NR/DNA complexes.

Gene expression on addition of PEI-NR/DNA complexes to cultured cells is shown in Fig. 5 (bars). Transfection efficiency has improved by formation of the complexes. When plasmid DNA formed complexes with 12.5 μM PEI-NR (final PEI-NR concentration in medium: 1 μM), the transfection efficiency was about 40 times higher

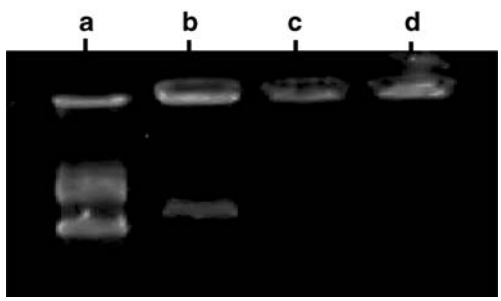

Fig. 4 Agarose gel (1% w/v) electrophoresis of plasmid DNA in the absence (**a**) and the presence (**b–d**) of PEI-NRs. Plasmid DNA (0.5 mg/ml, 10 μl) was mixed with 10 μl of PEI-NR solution ((**b**) 3.125, (**c**) 6.25, (**d**) 12.5 μM (Au atoms)). Final PEI-NR concentrations: (**b**) 0.25, (**c**) 0.5, (**d**) 1 μM. The plasmid DNA was stained with SYBR® Green I (Molecular Probes)

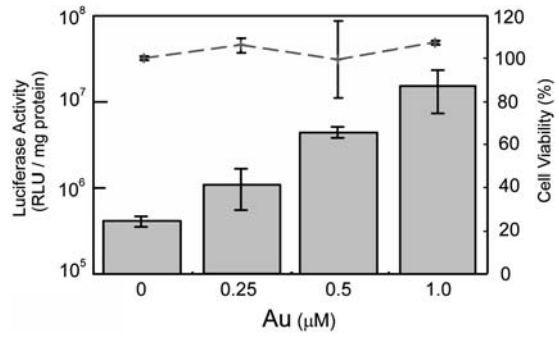

Fig. 5 Transfection efficiency (bars) and cell viability (line) with HeLa cells for PEI-NR/DNA complexes. Plasmid DNA containing the firefly luciferase reporter gene (0.5 mg/ml, 20 μl) was mixed with 20 μl of PEI-NR solution (3.125–12.5 μM (Au atoms)). Final PEI-NR concentrations: 0.25, 0.5 and 1 μM

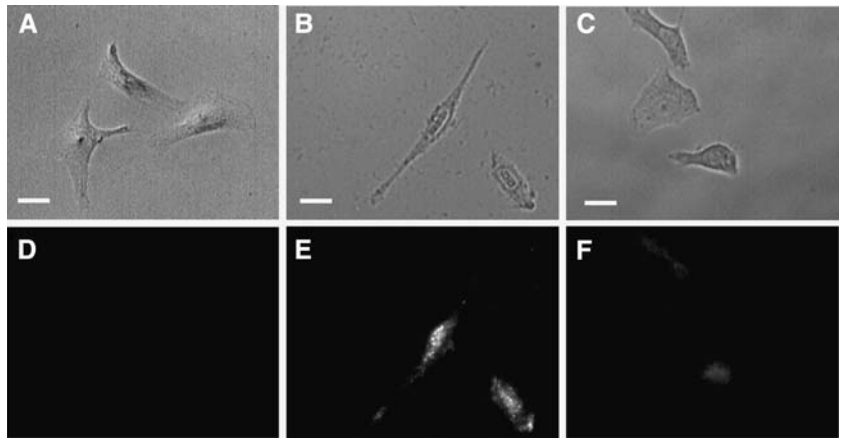

Fig. 6 Microscopic images (**A**–**C**) and light scattering images (**D**–**F**) of HeLa cells; in the absence of NRs (**A**, **D**) and the presence of NRs ((**B**, **E**) PEI-NRs, (**C**, **F**) PC-NRs). The cells were incubated with NRs (final concentration: 1 μM) for 3 h. Near-IR scattered light was detected selectively using an IR filter (HOYA R72). Scale bar: 10 μm for all images

than that of naked DNA. When PEI-NRs were precipitated by centrifugation (6,500*g*, 10 min), the supernatant showed no enhancement of gene expression under the same experimental condition. Thus, it was obvious that the gene expression in Fig. 5 resulted from PEI-NR/DNA complexes. In addition, under these transfection conditions, the PEI-NR/DNA complexes showed negligible cytotoxicity (line in Fig. 5). It was remarkable that the PEI (25 kDa) showed such low cytotoxicity. High molecular weight PEIs (>2,000 Da) frequently show high cytotoxicity in return for higher transfection activity (Alexis et al. 2006; Tang et al. 2006). When lower molecular weight PEI (for example, 800 Da) was used for the layer-by-layer deposition, the resultant PEI-modified NRs showed no transfection activity. PC-NRs also showed no transfection despite PC-NRs formed complexes with plasmid DNA and interacted with cultured cells (Takahashi et al. 2005, 2006a). To achieve gene delivery using the surface-modified NRs, the ''balance'' of several parameters (cytotoxicity, colloidal dispersion, cellular uptake efficiency, etc.) must be optimized. The surface modification with BSA and PEI gave a balanced surface condition to achieve efficient transfection without inducing cell death. Thus, PEI-NRs can be used as a transfection vehicle for in vitro gene delivery.

The unique optical property of NRs enables light scattering imaging (Orendorff et al. 2006; Huang et al. 2006). Figure 6 shows microscopic images (A–C) and near-IR light scattering images (D–F) of HeLa cells in the absence and presence of two kinds of NRs (PEI-NRs, PC-NRs). In the absence of NRs (A), hardly any scattered light was observed from HeLa cells (D). This means that scattered light from the cell organelles and membrane was negligible under the experimental conditions used. In the presence of PEI-NR/DNA complexes (B), the cell image could be seen by scattered light (E). This indicates that the PEI-NRs were accumulated in and/or on the cells, to make individual cells identifiable (El-Sayed et al. 2005). In the presence of PC-NRs (C), the light scattering was much weaker than in the presence of PEI-NRs (F), in spite of the same amount of NR being used as for the case in (E). This indicates that the surface modification increased cellular binding and uptake, resulting in the transfection activity of the NRs.

Conclusion

This is a preliminary work realizing gene delivery using surface-modified NRs. A layer-by-layer technique had achieved the surface modification of PC-NRs with BSA and PEI without forming aggregation. The surface-modified NRs with high colloidal stability retained the near-IR property in biochemical conditions and showed cellular uptake activity. The transfection efficiency was smaller than that of PEI/DNA complexes (Boussif et al. 1995; Godbey et al. 1999; Kursa et al. 2003). Thus, further development to achieve a ''fine-tuned'' surface is needed; however, NRs having uptake activity is advantageous to biochemical

and biomedical applications using the near-IR region. For example, use of near-IR light realizes controlled release of plasmid DNA from NR/DNA complexes (Takahashi et al. 2005). If the photoreaction can be used for gene delivery, gene expression will be enhanced by near-IR light. Such a photochemical enhancement system could be useful for a new type of gene delivery system. Moreover, near-IR scattering imaging was advantageous in monitoring the distribution of NRs in living cells without use of fluorescent molecules, which tend to be quenched by the intense excitation light required for microscopic fluorescent observation. Surface-modified NRs with a fine-tuned surface will open up a new type of gene delivery and probing system.

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