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Biosensing, Cytotoxicity, and Cellular Uptake Studies of Surface-Modified Gold Nanorods

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Herein we report biorecognition studies of protein IgG using biocompatible gold nanorods as molecular probes. Surface modification of cetyltrimethylammonium bromide (CTAB)-stabilized gold nanorods was carried out by using poly(styrenesulfonate) (PSS) to reduce the toxicity of as-synthesized gold nanorods caused by free CTAB. ζ potential analysis confirmed charge reversal on the surface of gold nanorods caused by the PSS coating. Surface plasmon resonance exhibited by gold nanorods has been employed as a tool for analyzing the binding events for biomolecules. TEM results, showing the aggregation of gold nanorods, in addition to the shift in surface plasmon resonance peak in UV-vis absorption measurements, upon the interaction of biomolecules with gold nanorods, confirmed molecular binding. Morphological changes caused by the cellular uptake of gold nanorods before and after PSS modification have been observed. Cell viability studies using gold nanorods were performed to study the cytotoxic effects of these molecular probes.

1. Introduction

Recently a great advancement was observed in utilizing metal nanoparticles, especially gold, for biomedical applications, owing to their unique shape/size-dependent properties, strong absorption/scattering of light, stability, and nontoxic nature.^{1–15} Among all, gold nanorods are found to be more popular and useful for potential applications such as biochemical sensing, biomedical diagnostics, and therapeutics due to possible tuning of their surface plasmon resonance (SPR) in the visible and near-infrared region, which is the potential window of the electromagnetic spectrum for in vivo applications. The origin of SPR of gold nanostructures, which includes contributions from scattering and absorption components, depends on the shape and size of the gold nanostructures. Substantial progress made in controlling the size and shape of nanoparticles by confined growth offers a unique opportunity to tune the SPR of gold nanostructures in the visible and near-infrared region.^{16–20} In principle, gold nanorods exhibit two SPR bands, namely, the transverse band (corresponding to the oscillation of electrons along the short axis of the nanorod) and the longitudinal band (corresponding to the oscillation of electrons along the long axis of the nanorod). The longitudinal band of gold nanorods can be tuned from the visible to near-infrared region by increasing the aspect ratio of gold nanorods. The strong light scattering properties of gold nanorods have been applied mainly for optical microscopic imaging of cancer cells, and the absorption properties in the NIR region causing a localized hypothermic effect have been utilized for therapeutic purposes.^{3,4,6,8,9} However, changes in localized SPR due to alteration in the conditions of the surrounding environment of gold nanorods have been employed to analyze different biorecognition events at the molecular level.^{21–27} It was observed that the longitudinal band

is more sensitive to the changes in the environment of the gold nanorods as compared to the transverse band, making them ideal candidates for sensing and imaging applications.

Engineering of gold nanorods with novel properties by controlled synthesis for potential applications is very important. The most popular method for synthesizing gold nanorods involves the seed-mediated approach using cetyltrimethylammonium bromide (CTAB) surfactant as the shape-directing agent.^{16–20} However, it was observed that an excess of CTAB is toxic²⁸ and its removal from solution causes unwanted aggregation of the particles, which is a cause of concern for use of gold nanorods for various clinical applications. This necessitates the surface modification of as-synthesized gold nanorods to get biocompatible nanoparticles with a stable dispersion in an aqueous or a buffer medium. It is also essential to understand the properties of surface modified gold nanorods and their effects after in vivo administration for clinical applications. Thus, cellular uptake and cytotoxicity studies of gold nanostructures containing a variety of surface modifiers and stabilizers become necessary for potential biomedical applications. There are very few reports on cellular uptake and cytotoxicity studies on gold nanostructures.^{28–35} As such pure gold nanoparticles are expected to be safe, however, modifiers and stabilizers used during the chemical synthesis make them toxic. Studies on cellular uptake of gold nanoparticles suggest that nanoparticles are readily endocytosed by mammalian cells and the kinetics depends on the shape and size of the nanoparticles.^{36,37}

The objective of the present investigation is to study the cytotoxicity, cellular uptake, and biosensing properties of bioconjugated gold nanorods, which will be useful for future biomedical applications. In this study, bioconjugated nontoxic gold nanorods were utilized as molecular probes for the detection of goat IgG by the localized surface plasmon resonance (LSPR) method. Surface modification of CTAB-stabilized gold nanorods was carried out by using poly(styrenesulfonate) (PSS) to reduce the toxicity of as-synthesized gold nanorods caused by an excess of CTAB. Gold nanorods after surface modification were

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characterized by the ζ potential to ensure the surface coating by PSS. Surface-modified gold nanorods were functionalized by anti-g-IgG antibodies, which were further utilized for rapid and sensitive detection of g-IgG by localized surface plasmon absorption. The *in vitro* cell viability and cellular uptake studies were performed by using normal human gingival epithelialoid cells (S-G) and oral cancer cells isolated from a Taiwan patient (TW 2.6).

2. Experimental Section

2.1. Chemicals and Materials. Hydrogen tetrachloroaurate(III) hydrate ($\text{HAuCl}_4 \cdot 0.3\text{H}_2\text{O}$), trisodium citrate dehydrate (99%), silver nitrate (AgNO_3) (99%), ascorbic acid (AA) (99%), and CTAB (99%) were obtained from Acros Organics and used without further purification. PSS with MW 14 000 was purchased from Alfa Aesar. The peroxide-conjugated AffiniPure donkey anti-goat IgG and pure goat IgG molecules were purchased from Jackson Immuno Research Laboratories, Inc. The water used throughout this investigation was reagent-grade water, produced using a Milli-Q SP ultrapure water purification system from Nihon Millipore Ltd., Tokyo.

2.2. Preparation of Au Seeds. Gold nanorods were prepared using a previously reported procedure with slight modification¹⁷ and scaled up to increase the quantity. Gold seed particles were prepared by adding 0.25 mL of an aqueous 0.01 M solution of HAuCl_4 to 7.5 mL of a 0.1 M CTAB solution in a test tube followed by gentle inversion. The solution appeared bright brown-yellow in color. Then 0.6 mL of an aqueous 0.01 M NaBH_4 solution was added all at once, followed by rapid inversion mixing for 2 min. The solution developed a pale brown-yellow color. Then the test tube was kept in a water bath maintained at 25 °C for future use. This seed solution was used 2 h after its preparation.

2.3. Preparation of Au Nanorods. For the growth of gold nanorods, 9.44 mL of 0.10 M CTAB solution, 0.4 mL of 0.01 M HAuCl_4 solution, and 0.06 mL of 0.01 M AgNO_3 solution were added in that order, one by one, to a test tube, followed by gentle mixing by inversion. The solution at this stage turned bright brown-yellow in color and upon further addition of 0.06 mL of 0.10 M AA became colorless. Finally, 0.04 mL of seed solution was added, and the reaction mixture was gently mixed for 10 s and left undisturbed for at least 3 h. After the growth of gold nanorods, the excess of CTAB was removed by repetitive centrifugations at 13 000 rpm for 12 min, and finally the concentrated colloidal GNRs were redispersed in water.

2.4. Surface Modification and Biosensing Studies. The surface modification of GNRs with PSS has been carried out using a previously reported procedure.²⁶ In brief, 0.1 mL of GNR solution was diluted to 10.0 mL. To this solution was added dropwise 10 mL of 2 g/L PSS prepared in a 6 mM NaCl solution (which was sonicated previously for 30 min), and the resulting solution was stirred vigorously for 3 h. After the incubation of GNRs with PSS for 3 h, the solution was centrifuged twice at 10 000 rpm to remove excess polyelectrolyte and redispersed in 10 mL of PBS buffer (pH 7.6). The molecular probes of gold nanorods were obtained by incubating PSS-coated gold nanorods with an excess amount of goat anti-IgG solution (50 mg/L in phosphate-buffered saline (PBS) solution) for 30 min, followed by centrifugation and redispersion twice into PBS solution to remove the unbound antibodies. To avoid the nonspecific binding of these molecular probes, a blocking solution of 50 mg/L bovine serum albumin (BSA) was employed. Gold nanorod-based molecular probes were incubated with the blocking solution for 15 min followed by centrifugation to remove excess BSA and dispersion in PBS solution. These

TABLE 1: ζ Potentials of Gold Nanorods

gold nanorods	ζ potential (mV)
gold nanorods stabilized with CTAB	+47.6 ± 1.9
PSS-capped gold nanorods	-11.7 ± 3.8

molecular probes were further used for detection of goat IgG, where a 0.5 mL solution of goat anti-IgG/nanorod conjugates was mixed with 1 mg of goat IgG.

2.5. Characterization. The characterization of gold nanorods has been carried out by using different experimental techniques. Absorption spectra of gold nanorods were taken on a Shimadzu spectrometer in plastic cuvettes with a 1 cm path length. For transmission electron microscopy, colloids of gold nanorods were dried on a carbon film grid and imaged at 100 kV (JEM 2010, JEOL). The surface charge of the gold nanorods before and after PSS coating was analyzed using a Malvern Zetasizer Nano ZS.

2.6. Cytotoxicity and Cellular Uptake Studies. Considering the increasing applications of gold nanorods in biomedical fields, *in vitro* cell viability studies were performed in the presence of gold nanorods. Normal human gingival epithelialoid cells (S-G) and oral cancer cells isolated from a Taiwan patient (TW 2.6) were used for cytotoxicity and cellular uptake studies. Cell cytotoxicity was evaluated by the colorimetric MTS assay. Briefly, the cells (2000 cells/90 μL) with 10 μL of gold nanorods were seeded into different wells and were incubated for 72 h followed by the addition of 20 mL of MTS in each well. The optical density (OD) of the resultant solution was determined ($\lambda = 490$ nm) by using a microplate absorbance reader (SpectraMAX 340pc, Molecular Devices, California).

For cellular uptake studies, normal human gingival epithelialoid cells (S-G) and oral cancer cells isolated from a Taiwan patient (TW 2.6) were incubated with gold nanorods. After a 6 h incubation period, medium containing gold nanorods not taken up by the cells was discarded; the cells were washed thoroughly three times with PBS, scraped from the culture dish, and centrifuged into a small pellet. These cell pellets were fixed in a 0.1 M PBS solution containing 2.5% gluteraldehyde and 4% paraformaldehyde for 1 h. They were then rinsed with 0.1 M PBS, embedded in 2% agarose gel, and postfixed in 4% osmium tetroxide solution for 1 h in the same buffer. The cells were dehydrated in an ethanol series and embedded in epoxy resin. The resin was polymerized at 60 °C for 48 h. Ultrathin sections (100 nm) were cut and stained with standard uranyl acetate and lead citrate and imaged under a Philips Technai 20 electron microscope.

3. Results and Discussion

Gold nanorods were synthesized by a seed-mediated growth method using CTAB, as described in the Experimental Section. The as-synthesized gold nanorods with an average aspect ratio of ~2–3 were fairly uniform in shape and were highly dispersed in water without aggregation. CTAB forms rodlike micelles in the solution above its critical micelle concentration and forms bilayers on the surface of gold nanorods, which results in a stable dispersion of gold nanorods. The nanorods capped with a bilayer of CTAB are positively charged. However, CTAB is known for its cytotoxicity,²⁸ hence, it is important to mask the CTAB layer for future biomedical applications. The CTAB-coated gold nanorods were further covered with PSS by electrostatic interactions to obtain the negatively charged gold nanorods. ζ potential analysis of gold nanorods was performed before and after PSS coating (Table 1). CTAB-stabilized gold nanorods

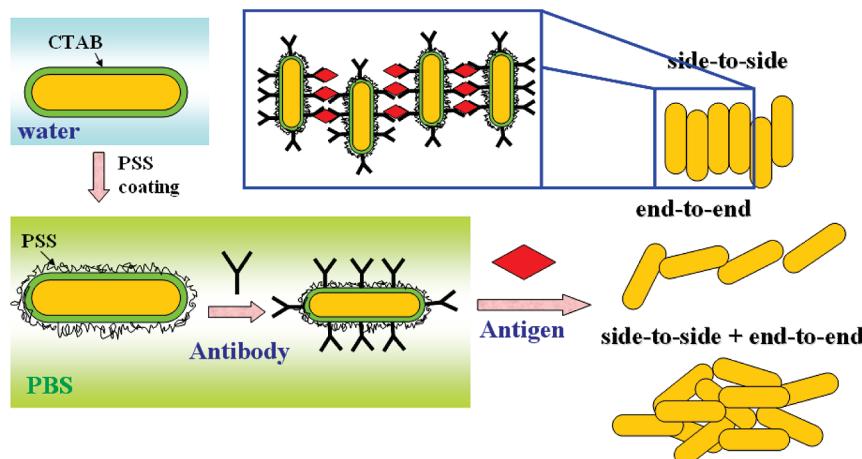


Figure 1. Schematic representation of bioconjugation of gold nanorods and the detection of g-IgG through the aggregation of gold nanorods.

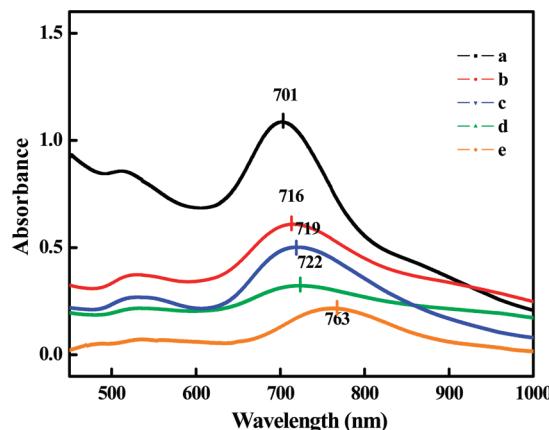


Figure 2. UV-vis absorption spectra of as-prepared gold nanorods (a), PSS-coated gold nanorods (b), PSS-coated gold nanorod/goat anti-g-IgG conjugates (c), gold nanorods interacted with BSA (d), and gold nanorods after incubation with g-IgG (e).

showed a positive charge on the surface due to the presence of quaternary amine hydrophilic head groups from adsorbed CTAB, whereas PSS-capped gold nanorods showed a negative charge on the surface due to the presence of anionic SO_3^- groups. Thus, ζ potential analysis confirmed successful coating of PSS on the surface of CTAB-capped gold nanorods. This surface modification of gold nanorods was found to be very effective in reducing the cytotoxicity of gold nanorods caused by an excess of CTAB (which is discussed in the last section) and formed a stable dispersion of gold nanorods for further attachment of biomolecules. The bioconjugation of gold nanorods and the detection of IgG are represented schematically in Figure 1.

Absorption spectra of gold nanorods at different stages of the analysis are shown in Figure 2. It is clear from the figure that there is a shift in the absorbance of gold nanorods at each stage. The SPR band of as-prepared gold nanorods is observed at 701 nm. However, the PSS-capped gold nanorods ($\lambda_{\text{max}} = 716 \text{ nm}$) show a red shift in the peak maxima of $\sim 15 \text{ nm}$ with peak broadening. It is known that the absorption and scattering properties of metal nanoparticles are strongly determined by the local dielectric function. Therefore, the red shift in the peak maxima is probably due to the change in the local refractive index from that of water to that of PSS. These observations are consistent with the previous reports on coating of gold nanorods with different polyelectrolytes.^{24,26} Further, PSS-capped gold nanorods on incubation with anti-g-IgG solution showed an SPR peak at 719 nm which almost remains the same for the antibody-

coated gold nanorods after the BSA masking. The BSA masking has been carried out to avoid the nonspecific binding of gold nanorods. The observation suggests the saturation of the surface of gold nanorods with anti-g-IgG. After addition of 1 mg of g-IgG to 0.5 mL of a gold nanorod molecular probe solution, the intensity was dramatically reduced accompanied by a red shift in the peak maxima to much longer wavelength (763 nm). It is believed that the large shift in the peak maxima after the binding of protein IgG to its antibodies may be due to the preferential assembly of gold nanorods caused by binding events. It has been also observed that, for all the stages, the longitudinal SPR band responded with a higher shift in the peak maxima as compared to that of the transverse band, which is consistent with the previous reports. This shows that the SPR longitudinal band is more sensitive in response for molecular binding events as compared to the SPR transverse band. Thus, the absorption measurements showed a simple form of molecular sensing based on changes in the local dielectric environment due to the binding of analyte molecules to gold nanorods, which results in the shift in the plasmonic extinction peak.

To verify the conclusions derived from absorption measurements, TEM images were taken for each step of bioconjugation and biorecognition, which are shown in Figure 3. It is clear from Figure 3a that the as-synthesized gold nanorods are monodispersed with a high yield (95%). Moreover, the PSS coating, antibody incubation, and BSA masking also retained the dispersity of the gold nanorods (Figure 3b-d). A sudden aggregation of gold nanorods was observed after treatment with g-IgG, which was preferentially oriented in a lateral (side-to-side and/or end-to-end) fashion (Figure 3e,f). These observations suggest that the interaction of biomolecules due to biorecognition results in the aggregation of gold nanorods. These data support the observations obtained from absorption measurements. Thus, it can be concluded that molecular binding events along with a change in the refractive index of the medium are responsible for the change in the peak position, intensity, and shape of the longitudinal surface plasmon band of gold nanorods as well as the aggregation of gold nanorods. The dependence of the absorption characteristics of gold nanorods on biorecognition events facilitates the use of gold nanorods as molecular probes for future biomedical applications. These observations are very useful in monitoring the target-specific binding events at the molecular level with the help of gold nanorods as molecular probes.

For clinical applications, it is very essential that the nanoparticles show efficient cellular uptake, easy clearance, and

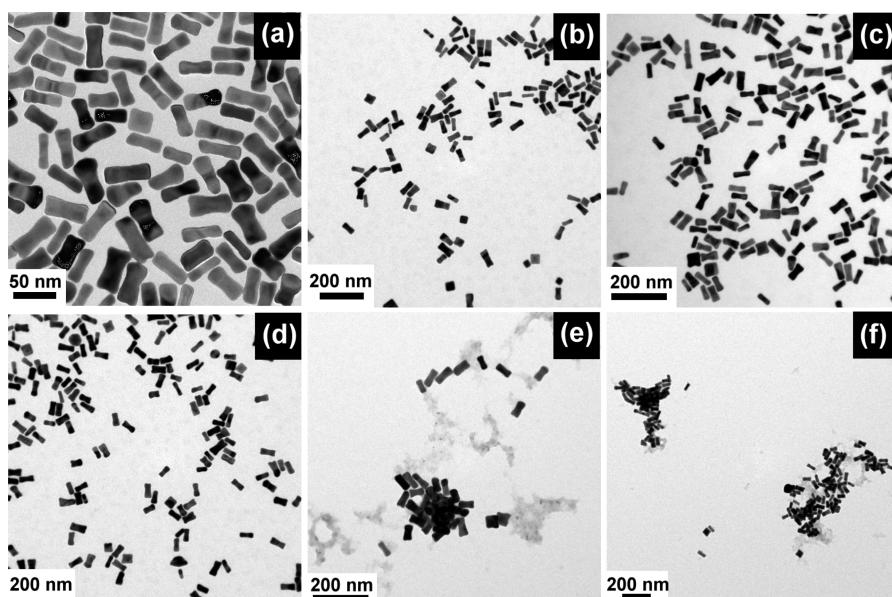


Figure 3. TEM images of as-prepared gold nanorods (a), PSS-coated gold nanorods (b), PSS-coated gold nanorod/goat anti-g-IgG conjugates (c), gold nanorods after treatment with BSA (d), and gold nanorods after binding to g-IgG (e, f).

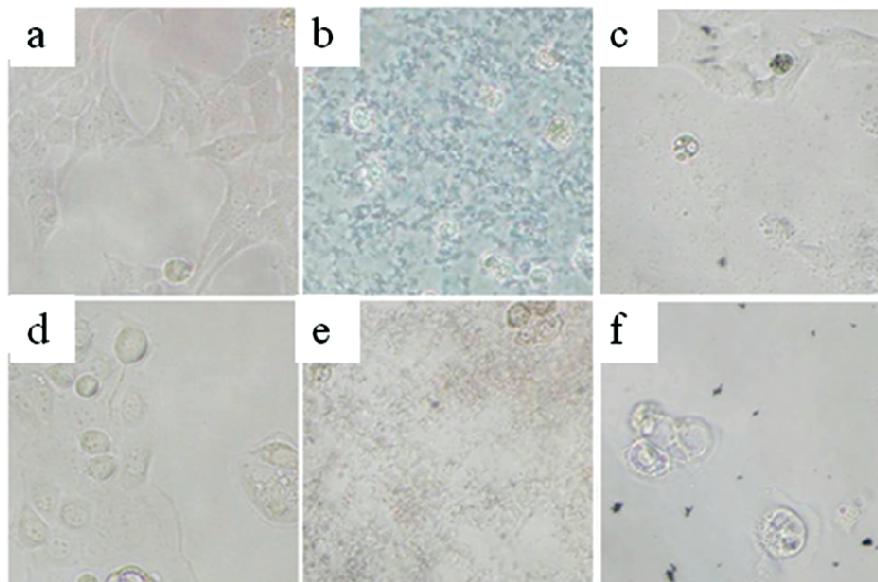


Figure 4. Microscopic images and cell survival of S-G (a–c) and TW 2.6 (d–f) cells after treatment with the desired samples. The cells were treated with medium (a, d), CTAB-capped gold nanorods (b, e), and PSS-capped gold nanorods (c, f) for 72 h.

nontoxicity. To examine this feasibility, we performed cellular uptake and cytotoxicity studies with gold nanorods. The cellular uptake images of gold nanorods before and after PSS coating, incubated with the S-G and TW 2.6 cells for 72 h, are shown in Figure 4. The S-G cells treated with CTAB-capped gold nanorods (Figure 4b) for 72 h revealed great morphological changes compared with those treated with culture medium alone (Figure 4a). The cell morphology was not changed after treatment with PSS-capped gold nanorods (Figure 4c). Notably, the effect of TW 2.6 cell treatment shows a similar observation. TW 2.6 cells treated with CTAB-capped gold nanorods (Figure 4e) for 72 h revealed great morphological changes compared with those treated with culture medium alone (Figure 4d). The cell morphology was not changed after treatment with PSS-capped gold nanorods (Figure 4f). After 72 h of incubation, PSS-capped gold nanorods were taken by S-G (Figure 4c) and TW 2.6 (Figure 4f) cells as visualized by the microscopic imaging of nanoparticles. The observed black dots (Figure 4c,f)

indicate the presence of PSS-conjugated gold nanorods after their internalization into cells. The uptake of gold nanoparticles is mediated by the adsorption of serum proteins onto the gold surface via the mechanism of receptor-mediated endocytosis.^{36,38} Since the size of PSS-capped gold nanorods is less than 100 nm, they are not clearly visible in the image; rather a few black dots appeared showing the intracellular localization of the gold nanorods. Unlike CTAB-capped gold nanorods, PSS-capped gold nanorods exhibit a negligible effect on cells after 72 h of incubation.

Figure 5 shows the cell viability results of S-G and TW 2.6 cells after 72 h of exposure to CTAB-capped gold nanorods and PSS-capped gold nanorods. Similar tests were also carried out with the CTAB-capped ϕ -shaped (fusiform) gold nanoparticles for comparison. The synthesis of ϕ -shaped (fusiform) gold nanoparticles was carried out using the procedure in our previous report.³⁹ It was observed that CTAB-capped gold nanorods and CTAB-capped ϕ -shaped nanoparticles exert a higher cytotoxic

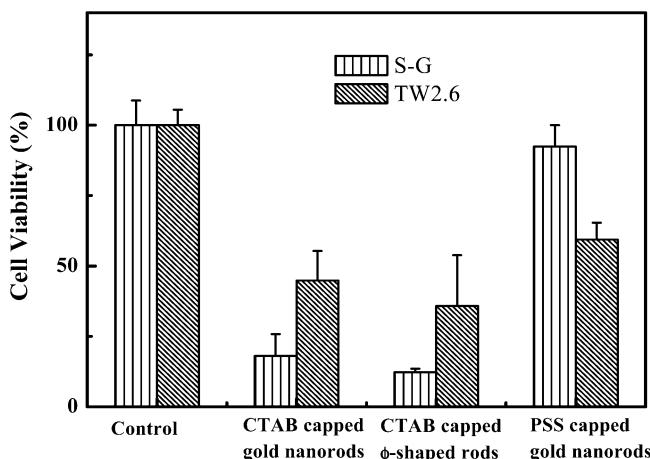


Figure 5. Comparison of cell viability assays for CTAB-capped gold nanorods, CTAB-capped ϕ -shaped (fusiform) gold nanoparticles, and PSS-capped gold nanorods after treatment with S-G and TW 2.6 cells for 72 h.

effect than PSS-capped gold nanorods on both the cell lines, suggesting that PSS coating on the surface of the gold nanorods significantly decreases the cytotoxicity of the nanorods. Thus, the cellular uptake and cell viability studies suggest that the PSS-capped gold nanorods are suitable for in vivo applications.

4. Conclusions

Gold nanorods synthesized by the seed-mediated method in the presence of CTAB have been stabilized using PSS, conjugated to the antibodies, and characterized for cytotoxicity, cellular uptake, and detection of protein, IgG. The positively charged stabilizing surfactant bilayer on the surface of gold nanorods has been replaced by poly(styrenesulfonate) to reduce the toxicity of the gold nanorods caused by excess CTAB and to obtain a stable dispersion of the nanorods in PBS buffer. The reversal of the surface charge on the nanorods before and after modification with PSS has been confirmed by ζ potential analysis. Selective binding of antigen and antibodies was demonstrated by a change in the localized surface plasmon absorption of gold nanorods, which was further confirmed by TEM results showing aggregation of the nanorods with preferential orientation in a lateral (side-to-side and/or end-to-end) fashion due to the interaction of biomolecules. Absorption measurements showed a simple form of molecular sensing based on changes in the environment of the medium due to the binding of analyte molecules to the gold nanorods, which results in a shift in the plasmonic extinction peak. Surface coating of nanorods with PSS significantly increased the cell viability and showed easy intracellular uptake of the nanorods, which suggests their possible use for different biomedical applications. This rapid, sensitive, and label-free approach for detection of molecular binding events using surface-modified gold nanorods may provide a novel optical multiplex biosensor platform and may have broad potential applications in immunoassay and disease diagnosis.

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