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# Challenge in understanding size and shape dependent toxicity of gold nanomaterials in human skin keratinocytes

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ABSTRACT

As the nanotechnology field continues to develop, assessing nanoparticle toxicity is very important for advancing nanoparticles for biomedical application. Here we report cytotoxicity of gold nanomaterial of different size and shape using MTT test, absorption spectroscopy and TEM. Spherical gold nanoparticles of different sizes are not inherently toxic to human skin cells, but gold nanorods are highly toxic due to the presence of CTAB as coating material. Due to toxicity of CTAB, and aggregation of gold nanomaterials in the presence of cell media, we have demonstrated that it is difficult to understand the cytotoxicity of gold nanomaterials individually.



## 1. Introduction

The nanoscience revolution that sprouted throughout the 1990s is having promises to benefit society [1–24]. Since their size scale is similar to that of biological molecules (e.g., proteins, DNA) and structures (e.g., viruses and bacteria), enormous interest is growing to exploit nanoparticles for various biomedical applications [1–24]. Due to valuable size and shape dependent properties, gold nanoparticles have been exploited as tools in biology for the last fifteen years [1–24]. Their high electron density has made them popular labels in electron microscopy, and because their surface properties enable a great variety of functionalization procedures. Having extremely small size with highest possible surface area enables them to access a variety of biological environments. These advantages, along with the universal biocompatibility, gold nanoparticles are promising to be used as vehicles for drug and gene delivery. Furthermore, gold nanoparticles have recently been demonstrated in cell imaging, targeted drug delivery, and cancer diagnostics and therapeutic applications [4–24]. As nanotechnology field continues to develop, studies on the cytotoxicity of nanoparticles, with respect to their size and shape, are required in order to advance nanotechnology for biomedical applications. This will be important for assessing nanoparticle toxicity, for advancing nanoparticles for imaging, drug delivery, and therapeutic applications and for designing multifunctional nanoparticles. Though nanoparticle production has been estimated to increase from 2300 tons produced today to 58 000 tons by 2020, it is surprising that knowledge on the toxicity effect of nanoparticle exposure is in infancy and also reported results are confusing [25–30]. Driven by the need, we report the systematic study of cytotoxicity of gold nanomaterial of different size and shape.

## 2. Experimental

Gold nanoparticles of different particle sizes were synthesized using the citrate reduction method as reported recently [9–16].

Transmission electron microscope (TEM) images and UV–Vis absorption spectra were used to characterize the nanoparticles, as we reported recently [9–16]. The particle concentration was measured by UV–Vis spectroscopy using molar extinction coefficients at the wavelength of maximum absorption of each gold colloid as reported recently [$\varepsilon_{(15)\ 518\ nm} = 3.6 \times 10^8\ cm^{-1}\ M^{-1}$, $\varepsilon_{(30)\ 530\ nm} = 3.0 \times 10^9\ cm^{-1}\ M^{-1}$, $\varepsilon_{(40)\ 533\ nm} = 6.7 \times 10^9\ cm^{-1}\ M^{-1}$, $\varepsilon_{(50)\ 535\ nm} = 1.5 \times 10^{10}\ cm^{-1}\ M^{-1}$, $\varepsilon_{(60)\ 540\ nm} = 2.9 \times 10^{10}\ cm^{-1}\ M^{-1}$, and $\varepsilon_{(80)\ 550\ nm} = 6.9 \times 10^{10}\ cm^{-1}\ M^{-1}$] [9–16].

Gold nanorods were synthesized using a seed-mediated, surfactant-assisted growth method in a two-step procedure [10,14,16,23]. Colloidal gold seeds (~1.5 nm diameter) were first prepared by mixing aqueous solutions of hexadecylcetyltrimethylammonium bromide (CTAB, 0.1 M, 4.75 mL) and $HAuCl_4$ (0.01 M, 0.2 mL) followed by addition of an aqueous solution of $NaBH_4$ (0.01 M, 0.6 mL). The colloidal gold seeds were then injected into an aqueous growth solution of CTAB (0.1 M, 4.75 mL), silver nitrate (0.01 M, varying amounts of silver nitrate between 20 and 120 mL were used to achieve the desired nanorod aspect ratio), $HAuCl_4$ (0.01 M, 0.2 mL), and ascorbic acid (0.1 M, 0.032 mL). Nanorods were purified by several cycles of suspension in ultrapure water, followed by centrifugation. Nanorods were isolated in the precipitate, and excess CTAB was removed in the supernatant. Nanorods were characterized by TEM and absorption spectroscopy as shown in Fig. 1.

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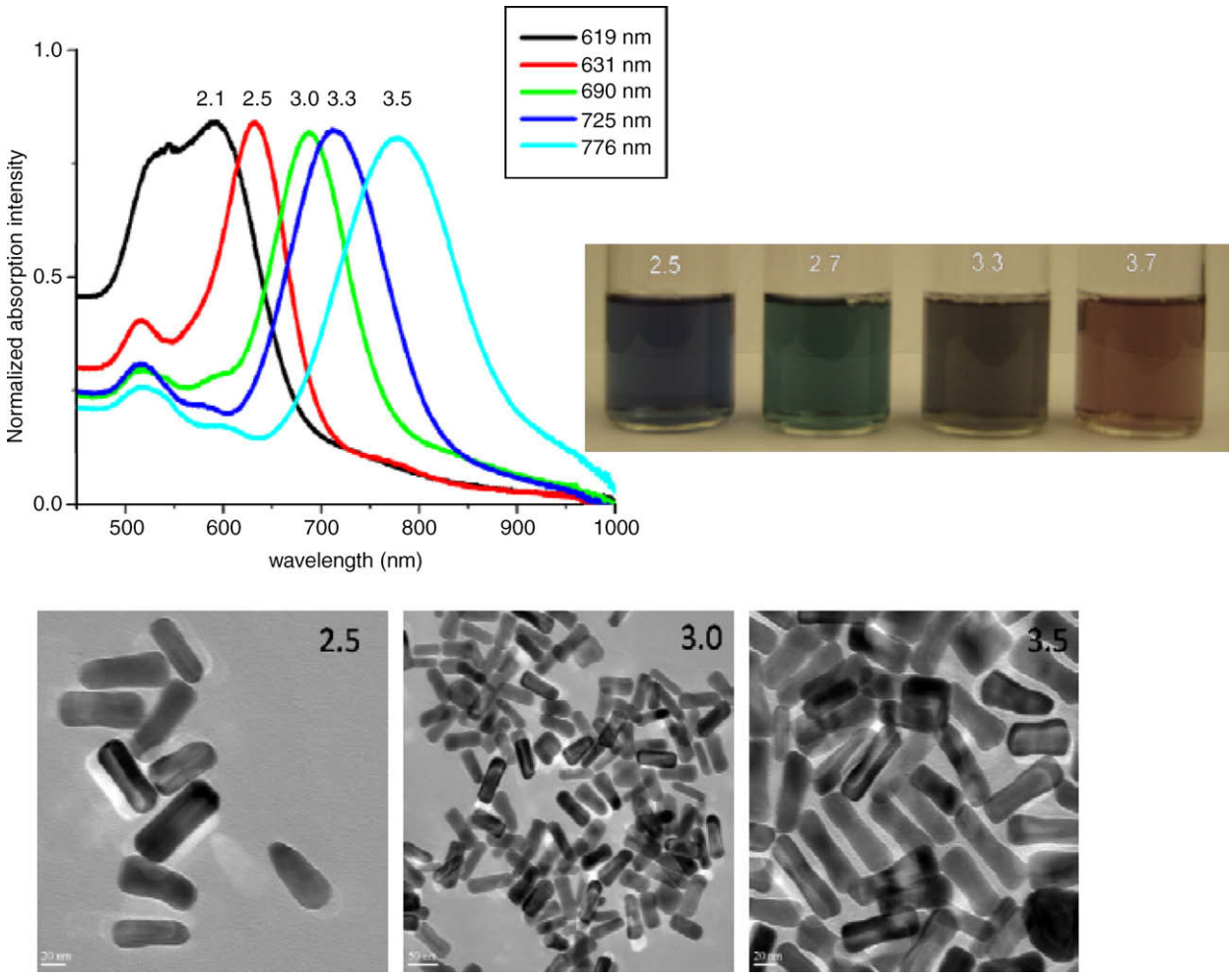


**Fig. 1.** Absorption spectra, photograph and TEM images of gold nanorods of different aspect ratios.

To explore the cytotoxicity of gold nonmaterials, we have used human skin cell line, HaCaT keratinocytes, a transformed human epidermal cell line obtained from Dr. Norbert Fusening of the germany cancer research center, Heidelberg, Germany. The HaCaT cells were grown in Dulbecco's Modified Eagle's Medicum (DMEM) with 10% FBS in 25 $cm^2$ culture flasks cultured in a humidified incubator with 5% $CO_2$ at 37 °C. After cells grew to the desired concentration, they were centrifuged at 2000 rpm for 5 min. The cells were washed twice with 1 × PBS and resuspended in DMEM to reach a final cell concentration of $1 \times 10^5$ cells/mL for further treatment. After treating the cells with gold nanomaterials at different time intervals, the cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Viable cells are capable of metabolizing MTT while dead cells are not. 10 μL of nanomaterial were added in 90 μL cell media and incubated for 24 h at 37 °C with 5% $CO_2$. After treatment, the medium was removed and the culture wells were washed in PBS buffer. Then we added 50 μL of 5 mg/mL MTT solution and incubated for 60 min. The cells were then centrifuged and the suspension was discarded. 200 μL DMSO was added to each well and incubated for 10 min. The absorbance at 540 nm was recorded using Multiskan Ascent Plate Reader with the Ascent software (Labsystems).

## 3. Results and discussion

The cell viabilities for gold nanoparticles of different sizes are shown in Fig. 2. Chithrani et. al. [29] have measured the intracellular uptake of different sizes and shapes of colloidal gold nanoparticles. Their results show that gold nanoparticles and nanorods are capable of entering the cells. The cellular uptake increases with incubation time until it is saturated at 8 h.

To make sure that cellular uptake reaches maximum, all our results are for 24 h incubation. As shown in Fig. 2A, no difference was found in cell viability between cell with and without gold nanoparticles of different sizes. This indicates that spherical gold nanoparticles of different sizes are not inherently toxic to human cells at our test concentration. To further confirm this, we also continuously exposed the cell for 48 h and no toxicity was observed. Connor et al. [28] and Takahasi et al. [30] have shown that nanoparticles or nanorods are usually clustered inside the cell. They concluded that cell death might be the result of a harmful influence by the aggregates. To understand whether gold nanomaterials are only aggregated inside the cell or may be aggregated outside the cell and then entered as aggregate, we did a time dependence study of gold nanoparticle's surface plasmon resonance band (SPB) between 510–570 nm. The SPB origin is attributed to the collective oscillation of the free conduction electrons induced by an interacting electromagnetic field. These resonances are also denoted as surface plasmon's. Our experiments indicate that the shift in plasmon band energy to longer wavelengths by about 150–400 nm (Fig. 2B) in the presence of DMEM, which indicated strong aggregation of gold nanoparticles (TEM image, Fig. 2C). This aggregation is due to the presence of high concentration of sodium salt in DMEM. To understand whether other compo-

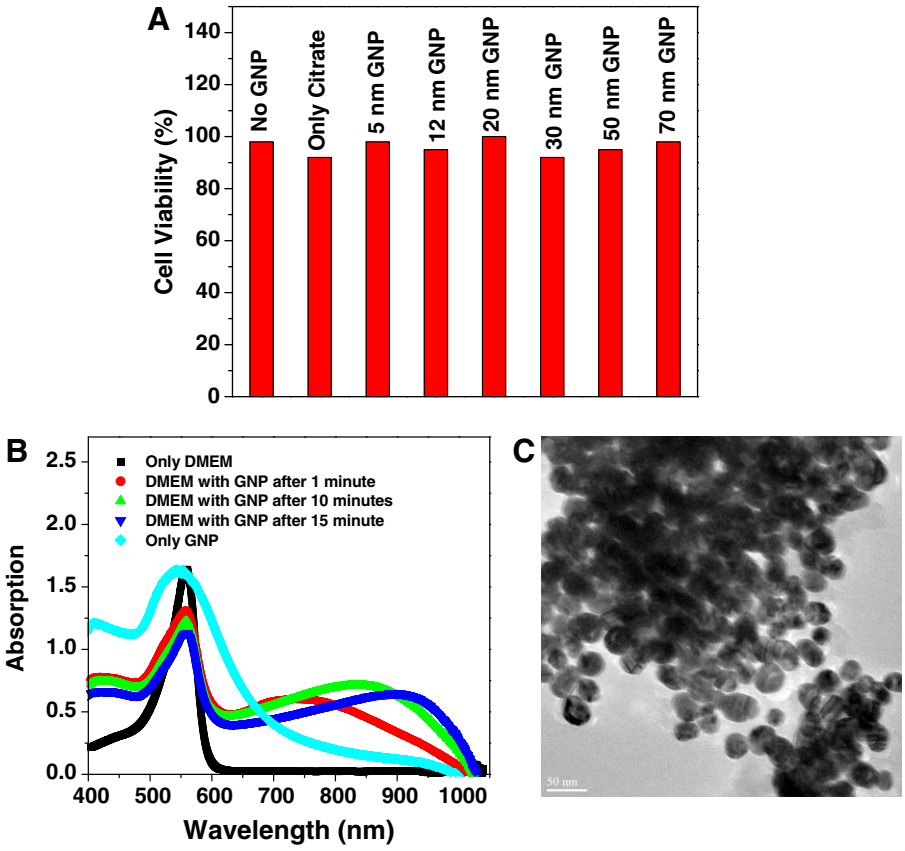


**Fig. 2.** (A) Viability of cells incubated with gold nanoparticle of different sizes. (B) Time dependent absorption change in the presence of DMEM of 30 nm gold nanoparticles. (C) TEM image for gold nanoparticle in the presence of cell media.

nents of DMEM media are also responsible for aggregation, we performed aggregation experiment with simulated media without sodium salt. We have not observed any aggregation even after 24 h. Even experiments with different sodium salt concentration indicate that for simulated media with sodium salt concentrations less than 0.1 M, there was no aggregation. Our experiments show that the plasmon band shifted to longer wavelengths with time, indicating bigger cluster formation. Therefore, our results show that nanoparticles aggregated outside the cell and may enter the cell as aggregated form. It is known that unique properties of nanoparticles change as it forms bigger clusters and, as a result, our experiments indicate that it is really challenging to study the gold nanoparticle toxicity individually in cell media.

Fig. 3A shows the cell viabilities for gold nanorods of aspect ratio 3. Our experiment indicates that gold nanorod is highly toxic. It was surprising that gold nanoparticles are not toxic, whereas gold nanorods exhibit very high toxicity, though gold nanorods are synthesized from gold nanoparticles of small size using a seed-mediated, surfactant-assisted growth method. CTAB is a unique surfactant used for the synthesis of nanorods and is widely used. CTAB selectively forms a tightly packed bilayer on the side faces of nanorods, which leads the ends of the rods to be more exposed to facilitate anisotropic growth along the longitudinal axis. Since the extra chemical present in gold nanorod that is not in gold nanoparticle is CTAB, we studied the toxicity of CTAB (Fig. 3A).

Our results indicate that CTAB alone shows similar toxicity at the concentration of 100 μM. Concentration dependent study shows that CTAB is toxic even at 10 μM concentration (data not shown in Fig. 3A). In gold nanorods, there are nanorod-bound CTAB as well as excess free CTAB in solution, which have not been used during nanorod formation. The excess CTAB left in the solution can be removed by centrifugation. Our data indicate that cell viability increases with centrifugation. As we discussed before, we started with 0.1 M, 4.75 ml, CTAB for gold nanorod synthesis. After nanorod formed, the total volume was 120 ml (CTAB diluted 25 times). To remove the excess CTAB from the solution, we centrifuged several times. For each centrifugation, we have diluted nanorod solution 10 times. So after three centrifugations, we have diluted CTAB by 25 000 times. Since we do not know how much CTAB has been used for nanorod formation, we estimated that maximum CTAB in solution is <0.1 μM. CTAB-coated nanorods are positively charged. Their mutual repulsion prevents aggregation so that gold nanorods are stable in solution even for several months.

As shown in Fig. 3A, 1 μM CTAB is not toxic. Therefore, we do not expect toxicity after three centrifugations. Our experimental data indicate there is 40% cell death even after three centrifugations (Fig. 3A). We believe this is due to the fact that gold nanorod-bound CTAB layer will eventually enter solution in the presence of cell media due to aggregation of nanorods as shown in Fig. 3B and C. This is due to the screening effect of the sodium salts present in DMEM, leading to more linked particles and hence larger damping of the surface plasmon absorption of Au nanorod surfaces. The aggregated gold nanorods in cell media make it difficult to monitor their toxicity. We tried to remove further CTAB from the nanorod-bound CTAB layer by performing 4–5 times centrifugation, and our experimental results shows that nanorods are not stable if CTAB is removed further by centrifuging it more than 3 times. We believe that this is due to the lack of repulsive interaction among individual nanorods and, as a result, nanorods break to nanoparticles and some nanorods undergo aggregation.

To protect from CTAB which is bound with gold nanorod, we have exposed the nanoparticles to poly(styrenesulfonate) (PSS)

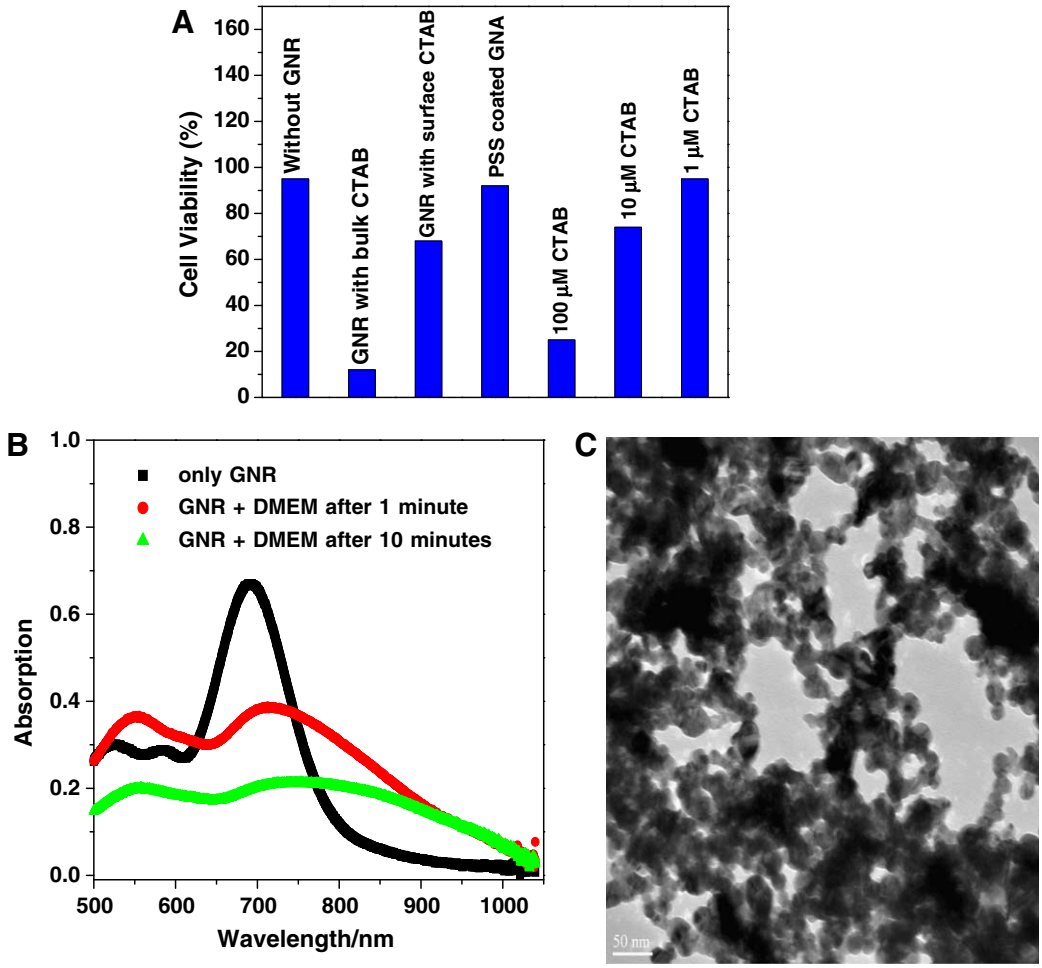


**Fig. 3.** (A) Viability of cells incubated with gold nanorod (GNR, aspect ratio 3.0) with CTAB, only CTAB and PSS coated GNR. (B) Time dependent absorption change in the presence of DMEM for gold nanorod with aspect ratio 3.0. (C) TEM data for gold nanorod in presence of cell media.

polyelectrolyte solution. The extra PSS in solution was separated by centrifugation of the nanorod solution at 8000 rpm. The pellet was redispersed in *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) solution. The nanorods prepared by seed-mediated surfactant (CTAB) methods are positively charged. Our zeta potential measurement using Zetasizer NanoZS shows that positively charged surface (Zeta potential 58.7 mV) of the nanorods changed to a negatively charged surface (Zeta potential −69.8 mV) when exposed to PSS. Absorption spectra measurement indicate that $\lambda_{max}$ remains about same after PSS coating, which indicate there are no aggregation during coating process, which is also confirmed by TEM experiment. Our TEM data indicated that the PSS coated outside the CTAB bilayer (as shown in Fig. 4A).

Fig. 4 shows the cell viability for PSS-coated gold nanorods with different aspect ratios. Our experiment indicates that PSS coated gold nanorod is not toxic. Therefore, the toxicity of gold nanorod

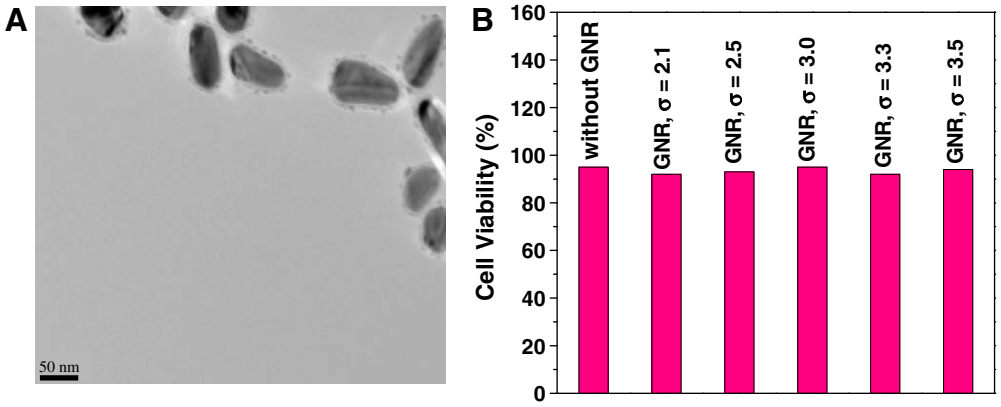


**Fig. 4.** (A) TEM data for PSS-coated gold nanorod. (B) Viability of cells incubated with PSS-coated gold nanorod (3.2 aspect ration) of different aspect ratios.

is due to the presence of CTAB and replacing CTAB with biocompatible and functionalization friendly stabilizing agents like PSS is essential for the use of gold nanorod in living cells.

In conclusion, the cytotoxicity of gold nanomaterials of different sizes and shapes is explored in this letter. Our data demonstrated that spherical gold nanoparticles of different sizes are not inherently toxic to human skin cells, but gold nanorods are highly toxic due to the presence of CTAB as coating material. However, further PSS coated gold nanorods coated with CTAB is not toxic. So coating CTAB with biocompatible and functionalization friendly stabilizing agents is essential for using of gold nanorods in living cells. Our result points out that it is difficult to understand the cytotoxicity of gold nanomaterials individually, due to the presence of CTAB in gold nanorod as well as aggregation of gold nanomaterial in the presence of cell media.

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## References

[1] S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L. Vander Elst, R. Muller, Chem. Rev. 108 (2008) 2064.
[2] N.L. Rosi, C.A. Mirkin, Chem. Rev. 105 (2005) 1547.
[3] A. Kumar, P.K. Vemula, P.M. Ajayan, G. John, Nature Mater. 7 (2008) 236.
[4] W. Jiang, B.Y.S. Kim, J.T. Rutka, W.C.W. Chan, Nature Nanotechnology 3 (2008) 145.
[5] A.P. Alivisatos, Nat. Biotechnol. 22 (2004) 47.
[6] J. Xiang, W. Lu, Y. Hu, Y. Wu, H. Yan, C.M. Lieber, Nature 441 (2006) 441.
[7] J.M. Nam, C.S. Thaxton, C.A. Mirkin, Science 301 (2003) 301, 1884.
[8] X. Gao, Y. Cui, R.M. Levenson, L.W.K. Chung, S. Nie, Nat. Biotechnol. 22 (2004) 969.
[9] G.K. Darbha, A.K. Singh, U.S. Rai, E. Yu, H. Yu, P.C. Ray, J. Am. Chem. Soc. 130 (2008) 8038.
[10] G.K. Darbha, U.S. Rai, A.K. Singh, P.C. Ray, Chem. Eur. J. 14 (2008) 3896.
[11] S.R. Dasary, U.S. Rai, H. Yu, Y. Anjaneyulu, M. Dubey, P.C. Ray, Chem. Phys. Lett., ASAP Article. 2008.
[12] P.C. Ray, Angew. Chem. 45 (2006) 1151.
[13] G.K. Darbha, A. Ray, P.C. Ray, ACS Nano. 1 (2007) 208.
[14] V.S. Tiwari, T. Oleg, G.K. Darbha, W. Hardy, J.P. Singh, P.C. Ray, Chem. Phys. Lett. 446 (2007) 77.
[15] T.L. Jennings, M.P. Singh, G.F.J. Strouse, Am. Chem. Soc. 128 (2006) 5462.
[16] P.C. Ray, A. Fortner, G.K. Darbha, J. Phys. Chem. B. 110 (2006) 20745.
[17] T.L. Jenning, J.C. Schlatterer, M.P. Singh, N.L. Greenbaum, G.F. Strouse, Nano Lett. 6 (2006) 1318.
[18] D.S. Seferos, D.A. Giljohann, D.H. Hill, A.E. Progodich, C.A. Mirkin, J. Am, Chem. Soc. 129 (2007) 15477.
[19] L.R. Skewis, M.B. Reinhard, Nano Lett. 8 (2008) 208.
[20] X. Huang, I.H. El-Sayed, W. Qian, M.A.J. El-Sayed, Am. Chem. Soc. 128 (2006) 2115.
[21] A.J. Bonham, G. Braun, I. Pavel, M. Moskovits, N.O. Reich, J. Am. Chem. Soc. 129 (2007) 14572.
[22] S. Lan, Y. Chenxu, I. Joseph, Ananl. Chem. 80 (2008) 3342.
[23] X. Huang, I.H. El-Sayed, W. Qian, M.A. El-Sayed, Nano Lett. 7 (2007) 1591.
[24] G.H. Chan, J. Zhao, E.M. Hicks, G.C. Schatz, R.P. Van Duyne, Nano Lett. 7 (2007) 1947.
[25] N. Lewinski, V. Colvin, R. Drezek, Small 4 (2008) 26.
[26] T.S. Haunk, A.A. Chazani, W.C.W. Chan, Small 4 (2008) 153.
[27] T.B. Huff, M.N. Hansen, Y. Zhao, Ji.-X. Cheng, A. Wei, Langmuir 23 (2007) 1596.
[28] E.E. Connor, J. Mwamuka, A. Gole, C.J. Murphy, M.D. Wyatt, Small 1 (2005) 325.
[29] B.D. Chithrani, A.A. Ghazani, W.C.W. Chan, Nano Lett. 6 (2006) 662.
[30] H. Takahashi, Y. Niidome, T. Niidome, K. Kaneko, H. Kawasaki, S. Yamada, Langmuir 22 (2006) 2.