Published in final edited form as:

Angew Chem Int Ed Engl. 2010 March 8; 49(11): 1976–1980. doi:10.1002/anie.200906584.

A Simple Spectroscopic Method for Differentiating Cellular Uptakes of Gold Nanospheres and Nanorods from Their Mixtures**

Dr. Eun Chul Cho, Yang Liu, and Prof. Younan Xia

Department of Biomedical Engineering, Washington University, St. Louis, MO 63130, USA

Younan Xia: xia@biomed.wustl.edu

Abstract

This paper presents a simple spectroscopic method for differentiating cellular uptakes of two different types of Au nanostructures (i.e., nanospheres and nanorods) when they are supplied as a mixture. Because Au nanospheres and nanorods have distinctive surface plasmon resonance peaks, we can simultaneously measure their concentrations in a culture medium by UV-Vis spectroscopy. For the uptake of Au nanostructures by SK-BR-3 breast cancer cells, we found that the surface functional group had a stronger influence than the shape. We also found that the cellular uptake of one type of Au nanostructure could be affected by the presence of another type of nanostructure in the medium. This study not only suggests a simple way to compare the cellular uptakes of more than one type of Au nanostructures without incubating them separately but also allows one to investigate the influence of one type of Au nanostructures on the uptake of another type.

Keywords

cellular uptake; gold nanospheres; gold nanorods; spectroscopic method

Owing to their unique optical (scattering and absorption) properties, Au-based nanostructures have recently been applied to many applications related to biomedical research.^[1] For example, Au nanostructures have been demonstrated as either contrast agents for optical imaging (and therefore cancer diagnosis)^[2–6] or therapeutic agents for photothermal ablation of cancer by making use of the scattering or absorption component.^[7–9] In addition, Au nanostructures have been exploited as probes or carriers to evaluate the delivery efficacy of nanomedicine into target cells or tissues.^[8–11] This is because Au nanostructures of various sizes and shapes can now be synthesized in relatively large quantities; their surfaces can be readily derivatized with different functional groups through the well-established Au-thiolate chemistry; and the content of Au in a specific amount of cells or tissue can be accurately determined with high sensitivity using inductively-coupled plasma mass spectrometry (ICP-MS).^[10,11] However, ICP-MS simply cannot differentiate different types of Au nanostructures in a mixture. As a result, one has to conduct a large number of *in vitro* or *in vivo* experiments separately when one wants to

**This work was supported in part by an NIH Director's Pioneer Award (5DP1 OD000798) and startup funds from Washington University in St. Louis. E.C.C. was also partially supported by a fellowship from the Korea Research Foundation (KRF-2007-357-D00070) funded by the Korean Government. Part of the work was performed at the Nano Research Facility (NRF), a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the NSF under award ECS-0335765. The NRF is a part of the School of Engineering and Applied Science at Washington University in St. Louis.

Correspondence to: Younan Xia, xia@biomed.wustl.edu.

Supporting information for this article is available on the WWW under <http://dx.doi.org/>.

compare Au nanostructures with different sizes, shapes, and/or surface chemistries. Since cell culture or animal studies are highly sensitive to experimental conditions, doing experiments separately (even side by side) are expected to introduce errors. On the other hand, one has to mix different types of Au nanostructures together in order to observe any possible interference between the uptakes of different nanostructures. In these regards, it will be a great advantage to supply different types of Au nanostructures as a mixture and then follow their cellular uptakes simultaneously in the same experiment.

Here we introduce a spectroscopic method for simultaneously evaluating cellular uptakes of two different types of Au nanostructures from their mixtures, and thus to compare their uptakes without incubating them independently with cells (Figure 1a). We chose Au nanospheres and nanorods to compare the uptakes of Au nanostructures with different shapes by a breast cancer cell line, SK-BR-3. Because Au nanospheres and nanorods have distinctive optical signatures, one can take advantage of these differences to conveniently and simultaneously measure their individual concentrations in a solution using UV-Vis absorbance spectroscopy. Therefore, by culturing cells with a medium containing a mixture of Au nanospheres and nanorods, as well as modified with various ligands, one can decipher the role of shape on cellular uptake and investigate the interplay between two different types of nanostructures in a cellular uptake process.

We synthesized Au nanospheres of 17.1 ± 1.6 nm in diameter by reducing HAuCl_4 with sodium citrate (Figure 1b). We followed the protocol by Nikoobakht and El-Sayed for the preparation of Au nanorods that were 20.0 ± 2.9 nm and 50.0 ± 4.0 nm in width and length, respectively (Figure 1c).^[12] Note that the diameter of nanospheres was approximately the same as the width of nanorods to single out the role of shape (or aspect-ratio). The surfaces of the as-prepared Au nanospheres and nanorods were covered by citrate ions and hexadecyltrimethylammonium bromide (CTAB), respectively. It is worth pointing out that at least three rounds of repeated washing and centrifugation were required in order to achieve over 90% cell viability for the Au nanorods; otherwise the cell viability would drop considerably as a result of the CTAB remaining in the dispersion medium.^[11c] We also modified the surfaces of the Au nanostructures with poly(ethylene glycol) (PEG) and antibodies in an effort to resolve the effect of shape on cellular uptake while the surface functional groups were the same. For PEG modification, methoxy-terminated PEG-thiol (mPEG-SH, Mw 5,000) was added to a dispersion of the Au nanostructures, followed by incubation for 12 h and removal of the unreacted PEG-thiol by centrifugation. We selected anti-HER2 as the targeting antibody because SK-BR-3 cells are known to overexpress HER2 receptors on the surface. The surface modification involved two steps: succinimidyl propionyl PEG disulfide (Mw 5,000) was first grafted to the surface of the Au nanostructures, followed by the addition of anti-HER2. Strictly speaking, “anti-HER2” actually represents “anti-HER2-PEG”. As for cell culture medium, we chose Dulbecco's Modified Eagle's Medium (DMEM) without phenol red because phenol red showed a strong absorption at 550 nm, which overlaps with the absorbance peaks of the Au nanostructures (see Figure S1). We recorded UV-Vis absorbance spectra of the Au nanostructures dispersed in the culture medium after they had been incubated at 37 °C under 5% CO_2 for 24 h (Figure 1, d and e). The spectra for the Au nanospheres with three types of surface groups did not show much difference after the surface modification and incubation steps. By contrast, the spectra from the Au nanorods were slightly changed by these treatments, probably due to some minor etching. Therefore, for every mixture of Au nanospheres and nanorods we prepared, we had to obtain a calibration curve in order to precisely determine the amount of Au nanospheres and nanorods taken up by cells from the mixture.

We prepared three types of mixtures (see the table in Figure 1a): *i*) the as-prepared, with the surface covered by citrate for Au nanospheres and CTAB for Au nanorods; *ii*) the PEG-

modified; and iii) the anti-HER2 conjugated. Each mixture contained 60 pM (particle concentration) of nanospheres and nanorods, respectively. At this relatively low concentration, all types of mixtures were found to be not toxic to the cells (see Figure S2), where over 90% of the cells remained viable after incubation for 24 h relative to the control experiment conducted in the absence of Au nanostructures.

We first obtained separate calibration curves for the Au nanospheres and nanorods from their mixtures. Figure 2a shows atypical example of the spectra, whose absorbance peaks can be used to calculate the concentrations of the as-prepared Au nanospheres and nanorods in the mixture. As shown in Figure 1e, the as-prepared Au nanorods had longitudinal and transversal peaks at 652 and 515 nm, respectively. Since the longitudinal peak of the Au nanorods did not overlap with the peak of the nanospheres (523 nm), we could easily obtain a calibration curve for the Au nanorods from the absorbance (A_{rods}) at 652 nm for the mixture. However, there is an overlap between the peak of Au nanospheres and the transverse peak of the Au nanorods. We have to obtain the absorbance of the Au nanospheres (A_{spheres}) by subtracting the absorbance of nanorods from the total absorbance at 515 nm recorded for the mixture (A_{mix}), that is, $A_{\text{spheres}} = A_{\text{mix}} - A_{\text{rods}}/R$. Here R is the ratio (a fixed number for a specific sample) of the absorbance at 652 nm to that at 515 nm for the nanorods, which could be obtained from the UV-Vis spectrum of pure Au nanorods (Figure 1e). Using this procedure, we obtained calibration curves for the as-prepared Au nanostructures with quite good linearity ($R^2=0.99$ for both types of nanostructures, Figure 2b). Similarly, we obtained calibration curves for samples of PEG- and anti-HER2-modified Au nanospheres and nanorods in the form of mixtures (Figure S3).

We then conducted cell uptake studies with the mixtures of Au nanospheres and nanorods. In a typical process, 1.0 mL of culture medium containing a mixture of Au nanospheres and nanorods (60 pM for each, on the basis of particles) was incubated with the SK-BR-3 cells at 37 °C in a 12-well culture plate. After 24 h, we removed the medium from the culture plate and added 0.5 mL of fresh medium (without Au nanostructures) to wash off the loosely-bound Au nanostructures on the cell surface. We once again removed the medium from the well and repeated the previous step. We recorded UV-Vis spectra of the media at every step (Figure 3): before incubation (denoted *before*), after incubation (denoted *after*), and for the combination of all the media after incubation and the two washing media (denoted *after + wash*). For the combination of incubation medium and two washing media, the concentration of Au nanostructures in the media was lowered as a result of dilution. For this reason, we normalized these spectra by considering the added volume (1 mL) of the washing media. From the figures, it is clear that the peak intensities of the media were reduced after incubation, but the degree of decrease was dependent upon the ligands attached to the Au nanostructures. In addition, the peak intensities of the media after washing steps were a little bit higher than those without washing steps, indicating that some loosely-bound Au nanostructures were removed from the cell surfaces during the washing.

Based on the spectra in Figure 3 and the calibration curves in Figure 2b, we could quantify the uptakes of Au nanostructures by the SK-BR-3 cells for the three types of mixtures (Figure 4a). We also determined the number of Au nanospheres and nanorods when they were independently incubated with the cells under similar conditions (Figure 4b, and Figures S4-S6). Before discussing the results, it is necessary to demonstrate the reliability of this spectroscopic method. For this purpose, the data obtained from the UV-Vis spectroscopic measurements were compared with the data determined by ICP-MS, where the Au nanospheres and nanorods were incubated separately with the cells (see Table S1). Although the absolute values from the UV-Vis measurements were 1.1–3.5 times higher than those from ICP-MS, we found similar trends in the uptake of the Au nanostructures from both

methods. This proves that the spectroscopic method, in addition to ICP-MS, could become a useful tool for quantifying the cellular uptakes of the Au nanostructures.

From Figure 4, we found that the uptake of the as-prepared Au nanospheres was influenced by the mode of incubation. When the as-prepared Au nanospheres were incubated together with the as-prepared Au nanorods, their uptake by the cells were decreased significantly, compared with the uptake in the case of being incubated independently. In contrast, for the PEG- and anti-HER2 modified Au nanostructures, there was no significant difference between the modes of incubation in both the absolute values and the trends of uptake by the cells. This result implies that, depending on the surface chemistry, the uptake of Au nanospheres could be interfered by nanorods when the two types of nanostructures are supplied as a mixture. In addition, we did not observe any trend relating the shape of Au nanostructures to their uptake by the cells regardless of incubation mode, indicating the surface chemistry might be a more important factor affecting the uptake of Au nanostructures.^[13]

From the viewpoint of surface chemistry, it is worth pointing out that the citrate-stabilized, as-prepared Au nanospheres (negatively charged) were taken up by the cells similarly to the CTAB-stabilized Au nanorods (positively charged) when they were incubated independently. Seeking explanations for this result, we further investigated the surface charges of the Au nanostructures after they were incubated in the culture medium at 37 °C for 1 h (see Table S2). Most importantly, the surface charges of the as-prepared Au nanorods significantly changed from positive (+20 mV) to negative values (−16 mV), indicating that the CTAB on the as-prepared Au nanorods was probably displaced by the serum proteins in the medium. It is possible that the physically bound stabilizers (CTAB and citrate) could be displaced by ligands that could bind to the Au nanostructures more strongly.^[11c,14] The surface charges of citrate-stabilized nanospheres were not changed substantially upon suspension in the culture medium; this might be mainly due to the displacement of citrate by similarly-charged proteins.^[15] Chithrani *et al.* also suggested that the citrate-stabilized Au nanospheres could be modified by serum proteins, which could induce the uptake of the Au nanospheres by cells via receptor-mediated endocytosis.^[10c] Taken together, the similar cellular uptake of as-prepared Au nanospheres to that of the as-prepared nanorods might be mainly due to their surfaces being modified with the serum proteins in the medium. However, it is still not clear why the as-prepared Au nanospheres were suppressed in their uptake when they co-existed with the as-prepared Au nanorods. We speculate that, for some reasons such as difference in binding affinity for the two nanostructures, the as-prepared Au nanorods first occupy the surface of the cells, thereby preventing the subsequent uptake of Au nanospheres. The surface charges of PEG- and anti-HER2-modified Au nanostructures were also influenced by serum proteins. However, the proteins are assumed to bound loosely to the chemically bonded ligands due to steric repulsion,^[16] and probably do not influence the uptake of these Au nanostructures by the cells.

In summary, we have demonstrated that the spectroscopic method could be very useful in differentiating the uptakes of Au nanospheres and nanorods when they were supplied as mixtures. Utilizing the different optical signatures of these two types of Au nanostructures, we could single out the cellular uptake of one particular type of Au nanostructure from the mixture. We found that the uptake of Au nanostructures by the cells had a stronger dependence on the surface ligand than on the shape of the nanostructures. In addition, depending on their surface chemistries, the uptake of one Au nanostructure could be influenced by the other kind of Au nanostructure in a mixture. This study should shed some light on the delivery mechanisms of nanoparticle-based carriers when more than two types of carriers are mixed together. In addition, we suggest that this method can provide a simple

way for quantifying the uptake of Au (and other materials) nanostructures by cells without using a specialized facility such as ICP-MS.

Experimental Section

Procedures for the synthesis of Au nanostructures, surface modification, and cell culture are described in the supporting information.

References

1. a) Jain PK, Huang X, El-Sayed IH, El-Sayed MA. *Acc Chem Res.* 2008; 41:1578. [PubMed: 18447366] b) Skrabalak SE, Chen J, Sun Y, Lu X, Au L, Cobley CM, Xia Y. *Acc Chem Res.* 2008; 41:1587. [PubMed: 18570442] c) Murphy CJ, Gole AM, Stone JW, Sisco PN, Alkilany AM, Goldsmith EC, Baxter SC. *Acc Chem Res.* 2008; 41:1721. [PubMed: 18712884]
2. El-Sayed MA. *Acc Chem Res.* 2001; 34:257. [PubMed: 11308299]
3. a) Mohamed MB, Volkov V, Link S, El-Sayed MA. *Chem Phys Lett.* 2000; 317:517. b) Hu M, Chen J, Li ZY, Au L, Hartland GV, Li X, Marqueze M, Xia Y. *Chem Soc Rev.* 2006; 35:1084. [PubMed: 17057837]
4. a) Métraux GS, Cao YC, Jin R, Mirkin CA. *Nano Lett.* 2003; 3:519. b) Skrabalak SE, Au L, Li X, Xia Y. *Nat Prot.* 2007; 2:2182. c) Siekkinen AR, McClellan JM, Chen J, Xia Y. *Chem Phys Lett.* 2006; 432:491. [PubMed: 18496589]
5. a) Zagaynova EV, Shirmanova MV, Kirillin MY, Khlebtsov BN, Orlova AG, Balalaeva IV, Sirotkina MA, Bugrova ML, Agrba PD, Kamensky VA. *Phys Med Biol.* 2008; 53:4995. [PubMed: 18711247] b) Chen J, Saeki F, Wiley BJ, Cang H, Cobb MJ, Li ZY, Au L, Zhang H, Kimmey MB, Li XD, Xia Y. *Nano Lett.* 2005; 5:473. [PubMed: 15755097] c) Alric C, Taleb J, Duc GL, Mandon C, Billotey C, Meur-Herland AL, Brochard T, Vocanson F, Janier M, Perriat P, Roux S, Tillement O. *J Am Chem Soc.* 2008; 130:5908. [PubMed: 18407638]
6. a) Kim K, Huang SW, Ashkenazi S, O'Donnell M, Agarwal A, Kotov NA, Denny MF, Kaplan MJ. *Appl Phys Lett.* 2007; 90:223901. b) Agarwal A, Huang SW, O'Donnell M, Day KC, Day M, Kotov M, Ashkenazi S. *J Appl Phys.* 2007; 102:064701. c) Yang X, Skrabalak SE, Li ZY, Xia Y, Wang LV. *Nano Lett.* 2007; 7:3798. [PubMed: 18020475] d) Song KH, Kim C, Cobley CM, Xia Y, Wang LV. *Nano Lett.* 2009; 9:183. [PubMed: 19072058]
7. a) Adler DC, Huang SW, Huber R, Fujimoto JG. *Opt Exp.* 2008; 16:4376. b) van Dijk MA, Tchegobtareva AL, Orrit M, Lippitz M, Berciaud S, Lasne D, Cognete L, Lounisc B. *Phys Chem Chem Phys.* 2006; 8:3486. [PubMed: 16871337]
8. a) Li JL, Day D, Gu M. *Adv Mater.* 2008; 20:3866. b) Au L, Zheng D, Zhou F, Li Z-Y, Li X, Xia Y. *ACS Nano.* 2008; 2:1645. [PubMed: 19206368] c) Chen J, Wang D, Xi J, Au L, Siekkinen A, Warsen A, Li ZY, Zhang H, Xia Y, Li X. *Nano Lett.* 2007; 7:1318. [PubMed: 17430005]
9. a) Hauck TS, Jennings TL, Yatsenko T, Kumaradas JC, Chan WCW. *Adv Mater.* 2008; 20:3832. b) Sonavane G, Tomoda K, Makino K. *Coll Sur B.* 2008; 66:274. c) Ghosh P, Han G, De M, Kim CK, Rotello VM. *Adv Drug Del Rev.* 2008; 60:1307. d) Rosi NL, Mirkin CA. *Chem Rev.* 2005; 105:1547. [PubMed: 15826019] e) Ghosh PS, Kim CK, Han G, Forbes NS, Rotello VM. *ACS Nano.* 2008; 2:2213. [PubMed: 19206385]
10. a) Chithrani BD, Chan WCW. *Nano Lett.* 2007; 7:1542. [PubMed: 17465586] b) Jiang W, Kim BYS, Rutka JT, Chan WCW. *Nat Nanotech.* 2008; 3:145. c) Chithrani BD, Ghazani AA, Chan WCW. *Nano Lett.* 2006; 6:662. [PubMed: 16608261] d) Xu C, Tung GA, Sun S. *Chem Mater.* 2008; 20:4167. [PubMed: 19079760]
11. a) Cho EC, Xie J, Wurm PA, Xia Y. *Nano Lett.* 2009; 9:1080. [PubMed: 19199477] b) Hauck TS, Ghazani AA, Chan WCW. *Small.* 2008; 4:153. [PubMed: 18081130] c) Alkilany AM, Nagaria PK, Hexel CR, Shaw TJ, Murphy CJ, MDW. *Small.* 2009; 5:701. [PubMed: 19226599]
12. Nikoobakht B, El-Sayed MA. *Chem Mater.* 2003; 15:1957.
13. Cho EC, Au L, Zhang Q, Xia Y. *Small.* in press. 10.1002/smll.200901622
14. Ohnuma A, Cho EC, Jiang M, Ohtani B, Xia Y. *Langmuir.* 2009; 25:13880. [PubMed: 19621917]

15. a) Peters T Jr. Adv Protein Chem. 1985; 37:161. [PubMed: 3904348] b) Aisen P, Leibman A, Reich HA. J Biol Chem. 1966; 241:1666. [PubMed: 5945842]
16. a) Mcpherson, TB.; Lee, SJ.; Park, K. Proteins at Interfaces II. Horbett, TA.; Brash, JL., editors. American Chemical Society; Washington DC: 1995. p. 396b) Feldman K, Halhner G, Spencer ND, Harder P, Grunze M. J Am Chem Soc. 1999; 121:10134.c) Sigal GB, Mrksich M, Whitesides GM. J Am Chem Soc. 1998; 120:3464.

Figure 1.

a) A schematic showing experiments for the cellular uptake of Au nanospheres and nanorods supplied as a mixture. The surface of the Au nanostructures was derivatized with various chemical groups. We conducted the cell experiments with three types of mixtures: i) As-prepared, with the surface covered by citrate for nanospheres and CTAB for nanorods; ii) PEG-modified; iii) and anti-HER2-PEG (anti-HER2 for short) modified. b, c) TEM images of Au nanospheres and nanorods, respectively. d, e) UV-Vis spectra taken from suspensions of d) Au nanospheres and e) Au nanorods with various chemical groups on the surface. The nanostructures (60 pM) were suspended in DMEM, and the spectra were taken after storage at 37 °C under 5% CO₂ for 24 h. Abbreviations: CTAB, hexadecylammonium bromide; and PEG, poly(ethylene glycol).

Figure 2.

a) UV-Vis spectra of the as-prepared Au nanorods (dashed line) and a mixture of the as-prepared Au nanospheres and nanorods (solid line) dispersed in the cell culture medium. The concentration of the Au nanorods was 60 pM, and the mixture contained 60 pM of nanospheres and nanorods, respectively. b) Calibration curves of the as-prepared Au nanospheres and nanorods as a function of concentration. The plots were obtained from the UV-Vis spectra of the mixture. The absorbance at 652 nm was used for the Au nanorods (A_{rods}), while the absorbance at 515 nm was used for the Au nanospheres (A_{spheres}), by subtracting the absorbance due to the Au nanorods: $A_{\text{spheres}} = A_{\text{mix}} - A_{\text{rods}}/R$. Here A_{mix} is the absorbance of mixture at 515 nm, and R is the ratio of the absorbance at 652 nm to that at 515 nm shown in the UV-Vis spectra for Au nanorods (see Figure 1e).

Figure 3.

UV-Vis spectra of the culture media containing a mixture of Au nanospheres and nanorods (60 pM of each). Three types of mixture were used for the cell culture: a) as-prepared, b) PEG-modified, and c) anti-HER2 modified. We recorded spectra from the media before (denoted with *before*) and after incubating the media into the SK-BR-3 cells at 37 °C for 24 h (denoted with *after*). After incubation, the culture medium was removed from the well, and 0.5 mL of fresh medium was added to remove the loosely-bound Au nanostructures on the cell surface. This washing process was repeated one more time. We combined all the media (incubating medium + two washing media), and recorded UV-Vis spectrum (denoted with *after+wash*). We normalized the spectra for *after+wash* by considering the volume (1 mL) of the washing media.

Figure 4.

a) Uptakes of nanospheres and nanorods by SK-BR-3 cells when the Au nanospheres and nanorods were mixed (60 pM for each type) and then incubated with the cells at 37 °C for 24 h. b) Uptakes of nanospheres and nanorods by SK-BR-3 cells when the Au nanostructures (at a particle concentration of 60 pM) were incubated independently with the cells at 37 °C for 24 h. Each data point was obtained from six samples, and N is the number of Au nanostructures taken up per cell.