Nanoparticles

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

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Owing to their unique optical (scattering and absorption) properties, gold-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 and absorption components.[7–9] Furthermore, Au nanostructures have been exploited as probes or carriers to evaluate the delivery efficacy of nanomedicine into target cells or tissues.[8–11] This variety of applications is found 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 gold thiolate chemistry; and the content of Au in a specific number of cells or amount of 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, it is necessary to conduct a large number of in vitro or in vivo experiments separately to compare Au nanostructures with different sizes, shapes, or surface chemistries. Since cell culture and animal studies are highly sensitive to experimental conditions, doing experiments separately (even side-by-side) is expected to introduce errors. Moreover, different types of Au nanostructures must be mixed together for any possible interference between the uptakes of different nanostructures to be observed. 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.

Herein we introduce a spectroscopic method for simultaneous evaluation of the cellular uptake of two different types of Au nanostructures from their mixtures, and thus for comparison of 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. Gold nanospheres and nanorods have distinctive optical signatures, and these differences can be exploited 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, the role of shape on cellular uptake can

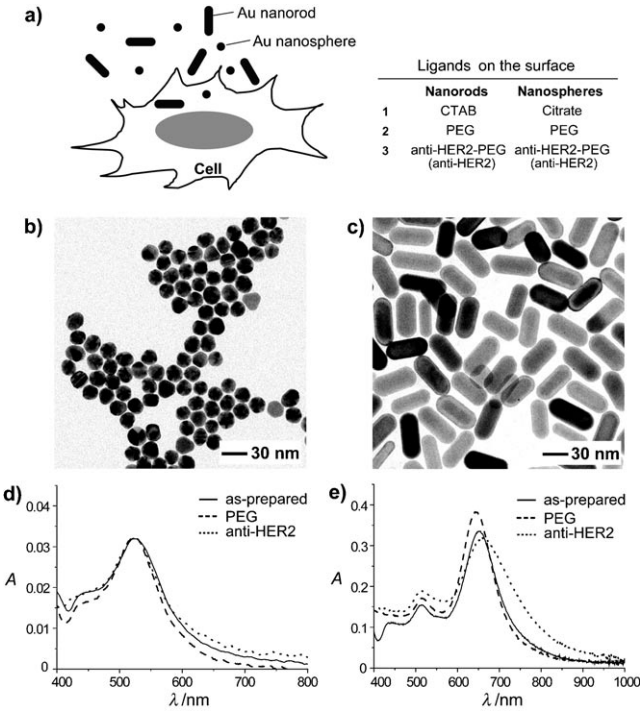

Figure 1. a) Schematic depiction of experiments for the cellular uptake of a mixture of Au nanospheres and nanorods. The surface of the Au nanostructures was derivatized with various chemical groups. We conducted the cell experiments with three types of mixtures: 1) As-prepared, with the surface covered by citrate for nanospheres and CTAB for nanorods; 2) PEG-modified; and 3) anti-HER2-PEG-modified (anti-HER2 for short). b,c) TEM images of Au nanospheres and nanorods, respectively. d,e) UV/Vis spectra taken from suspensions of Au nanospheres (d) and Au nanorods (e) 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% CO_2 for 24 h.

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be deciphered, and the interplay between two different types of nanostructures in a cellular uptake process can be investigated.

We synthesized Au nanospheres of (17.1 ± 1.6) nm diameter by reducing $HAuCl_4$ with sodium citrate (Figure 1b). We followed the protocol of 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 to achieve over 90% cell viability for the Au nanorods; otherwise the cell viability dropped considerably as a result of CTAB remaining in the dispersion medium.[11c] We also modified the surfaces of the Au nanostructures with poly(ethylene glycol) (PEG) and with 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 ≈ 5000) was added to a dispersion of the Au nanostructures; the mixture was incubated for 12 h, and the unreacted PEG thiol was removed 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 ≈ 5000) was first grafted to the surface of the Au nanostructures, and then anti-HER2 was added. 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 in the Supporting Information). 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 1d,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. In contrast, the spectra from the Au nanorods were slightly changed by these treatments, probably owing to some minor etching. Therefore, for every mixture of Au nanospheres and nanorods we prepared, we had to obtain a calibration to precisely determine the amount of Au nanospheres and nanorods taken up by cells from the mixture.

We prepared three types of mixtures (Figure 1a): 1) the as-prepared particles, with the surface covered by citrate for Au nanospheres and CTAB for Au nanorods; 2) the PEG-modified particles; and 3) the anti-HER2 conjugated particles. Each mixture contained 60 pM (particle concentration) each nanospheres and nanorods. At this relatively low concentration, all types of mixtures were found not to be toxic to the cells (see Figure S2 in the Supporting Information), 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 a typical example of the spectra, the absorbance peaks

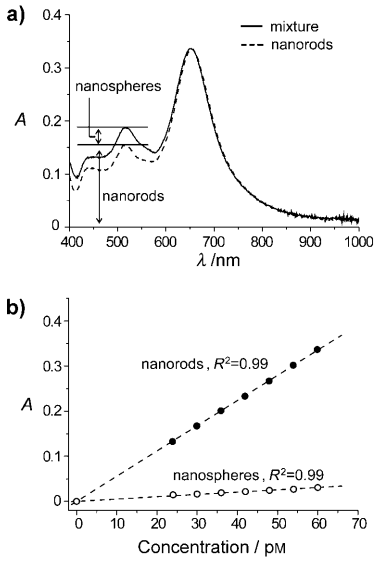

Figure 2. a) UV/Vis spectra of the as-prepared Au nanorods (– – –) and a mixture of the as-prepared Au nanospheres and nanorods (——) dispersed in the cell culture medium. The concentration of the Au nanorods was 60 pM, and the mixture contained 60 pM each nanospheres and nanorods. b) Calibration curves 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 arising from the Au nanorods: $A_{spheres} = A_{mix} - A_{rods}/r$. 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).

of which 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. As 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 at 652 nm (A_{rods}) 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, $A_{spheres} = A_{mix} - A_{rods}/r$. 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 in the Supporting Information).

We then conducted cell uptake studies with the mixtures of Au nanospheres and nanorods. In a typical process, 1.0 mL culture medium containing a mixture of Au nanospheres and nanorods (60 pM 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 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, after incubation, and for the combination of all the media after incubation and the two washing steps. 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. Furthermore, the peak intensities of the media after washing steps were a little bit higher than those without washing steps, thus indicating that some loosely bound Au nanostructures were removed from the cell surfaces during the washing.

On the basis of the spectra in Figure 3 and the calibration curves in Figure 2b, we could quantify the uptake 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 in the Supporting Information). 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 in the Supporting Information). 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 finding demonstrates that the spectroscopic method, in addition to ICP-MS, could become a useful tool for quantifying the cellular uptake 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

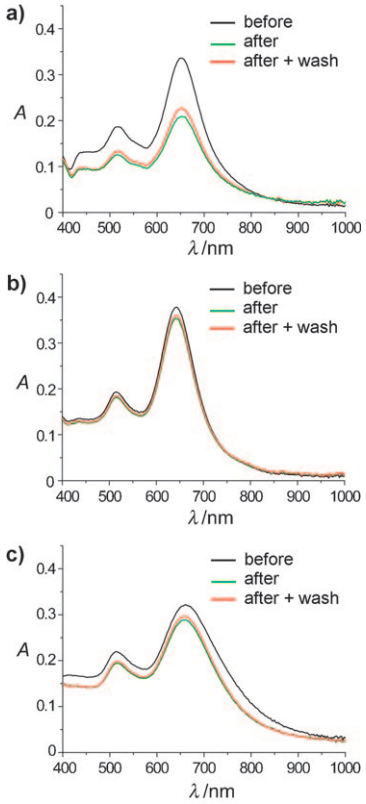

Figure 3. UV/Vis spectra of the culture media containing a mixture of Au nanospheres and nanorods (60 pM each). Three types of mixture were used for the cell culture: a) as-prepared, b) PEG-modified, and c) anti-HER2 modified. We recorded spectra from the media before (denoted "before") and after incubating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

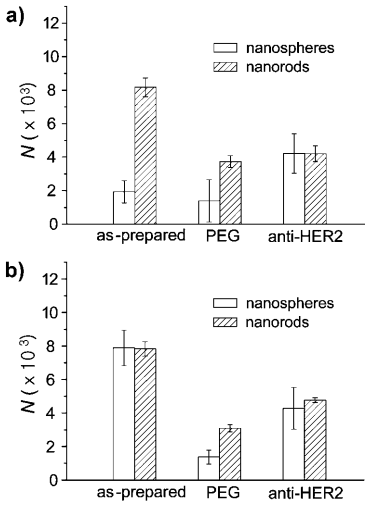

Figure 4. a) Uptake of nanospheres and nanorods by SK-BR-3 cells when the Au nanospheres and nanorods were mixed (60 pM each) and then incubated with the cells at 37 °C for 24 h. b) Uptake of nanospheres and nanorods by SK-BR-3 cells when the Au nanostructures (at a particle concentration of 60 pM) were 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 nanoparticles taken up per cell.

incubated together with the as-prepared Au nanorods, their uptake by the cells was decreased significantly, compared with the uptake when they were 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, Au nanorods might interfere with the uptake of Au nanospheres when the two types of nanostructures are supplied as a mixture. Moreover, we did not observe any trend relating the shape of Au nanostructures to their uptake by the cells regardless of incubation mode, thus indicating that 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 in the Supporting Information). Most importantly, the surface charges of the as-prepared Au nanorods significantly changed from positive (+20 mV) to negative values (−16 mV), thus 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 result 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 through 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 uptake of as-prepared Au nanospheres was suppressed when they were present in a mixture 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 bind loosely to the chemically bonded ligands owing to steric repulsion[16] and probably do not influence the uptake of these Au nanostructures by the cells.

In summary, we have demonstrated that this spectroscopic method could be very useful in differentiating the uptake 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 type of 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. Furthermore, we suggest that this method can provide a simple way for quantifying the uptake of nanostructures of both gold and other materials by cells without using a specialized technique such as ICP-MS.

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