Quantitative evaluation and visualization of size effect on cellular uptake of gold nanoparticles by multiphoton imaging-UV/Vis spectroscopic analysis

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Abstract. With ever-increasing applications of nanoscale materials in the biomedical field, the impact of nanoparticle size on cellular uptake efficiency, dynamics, and mechanism has attracted numerous interests but still leaves many open questions. A combined “multiphoton imaging-UV/Vis spectroscopic analysis” method was applied for the first time for quantitative visualization and evaluation of the cellular uptake process of different-sized (15-, 30-, 50-, and 80-nm) gold nanoparticles (AuNPs). Quantitative analysis of the size effect on cellular uptake behavior of AuNPs from a stack of three-dimensional multiphoton laser scanning microscopy images is obtained. The technique allows for differentiating AuNPs present in external and internal subcellular components, giving detailed information for elucidating cellular uptake dynamics without particle labeling. The data show that the internalization extent of AuNPs is highly dependent on particles’ sizes and incubation time. Due to sedimentation, 50- and 80-nm AuNPs are taken up to a greater extent than 15- and 30-nm particles after exposure for 24 h. However, the smaller particles’ uptake velocity is significantly faster in the first 10 h, indicating a disparity in uptake kinetics for different-sized AuNPs. The finding from this study will improve our understanding of the cellular uptake mechanisms of different-sized nanoparticles and has great implications in developing AuNP-based drug carriers with various sizes for different purposes. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.10.101505]

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1 Introduction

Recent progress in the biomedical applications of nanotechnology has demonstrated that the intracellular uptake efficiency or kinetics of nanoparticles is greatly affected by many factors, including some intrinsic properties of the particles themselves, such as size,\textsuperscript{1–7} shape,\textsuperscript{1,8–11} surface coating,\textsuperscript{5,6,12,13} sedimentation,\textsuperscript{14} and aggregation\textsuperscript{5,16} effects, as well as some features from the side of biological systems, for instance, cell species,\textsuperscript{17} cell cycle period,\textsuperscript{18} etc. Among all these factors, particle size has been identified as one of the most predominant features affecting cellular uptake mechanism, kinetics, and other bioreactivities in vivo or in vitro.\textsuperscript{2,9–24}

Despite remarkable advances made in this field, a critical debate with respect to the optimum particle size for internalization into cells with the highest efficiency is still ongoing and the data reported previously often conflict. For instance, it has been reported by several groups that particles with dimensions in a certain scale range, e.g., diameter of 50 or 100 or < 120 nm, were the optimum size (size range) for nanoparticles’ internalization,\textsuperscript{1,2,22} but these studies contradict each other and still suffer from a lack of convincing evidence. This is partly due to the fact that cell culture studies are highly sensitive to experimental conditions, including particle materials, surface stabilizers, exposure time, cell cycle status, and cell species, but mainly still due to the lack of a unified and standard approach to visualize and quantify particles within cells so far, which would be highly desirable. Cellular uptake behavior of nanoparticles is a sophisticated process occurring at both the cell surface (cell membrane) and the cell interior (cytoplasm, cell nucleus). Therefore, penetration dynamics as well as the particle’s distribution in each suborganelle are necessary for a deep understanding of the uptake mechanism. However, none of the existing quantitative approaches, e.g., transmission electron microscopy (TEM), inductively coupled plasma optical emission spectrometry (ICP-OES), can fulfill these aims. Therefore, developing a reliable tool to perform quantitative visualization of nanoparticles in cells while preserving the spatial information of cell organelles is highly needed.

In this paper, a systematic investigation of the size effect on the cellular uptake of nanoparticles was performed. Gold nanoparticles (AuNPs) with potential applications in biomedical, clinical, and pharmaceutical fields\textsuperscript{25–29} were applied as a model system since their size, shape, and surface features can be easily manipulated. In addition, their unique optical features offer another advantage, which can be used for label-free imaging.\textsuperscript{30,31} The main parameters of nanoparticles as well as the features of the biosystems exposed to particles, including particle materials, shape, coating ligands, concentration unit, and cell types and numbers, were all unified for the purpose of eliminating interference from the variance of experimental

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conditions. By virtue of the unique optical properties of gold, a novel semiquantitative approach was presented in this paper based on combining the use of a multiphoton laser scanning microscopy (MP-LSM) and UV/Vis spectroscopy for evaluation of the cellular uptake process of AuNPs with sizes below the resolution of optical microscopy, ranging from 15 to 80 nm. This approach can not only allow simultaneous acquisition of images as well as quantitative data with respect to the amount of AuNPs uptaken by cells, but also provides a capability to distinguish particle entry into cells from those only adsorbed on the cell membrane from three-dimensional (3-D) MP-LSM images, giving details in terms of the uptake process that cannot be achieved by other quantitative techniques applied at present, e.g., TEM. As revealed by the quantitative data, cellular uptake of AuNPs is a size- and time-dependent process.

In this report, A549 cells were exposed to four types of AuNPs with equal surface properties but differing in size and investigated through the multiphoton imaging-UV/Vis spectroscopic analysis method. We found the cellular uptake extent and kinetics for AuNPs differ greatly depending upon the size of particles and that sedimentation effects cannot be ignored in this process.

2 Materials and Methods

2.1 Nanoparticle Preparation

Colloidal AuNPs with a diameter of 15 nm were prepared by the Frens method, which is typically used to produce monodisperse spherical AuNPs with sizes of ~13 to 20 nm. Ten milligrams of tetrachloroaurate (HAuCl₄⋅3H₂O, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in 50 mL of ultrapure deionized water and heated to the boiling point. Then, 1 mL of a 37.34 mg/mL aqueous solution of sodium citrate (Na₃C₆H₅O₇⋅2H₂O, Sigma-Aldrich) was introduced under vigorous stirring to reduce the Au⁺⁺ to Au⁰. The solution was kept boiling for 10 min with sustained stirring. The resulting AuNPs were spherical with an average diameter of 15 nm. They were used for imaging and as gold seeds for further synthesis of AuNPs of increased size.

As a result of a portion of particles with unsatisfactory morphology produced in the synthesis process for AuNPs larger than 20 nm by the Frens method, a seeding growth method demonstrated by Brown and Natan was introduced here to prepare 30-nm AuNPs. Ten milliliters of 15-nm AuNPs solution was diluted by ultrapure deionized water to a volume of 40 mL as gold seeds. Then 1.77 mL of a freshly prepared 1 mg/mL aqueous solution of tetrachloroaurate was introduced while stirring. After 1 min, 0.306 µL of hydroxylamine (NH₂OH, 50%, solution in water, Sigma-Aldrich) was rapidly added to reduce the Au⁰ to Au⁺. Stirring continued for 10 min after the color turned reddish brown. Particles prepared by this method were spherical with an average diameter of 30 nm still stabilized with citrate. The water used in all experiments was prepared in a Millipore Milli-Q purification system (resistivity > 18.0 MΩ cm). Because some nonspherical and polydisperse particles were produced as byproducts on the second or third growth step, we purchased two batches of citric ligands stabilized AuNPs with sizes of 50 and 80 nm, respectively, from Ted Pella Inc. (Redding, California).

The gold core morphology and mean diameters of those AuNPs were determined by TEM (data not shown).

2.2 Nanoparticles Quantification by UV/Vis Spectroscopy

UV/Vis spectroscopy was carried out to determine AuNP concentration. Absorption spectra of AuNPs were recorded using a PerkinElmer diode array UV/Vis spectrophotometer (Lambda35, PerkinElmer, Rodgau, Germany). The spectra were collected using quartz cuvettes with a 1 cm optical path length. The precise concentration (number concentration) of each batch of gold nanodispersion can be obtained by the following equation:

\[
N = \frac{A_{450} \times 10^{14}}{d^2 \left( -0.295 + 1.36 \exp \left( -\frac{d^2 - 96.8}{18.2} \right) \right)},
\]

where \( N \) is the number concentration of AuNPs in number of particles/mL (nps/mL), \( A_{450} \) is the absorption at 450 nm, and \( d \) is the diameter of AuNPs.

2.3 Cell Culture Conditions

Human alveolar basal epithelial cells (A549 cells, CCL-185; ATCC, Manassas, Virginia) were taken as model cells. A549 were cultured in a 75-cm² flask in Roswell Park Memorial Institute (RPMI)1640 high glucose medium supplemented with 10% heat-inactivated fetal bovine serum without phenol red (PAA Laboratories GmbH, Pasching, Austria) at 37°C under 5% CO₂. The medium was changed every three days and subculture of the cells was performed when 80% confluence was reached.

2.4 Nanoparticle Uptake

Evaluation of the cellular uptake extent of AuNPs with various sizes was performed by MP-LSM techniques to detect the intrinsic MAIL signal from gold particles entrapped by cells. A549 cells were seeded into a 24-well flask (PAA Laboratories GmbH) with a density of 2 × 10⁴ cells per well and grown until 90% confluence. Prior to exposure to gold particles, the medium at each cell culture well was aspirated. Cells were washed with fresh medium three times and then the medium was aspirated again. 0.5 mL of gold suspension of various concentrations in RPMI solution plus 10% serum without phenol red was added into each well. The incubation of the cells was done at 37°C under 5% CO₂ for 24 h. The number concentrations for different-sized AuNPs were all set to a value of 7.5 × 10¹⁰ particles/mL, which could be fairly adjusted and visualized for both small and large particles.

After 24 h, the gold suspension was removed from each well. Cells were washed three times with phosphate-buffered saline (PBS) to remove excess AuNPs, fixed with 1% formalin, and then washed an additional three times with PBS. Afterward, 0.5 mL of a 1% Vybrant DiI cell-labeling solution (Invitrogen, Eugene, Oregon), a lipophilic membrane dye, was added for each well and incubated for 5 min at room temperature. Following DiI incubation, cells were washed three times with PBS and covered by a glass slide for visualization by MP-LSM.

2.5 Cellular Uptake Dynamics of AuNPs

Experiments for exploring the cellular uptake dynamic of AuNPs were carried out by combining MP-LSM and UV/Vis spectroscopic techniques.
A549 cells were propagated into 24-well plates with a density of $2 \times 10^4$ cells per well and grown until 90% confluence. Immediately before co-incubation with AuNPs, the medium of each cell culture well was aspirated. Cells were washed with fresh medium three times and afterward the medium was aspirated again. 0.5 ml of gold suspension in RPMI solution plus 10% serum without phenol red with concentrations of $5.8 \times 10^{11}$, $1.36 \times 10^{11}$, $3.0 \times 10^{10}$, and $4.4 \times 10^9$ particles per milliliter for 15, 30, 50, and 80 nm, respectively, was added into each well for incubation of the cells at 37°C under 5% CO$_2$ for defined time intervals. The number concentration of each sized AuNP was precisely set in a suitable range in which the UV/Vis absorption of colloidal gold shows a good linear relationship as a function of the particles’ concentration. Several positive controls (no cells present) as well as a negative control (no particles present) were set for reference groups incubated in the same conditions.

For each time point (1, 2, 4, 6, 8, 10, 12, and 24 h), medium co-incubated with cells was carefully removed from each well and placed in a clean cuvette for UV/Vis measurement. Cells were washed three times with PBS to remove excess AuNPs, fixed with 1% formalin, and then washed an additional three times with PBS. Afterward, 0.5 ml of a 1% Dil solution was added to each well and incubated for 10 min at room temperature. Following Dil incubation, cells were washed three times with PBS and covered by a glass slide for imaging by MP-LSM.

### 2.6 Confocal Imaging

The slides prepared above were visualized by an inverted confocal/multiphoton laser scanning microscope (Zeiss LSM510 META NLO system, Carl Zeiss, Jena, Germany) adapted with a pulsed infrared laser ($\lambda = 705$ to 980 nm, Chameleon XR, Coherent, Deisenhofen, Germany) for multiphoton excitation. Samples were excited with 778 and 543 nm. Images were captured with a 63 × /1.2 NA water immersion objective lens in multitracking mode at an acquisition resolution of 512 × 512 pixels. Z-stacks of the cell samples were acquired at 0.8-μm intervals. Image stacks were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/download.html).

Quantification of the number of AuNPs entrapped by cells throughout each image stack was carried out using the “analyze particles” function of the ImageJ software package, which permits visualization of either spherical or irregular-shaped objects throughout an image stack or a set of stacks and designates a spatial position along x, y, and z directions together with the intensity of the spot it represents. Prior to intensity and spot area measurements, only the tracks of AuNPs were selected. The threshold was optimized to a proper value of 25 in which the background signals were eliminated completely and only AuNPs can be visualized. A summary of the number as well as total area of the optical spots was provided by the software afterward.

Colocalization of AuNPs in the cell membrane was evaluated by analyzing a set of multichannel image stacks on a per cell level. Particles colocalized in cell membranes (green color area) and in the cells were counted respectively. Thus, the proportion of particles in the cell membrane for each cell can be determined.

### 3 Results and Discussion

#### 3.1 AuNPs Morphology

The average diameter of the gold core with a standard deviation as well as the zeta potential for each particle type is shown in the first two columns of Table 1.

For a certain sized AuNP, a characteristic absorption spectrum that has a distinct maximum absorption peak can be observed, shifting from 518 to 547 nm in wavelength with the increase of particle size from 15 to 80 nm. These features can be characterized conveniently by UV/Vis spectrophotometer and further exploited to measure their individual concentrations in solution by use of Eq. (1). The main parameters of their UV/Vis absorption spectra ($\lambda_{\text{max}}$ and $A_{\text{max}}$) and corresponding concentrations are shown in the last three columns of Table 1.

### 3.2 Single Particle Identification of Subresolution AuNPs and Threshold Setting

Due to the resolution limitation of classical microscopy techniques, a subresolution object exhibits an optical size in the image instead of its real physical size. The point spread function (PSF) yielding this size can be calculated from the following equations:35

$$r_{xy} = \frac{0.7\lambda}{\text{NA}},$$  \hspace{1cm} (2)

$$r_z = \frac{2.3\lambda n}{\text{NA}^2},$$  \hspace{1cm} (3)

where $\lambda$ is the wavelength of the emitted light, NA is the numerical aperture of the objective lens, and $n$ is the refractive index of the medium. The values of $r_{xy}$ and $r_z$ (equal the full width at half maximum) can be calculated by inputting proper constant values

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**Table 1** Selected gold nanoparticles (AuNPs) characteristics.

<table>
<thead>
<tr>
<th>Size range (nm)</th>
<th>Size of gold core (nm)$^a$</th>
<th>Zeta potential (mV)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$A_{\text{max}}$ (A.U.)</th>
<th>Conc. (number of NPs/ml)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>$15.59 \pm 0.77$</td>
<td>$-28.8$</td>
<td>518</td>
<td>1.7418</td>
<td>$2.9 \times 10^{12}$</td>
</tr>
<tr>
<td>30</td>
<td>$31.43 \pm 1.34$</td>
<td>$-28.5$</td>
<td>526</td>
<td>0.5354</td>
<td>$1.01 \times 10^{11}$</td>
</tr>
<tr>
<td>50</td>
<td>$51.98 \pm 2.58$</td>
<td>$-26.4$</td>
<td>530</td>
<td>1.2207</td>
<td>$4.5 \times 10^{10}$</td>
</tr>
<tr>
<td>80</td>
<td>$82.29 \pm 3.12$</td>
<td>$-39.1$</td>
<td>547</td>
<td>1.16</td>
<td>$1.1 \times 10^{10}$</td>
</tr>
</tbody>
</table>

$^a$Average diameter of gold core and standard deviation of each sized AuNPs are determined by TEM measurement (data not shown).

$^b$Concentration of AuNPs are calculated by Eq. (1).
into Eqs. (2) and (3). PSF of the current MP-LSM can be obtained by inputting relevant parameters into the equations. Considering an objective NA of 1.2, a broad emission spectrum for AuNP of ~530 to 640 nm, and a refractive index of 1.33, the theoretical lateral ($r_{xy}$) and axial ($r_z$) resolutions can be calculated as $r_{xy} = 320 \text{ nm}$ and $r_z = 1020 \text{ nm}$, respectively. Because AuNPs used in this study (15 to 80 nm) are below the resolution of the microscope, their real physical size cannot be resolved by microscopy. However, from the diffraction theory, we know a single subresolution particle presents in an MP-LSM image like a spot with diameter of ~320 nm. Based on this principle, an optical spot from an image with an area of $0.08 \mu m^2$ ($0.16 \mu m \times 0.16 \mu m \times 3.14$) can be regarded as the minimum resolvable spot unit representing one or more subresolution AuNP not exceeding this spot size.

Since a single spot could still contain a number of AuNPs, investigation on the signal intensity distribution of different-sized AuNPs is imperative. For instance, one spot unit might contain at least 1 to at most 455, 113, 41, or 16 AuNPs with sizes of 15, 30, 50, or 80 nm, respectively. Obviously, with the growth of the number of particles assembled in one spot unit, its contribution to image intensity increases as well.

The principal idea of this study is based on the assumption that one spot unit, which contains only one single AuNP, would emit a minimum signal with intensity at a low threshold level, whereas the spots containing a maximum number of particles emit light with the highest intensity. Through this principle, the number of monodisperse AuNPs entrapped by cells can be estimated by counting the number of AuNPs with emission intensities within a proper threshold regime extracted from a series of 3-D image stacks.

In order to generate strong and stable optical signals of AuNPs with a good signal-to-noise ratio, we set the excitation wavelength to $\lambda_{ex} = 788 \text{ nm}$ and the laser power to 1.66 mW at the samples under which the signals from small-sized particles (15 or 30 nm) can be distinguished from background noise at a threshold of 25 units. The background noise is practically determined by illuminating two sample areas containing A549 cells, one with and without the presence of AuNPs, respectively. Upon identical measurement setup, the two areas were irradiated sequentially. Then the background signals were recorded from the gold-free location and can be subtracted by shifting the threshold level (a value of 25). Likewise, the noise signals in the image of the gold sample can be eliminated with the same approach. Thus, we set the value of 25 as the lowest threshold to eliminate background noise.

As a result of the fact that the femtosecond laser power is fluctuating, the incident laser intensity passing through the objective varies between 1.59 and 1.72 mW with a periodical fluctuation of $\sim \pm 4\%$ measured by a power meter. Hence, the emission intensity induced under such irradiation, even from same-sized AuNPs, fluctuates. Furthermore, not only luminescence but also the signals from other sources, e.g., second harmonic generation and scattering light, contribute to the emission intensity. Therefore, we set the threshold scope at $\pm 4\%$ corresponding to the fluctuation of excitation power to avoid collecting agglomerated particles in this region by mistake. The threshold range for calculating individual AuNPs with smaller sizes (15 or 30 nm) was set from 25 to 34. Likewise, the threshold range for larger particles (50 or 80 nm) was set from 25 to 44 because their emission intensities were 1.8 times higher than the smaller ones.

Knowing that the signal is originating from a diffraction limited spot (unit spot), the emission with intensity in the lowest threshold level (25 to 34) is estimated to be generated from a single AuNP with a size of 15 or 30 nm. Likewise, the corresponding threshold range for individual AuNPs with larger sizes (50 or 80 nm) is set from 25 to 44. Applying the same principle, the emission light with an intensity larger than the lowest levels is estimated to be from agglomerated AuNPs. Figures 1(a) and 1(b) show the intensity distribution in each threshold regime for unit spots extracted from image stacks of various AuNPs (15, 30, 50, and 80 nm) exposed to A549 cells for 24 h. The data demonstrate that, on average, $>50\%$ of the optical unit spots distribute in the lowest threshold range, far more than in spots with higher threshold, indicating that the majority number of AuNPs still maintain a nonagglomerated state. On the other hand, the super bright unit spots (in threshold 235 to 255), which are estimated to contain the maximum numbers of single particles, account for very small proportion, only from 0.1 to 1.5%, proving that the population of agglomerated AuNPs plays a negligible role in affecting our study on the number

![Fig. 1](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics/101505-4/Fig_1.png)

**Fig. 1** Intensity distribution in each threshold regime of gold nanoparticles (AuNPs) with size of (a) 15 nm, 30 nm, (b) 50 nm and 80 nm.
of AuNPs entrapped by cells. Analyzing the ratio of the signal area from single particles (threshold: 25 to 44) to the total signal area (threshold: 25 to 255) for 80-nm AuNPs, no significant change was observed, indicating the absence of agglomeration (68 to 83% of the total signal area). This is also supported by Fig. 4. Hence, the cellular uptake extent of AuNPs can be evaluated quantitatively by analyzing the number of nonagglomerated AuNPs only, which are assumed to be emitting light with intensities in the lowest threshold range. This finding will greatly reduce the complexity and cost of calculation work on the evaluation process associated with vast known or unknown influencing factors.

3.3 AuNPs Concentration Unit Setting

Considering some of the conflicting results caused by the use of different particle concentration units among different research groups, selecting a suitable concentration unit of particles is a crucial issue. The most commonly used concentration unit is mass concentration (often expressed as mole concentration), which is on an equal mass-dose of raw materials basis as it is typically used for measurement of cytotoxicity. Concerning the fact that it is the individual particle that delivers the drugs or bimolecular into the target site of cells and exerts its function, being aware of particle number is more meaningful than only knowing the mass dose of the raw materials. Therefore, instead of mass concentration, number concentration was applied in our multiphoton work as a standard concentration unit. In the study of the cellular uptake rate for different-sized AuNPs (15, 30, 50, and 80 nm), the number of particles per unit volume in all sizes was kept constant before exposure to cells. For the study in the cellular uptake kinetics, however, the concentrations for different-sized particles were just set in a proper range to ensure the absorbance of the gold solution in the linear range of 0.2 to 0.8 Abs.

3.4 Cellular Uptake of AuNPs

Spherical AuNPs at four representative sizes (15, 30, 50, and 80 nm) were applied in this study. The other relevant parameters, including surface stabilizer, particle concentration, cell numbers, incubation time, etc., are all set identically as mentioned previously in the Method section, to avoid interference from the change of experimental conditions. Stability was ensured by TEM measurement before and after incubation in the medium (data not shown) and UV/Vis measurements (Fig. 4). No morphology changes or agglomeration of AuNPs were observed, demonstrating good stability of gold particles in cell growth medium. The cellular uptake process was monitored using MP-LSM. By counting the number of nonagglomerated particles internalized into cells from a stack of MP-LSM images, cellular uptake of different-sized AuNPs can be determined. Figures 2(a) to 2(d) show four representative micrographs derived from a z-series of MP-LSM images, illustrating AuNPs with various sizes are all able to gain intracellular access into A549 cells after 24-h exposure. AuNPs are detectable in slices at all levels from the 3-D images, suggesting their internalization in cells and not being only loosely adsorbed on the outer surface of the cell membrane. In Fig. 2(e), a bar graph of the number of AuNPs in/on cells per optical stack versus the diameter of the AuNPs shows cellular uptake is heavily dependent on the size of particles. Because it is difficult to precisely count the number of irregularly-shaped cells, we used the unit of AuNPs per optical stack instead of the number of particles per cell, allowing standardization of the measurements. The graph data highly coincide with the trend exhibited by Figs. 3(a) to 3(d), demonstrating the uptake rate increases greatly with increasing particle size and the maximum uptake occurs for a size of 80 nm. These results are also in agreement with Cho et al.’s study that the bigger AuNPs are passively taken up by a larger amount than smaller particles due to the influence of gravity (sedimentation). This assumption needs to be further confirmed in the following section of this article.

3.5 Kinetics of Particle Entry

We conducted the studies of cellular uptake kinetics by incubating A549 cells with various-sized AuNPs for different periods of time. The study was performed using two different approaches: (1) we counted the number of nonagglomerated AuNPs in cells through MP-LSM image analysis; (2) in parallel, we measured the content of AuNPs maintained in cell culture medium by UV/Vis method. MP-LSM image analysis revealed that the uptake kinetics of AuNPs greatly differs with the variation of particle size. As shown in Figs. 3(a) to 3(d), after 1-h incubation, the four types of AuNPs are all internalized by cells but to different degrees. For AuNPs of 15 nm, the uptake seems to be a two-step process, being slow in the beginning (~6 h) and with an increased number of internalized particles from 8 h on. After that the rate steadily increases until it reaches the highest value at 24 h. For AuNPs with sizes of 30 and 50 nm, similar trends are shown: the uptake extent increases slowly in the first 12 h before getting the highest value after 24 h. Differing from those kinetics, however, 80-nm AuNPs display a nearly constant uptake for the first 10 h. A significant increase of uptake is seen between 12 and 24 h, which is far higher than those at other time points. This disparity in uptake kinetics for different-sized AuNPs has never been reported previously and will be further explored later on in this study. Notably, the number of particles uptaken in Fig. 3 disagrees with the trend shown in Fig. 2. This difference in absolute numbers can be attributed to the different numbers of AuNPs applied in these two studies. Actually, normalizing the uptake values to the number of AuNPs can correct for this.

By exploiting the unique optical properties of AuNPs, UV/Vis spectroscopy as a macroscopic quantitative approach was applied here to verify whether the data obtained from MP-LSM image analysis can imply the real uptake extent of AuNPs in cells. Based on the Lambert-Beer law, the concentration of AuNPs has a linear correlation with the absorbance of their localized surface plasmon resonance (LSPR) peak. Through this way, the concentration of AuNPs in cell culture medium can be directly obtained. Thus, the number of AuNPs taken up by cells per well at each time point can be determined by subtracting the gold amount in the medium from the total gold dose applied. The initial concentration for each type of AuNP can be obtained by Eq. (1). Figures 4(a) to 4(d) show the UV/Vis spectra of the different-sized AuNPs dispersed in culture medium, before and after 1- and 24-h incubation with cells, respectively. It is clearly seen that the maximum peak intensities of AuNPs decrease gradually over time, indicating reduced AuNP concentration and, hence, possible uptake of AuNPs by cells. Because the range of the best-fit linear correlation between the concentration of AuNPs and the absorption of LSPR peak differs with the change of particle size, it is hard to keep an identical initial
concentration for all kinds of AuNPs. Thus, different initial concentrations for these four kinds of AuNPs were applied in this respect. Due to this reason, the investigation on the uptake percentage relative to the total amount of particles, instead of its absolute value, for each kind of AuNP is more reasonable.

Figures 5(a) to 5(d) summarize the percentage of cellular uptake per well for different types of AuNPs at each time point measured by UV/Vis analysis. Significantly, the uptake percentages increase over time for all types of AuNPs, illustrating it is a time-dependent process. Further, the uptake extent was also found to be sensitive to particle size. As the AuNPs become larger, their uptake processes slow down greatly for short incubation times. This result is consistent with previous data acquired by using MP-LSM image analysis method, confirming
these two methods are highly correlated to each other and both of them can imply the in vitro cellular uptake results of AuNPs. Notably, for uptake kinetics study, however, different numbers of particles were used for 15-, 30-, 50-, and 80-nm AuNPs, respectively, to keep the colloid gold in a proper concentration for UV/Vis measurement. But this is also reasonable because the dynamic cellular uptake process of AuNPs has similar mechanisms to the processes of absorption and metabolism of chemical drugs in human body, which do not depend on the initial concentration of drugs applied.\textsuperscript{38} In consequence, the numbers obtained for the MP-LSM study cannot be directly compared with the UV/Vis data in terms of absolute values. But the overall trend can be well analyzed, supporting that MP-LSM is a well-suited technique to investigate cellular uptake.

From these uptake graphs, there is one point worth mentioning. During most of the incubation time (0 to 12 h), the uptake extent of larger-sized AuNPs (50 or 80 nm) is far less than that of small ones; however, after a long time incubation (24 h), their uptake extent still reaches a high level being equivalent to that of smaller particles. To explain these different uptake behaviors for various-sized AuNPs, we can depict the whole uptake process in the following three steps: (1) AuNPs are transported from any arbitrary zone of the suspending medium to the contact zone (Fig. 6) and (2) adsorb on the surface of the cell membrane. Then the particles (3) penetrate into cells through protein-mediated or any other receptor-mediated pathway. Obviously, the uptake only occurs after AuNPs are transported to the contact zone. Therefore, the concentration of particles in the contact zone can be assumed as a critical factor for the cellular uptake process.

As illustrated in Fig. 6, AuNPs can be transported to the contact zone through diffusion and sedimentation. Considering the sedimentation effect caused by gravitational forces that highly depend on particles mass, which is proportional to the cubes of particles radius, it is imperative to investigate the sedimentation effect for different-sized AuNPs. Figure 7 gives a comparison of sedimentation for the four types of gold dispersions over a period of 48 h, exhibiting a clear disparity in sedimentation. For 15- and 30-nm AuNPs, their concentrations remain nearly identical relative to the initial concentration even after 48-h incubation. In contrast, for particles with sizes of 50 and 80 nm, a significant reduction in suspended particles was seen, particularly after a long period of incubation, such as 24 and 48 h. We note that after 24-h incubation, almost 7 and 12% of the gold concentration is lost due to sedimentation for 50- and 80-nm AuNPs, respectively. This reveals that sedimentation mainly contributes to the high penetration extent for bigger-sized AuNPs at 24 h. This result also very well explains the
unusual uptake rate of these two types of AuNPs at 24 h observed previously in Figs. 2 and 5.

3.6 Cell Membrane Penetration Ability of AuNPs

It is well known that the cell membrane is the major barrier separating the cell interior from the external phase and protecting the cell from all kinds of foreign materials. Hence, investigating the ability for different-sized particles to cross the cell membrane might be helpful for explaining their differences in cellular uptake kinetics. AuNPs with sizes of 15 and 80 nm were selected in this study because of their significant difference in uptake dynamics. Particles were visualized and quantified by MP-LSM analysis at a single cell level. Figure 8(a) shows a panel of typical 3-D MP-LSM images displaying $xy$ (left bottom) as well as $xz$ and $yz$ sections (left top and right bottom). This allows a view on the distribution of 80-nm AuNPs in one cell over 24-h incubation. Due to the cell membrane staining, AuNPs embedded in the cell membrane (marked with blue arrows) or present in the cell (marked with white arrows) can be easily differentiated, enabling us to precisely quantify the number of AuNPs localized in those two cell organelles separately. Figure 8(b) shows the ratio of the particles (15 or 80 nm) localized in the cell membrane relative to particles associated with the entire cell over the incubation time. 15-nm AuNPs exhibit a moderate declining trend from 40% at 1 h down to 22% at 24 h. In contrast, 80-nm AuNPs also display a declining trend but with a steep slope. The majority of particles (70 to 80%) observed in the first 6 h is localized in the cell membrane and not yet taken up. Even after 12 h, a big amount of AuNPs still failed to reach the cell interior ($\sim$40% particles at the membrane). After 24 h, however, their proportions are found to be down to 16% or even a bit below for the level of 15-nm particles. These observations can be explained in two respects: (1) due to its smaller diameter, 15-nm AuNPs have a superior membrane crossing ability relative to 80 nm. For this reason, we found a large portion of 80-nm AuNPs located in the cell membrane rather than 15-nm AuNPs during 12-h exposure; but (2) the sedimentation effect dramatically impacts the uptake extent for 80-nm AuNPs far more than 15-nm particles over time, confirmed.
Fig. 5 Quantification of the uptake percentage of AuNPs into cells relative to the total amount of particles applied per well at different time points by UV/Vis analysis. The initial gold amounts applied per cell growth well are (a) $2.9 \times 10^{11}$ particles (15-nm AuNPs); (b) $6.8 \times 10^{10}$ particles (30-nm AuNPs); (c) $1.5 \times 10^{10}$ particles (50-nm AuNPs); and (d) $2.2 \times 10^{9}$ particles (80-nm AuNPs). A549 cells were incubated with different-sized AuNPs at 37°C with defined concentration mentioned above, and at each time point, the suspension medium was collected and analyzed for gold concentration by UV/Vis.

Fig. 6 Representation of the cellular uptake process of colloidal gold. The uptake extent is determined by particles concentration in contact zone, which highly depends on the sedimentation and diffusion velocity of AuNPs.
by their high uptake percentages at 24 h expressed previously and further supported by the sedimentation data in Fig. 7. These results also give a clear answer to our assumption in respect of the uptake efficiency for different-sized AuNPs mentioned in last section: it is sedimentation effect rather than any other factors that cause the highest uptake extent for bigger AuNPs (50 or 80 nm). However, within a short period of exposure time, small-sized particles show advantages in uptake efficiency over their larger-sized counterparts. To our knowledge, these results have never been reported previously and will have important implications for deeply understanding the cellular uptake mechanism of nanoparticles with different sizes.

4 Conclusions

In conclusion, for the first time, a combined MP-LSM image-UV/Vis spectroscopic analysis method was applied in this paper for systematically investigating the size effect on cellular uptake process of AuNPs. Our study demonstrated differential cellular uptake rate and kinetics for a series of different-sized AuNPs. 50- and 80-nm AuNPs displayed faster sedimentation velocities over the smaller ones (15 and 30 nm), leading to their uptake extent in far higher levels than the latter after 24-h exposure to A549 cells. However, in a defined period of incubation time during which the sedimentation effect is slight (10 h), small particles exhibited superior penetration ability through cell membrane over the bigger-sized particles. This suggests the potential for controlling the cellular uptake extent and kinetics just by altering the size of the central AuNP nanoplatform.

Our studies also imply the sedimentation effects have to be considered as one plan to conduct cell or tissue entry experiments for nanoparticles with various sizes. Overall, these results are expected to help us in deeply understanding the mechanism and kinetics behind the cellular uptake process of different-sized particles, allowing us to optimize more efficient drug or gene nanocarriers by tuning their size for diagnostic and therapeutic applications.

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Fig. 7 Sedimentation effects of 15-, 30-, 50-, and 80-nm AuNPs at a series of time points from 0 to 48 h in cell grow medium.

![Fig. 7](image)

Fig. 8 (a) A stack of representative three-dimensional images of 80-nm AuNPs embedded into one A549 cell after 24-h incubation. A cross-section view (left bottom) and its two corresponding longitudinal section views (left top and right bottom) are displayed. (b) A comparison of the proportion of particles localized in cell membrane relative to the total cell during different periods of incubation time.

![Fig. 8](image)
References


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