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One-Pot Synthesis of Near-Infrared Fluorescent Gold Clusters for Cellular Fluorescence Lifetime Imaging

*Li Shang, Naghmeh Azadfar, Florian Stockmar, Winfried Send, Vanessa Trouillet, Michael Bruns, Dagmar Gerthsen, and G. Ulrich Nienhaus**

Dedicated to Professor Shaojun Dong on the occasion of her 80th birthday

A *facile strategy to synthesize water-soluble fluorescent gold nanoclusters (Au NCs) stabilized with the bidentate ligand dihydrolipoic acid (DHLA) is reported. The DHLA-capped Au NCs are characterized by UV–vis absorption spectroscopy, fluorescence spectroscopy, transmission electron microscopy, and X-ray photoelectron spectroscopy. The Au NCs possess many attractive features including ultrasmall size, bright near-infrared luminescence, high colloidal stability, and good biocompatibility, making them promising imaging agents for biomedical and cellular imaging applications. Moreover, their long fluorescence lifetime (>100 ns) makes them attractive as labels in fluorescence lifetime imaging (FLIM) applications. As an example, the internalization of Au NCs by live HeLa cells is visualized using the FLIM technique.*

1. Introduction

The synthesis of novel nanoscale materials with controllable physical and chemical properties continues to draw wide interest among scientists in many research fields.[1] Analogous to semiconductor quantum dots (QDs), metal nanoclusters with sizes comparable to the Fermi wavelength of the electron exhibit molecule-like properties of discrete electronic states and can display size-dependent photoluminescence.[2] Small gold nanoclusters (Au NCs) consisting of several Au atoms are chemically stable and exhibit bright fluorescence ranging from the visible to the infrared depending on the number of Au atoms in the cluster.[3] Owing to their ultrasmall size, good photostability and low toxicity, fluorescent Au NCs have recently been recognized as promising candidates for cell labeling and biosensing, and extensive research has been carried out on their synthesis[4] and bio-application.[5]

The stability of nanoparticles is heavily dependent on the chemical nature of the surface ligands and the interface between the inorganic core and the organic ligands.[6] Dissociation of organic ligands from the inorganic core lowers the colloidal stability of the nanoparticles. This process, which may occur during functionalization, processing or aging in biological media, has detrimental effects for their use in applications.[7] To achieve a robust stability of nanoparticles, the most direct and effective method is to use capping molecules forming strong chemical bonds to the surface atoms of the inorganic core. Ligands with at least two binding

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sites such as dihydrolipoic acid (DHLA) and its derivatives proved to endow metal[8] and semiconductor nanocrystals[9] with much enhanced stability. In addition to the stabilizing effect, the hydrophilic carboxyl group of DHLA can act as an anchor for immobilizing biological molecules either by electrostatic interactions[10] or covalent linkage.[11] Therefore, DHLA surface-capping of small-sized, fluorescent Au NCs may result in robust fluorophores that may find wide application in biological research. Indeed, Lin et al.[12] recently reported the synthesis of fluorescent DHLA-capped Au NCs (DHLA–AuNCs) via an etching-based strategy involving a multistep process.

Here we show that fluorescent DHLA–AuNCs can be synthesized in a one-pot strategy by simply reducing a mixture of lipoic acid (LA) and gold salt with sodium borohydride in aqueous solvent. The resulting DHLA–AuNCs exhibit bright near-infrared luminescence, high colloidal stability, and good biocompatibility. Their long luminescence lifetime (>100 ns), much longer than that of cellular autofluorescence and most organic dyes, makes them attractive as markers for cellular fluorescence lifetime imaging (FLIM) applications. In contrast to fluorescence intensity imaging, lifetime-based imaging is independent of fluorophore concentration and laser excitation intensity. Importantly, however, the fluorescence lifetime of fluorescent probes can be exquisitely sensitive to the local environment. Consequently, FLIM may provide contrast due to spatial variations of the lifetime and, thus, may yield valuable additional information.[13] Correspondingly, we further examined the capability of imaging DHLA–AuNCs in HeLa cells by the FLIM technique. Our results indicate that DHLA–AuNCs have great potential as robust fluorophores in biomedical applications, especially in combination with FLIM.

2. Results and Discussion

Fluorescent DHLA–AuNCs can be readily synthesized by reducing both LA and gold salt with $NaBH_4$ in aqueous solution. We observed that mixing $HAuCl_4$ with LA in basic solution led to a gradual fading of the light yellow color, indicating the formation of Au(I) complexes due to the interaction of gold ions with the disulfides of LA.[14] Upon adding the reductant $NaBH_4$, the colorless solution slowly turned pale brown. A bright red luminescence from the prepared solution under the UV lamp suggests that a luminescent species was formed during the reaction (**Figure 1**). We noticed that fluorescence did not appear for the colorless mixture of LA and $HAuCl_4$ prior to the addition of $NaBH_4$, or for an LA solution only in the presence of $NaBH_4$, thus suggesting that the luminescence originates from small Au NCs forming in the solution. Evidently, the strong reductant $NaBH_4$ is capable of reducing both gold ions and LA in the solution, from which small metallic nanoclusters capped with DHLA will eventually form.[11b]

Additional experiments were performed to optimize the DHLA–AuNC synthesis. The results suggest that it is important to control the molar ratio of LA to Au as well as the amount of $NaBH_4$ during the reaction to obtain brightly

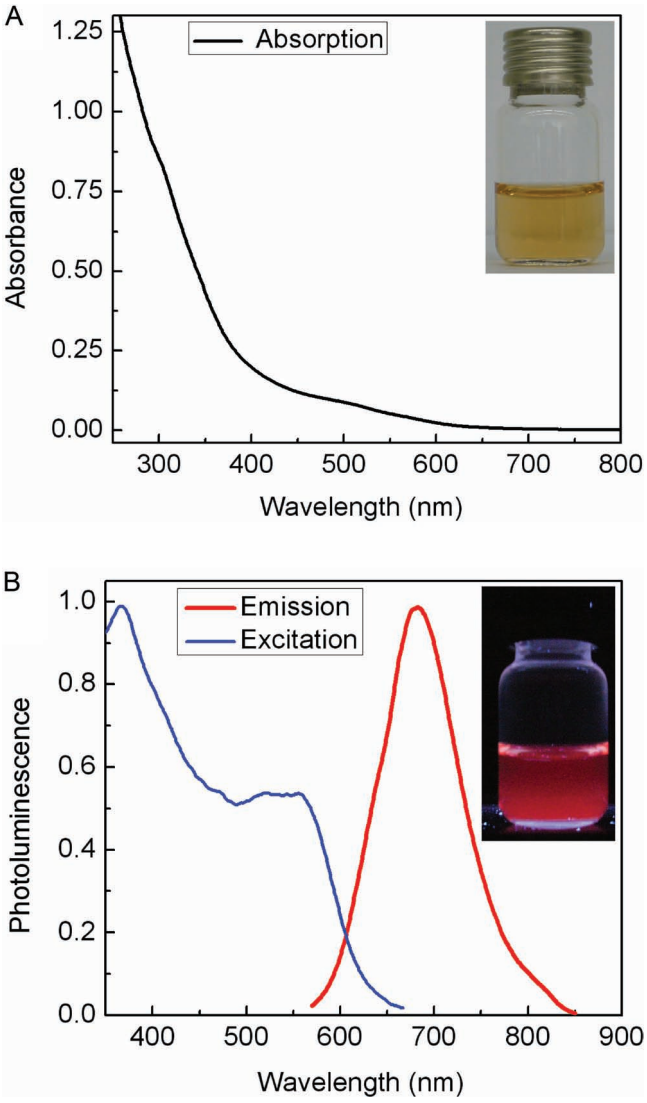

Figure 1. A) UV–vis absorption spectrum of an aqueous solution of DHLA–AuNCs. The inset shows a photograph of DHLA–AuNCs in room light. B) Fluorescence excitation (blue line, emission at 684 nm) and emission (red line, excitation at 550 nm) spectra of DHLA–AuNCs. The inset shows a photograph of DHLA–AuNCs under a UV light source emitting 365 nm light.

luminescent DHLA–AuNCs. At a constant Au concentration of 0.5 mM, we found that 1.5 mM LA and 1 mM $NaBH_4$ yielded the brightest Au NCs (Figure S1 and S2 in the Supporting Information(SI)). Adding NaOH prior to reduction is necessary to increase the solubility of LA in water, especially upon subsequent addition of acidic gold salt ($HAuCl_4$), and stabilize the formed clusters. Compared with the previously reported approach based on precursor-induced Au nanoparticle etching in the organic phase and subsequent ligand exchange with DHLA,[12] the strategy presented here appears simpler, saves time, and requires less reagents.

Figure 1A presents the absorption spectrum of the prepared DHLA–AuNCs. Unlike the UV–vis absorption spectra of larger Au nanoparticles, which display a strong surface plasmon resonance around 520 nm,[15] DHLA–AuNCs in aqueous solution show two weak absorption bands centered

on 505 and 560 nm, respectively. Glutathione-capped Au_{22} clusters were previously reported to possess similar spectral features in this region, which arise from mixed intraband (sp to sp) and interband (d to sp) transitions.[16] In contrast to large Au nanoparticles, ultrasmall Au NCs exhibit molecule-like properties including photoluminescence.[2a] Indeed, a strong fluorescence centered on 684 nm in the near-infrared region is observed from our DHLA–AuNCs in aqueous solution upon excitation at 550 nm (Figure 1B, red line). The excitation and emission spectra of DHLA–AuNCs are also similar to those of the recently reported mixed monolayer-protected Au_{22} clusters,[16c] indicating that Au_{22} may be the predominant species in the DHLA–AuNCs. Like semiconductor QDs, these Au NCs can be efficiently excited by a wide range of light wavelengths below 600 nm, whereas the emission maximum remains essentially unchanged (Figure S3 in the SI). Moreover, the broad emission band covering the red to near-infrared region makes the Au NCs useful markers for imaging in the 700–800 nm range, where tissue and blood optical properties are highly favorable for biomedical imaging.[17]

The quantum yield of our DHLA–AuNCs in aqueous solution (pH 9.0) was determined as ~0.6%, using Rhodamine 6G (QY = 0.95 in ethanol) as a reference. This value is thus comparable to that of DHLA–AuNCs synthesized by Lin et al.[12] The fluorescence emission of DHLA–AuNCs shows a gradual increase with increasing pH (Figure S4 in the SI), reflecting the important role of the ligands on the luminescence efficiency of Au NCs.[18] Fluorescence decays of DHLA–AuNCs in aqueous solution were measured with excitation at 470 nm. We obtained markedly non-exponential decay curves that required a sum of three exponentials for a satisfactory fit, with lifetimes (fractional weights) of 21.6 ns (56.3%), 132.5 ns (31.6%), and 675.8 ns (12.1%). The major component (21.6 ns) has been observed previously in the fluorescence decays of many fluorescent Au(0) nanoclusters and attributed to emission from singlet excited states.[3a,14,19] The long lifetime components (>100 ns) are characteristic of Au(I)–thiol complexes, suggesting contributions of such complexes to the luminescence of Au NCs due to ligand–metal charge transfer and Au(I)–Au(I) interactions.[20] Further reduction of the Au NCs with $NaBH_4$ caused a 30% decrease of the luminescence, accompanied by a slight increase in the absorption in the visible range (Figure S5 in the SI). These spectral changes confirm the existence of Au(I) complexes on the particle surface and their substantial contribution to the luminescence of Au NCs.[14,21]

High-resolution transmission electron microscopy (HRTEM) was used to measure the core size of DHLA–AuNCs, which revealed an average diameter of (1.4 ± 0.3) nm, as judged from image analysis of more than 200 individual particles (**Figure 2**A). The close-up in the inset shows lattice planes separated by about 0.235 nm, corresponding to the (111) lattice spacing of the face-centered cubic Au (0.23 nm).[22] The hydrodynamic diameter determined by dynamic light scattering (DLS) as (3.2 ± 0.1) nm (Figure 2B) is significantly larger than the TEM value because the DHLA shell contributes to the DLS diameter, whereas TEM reveals merely the core size of the particles. The very small

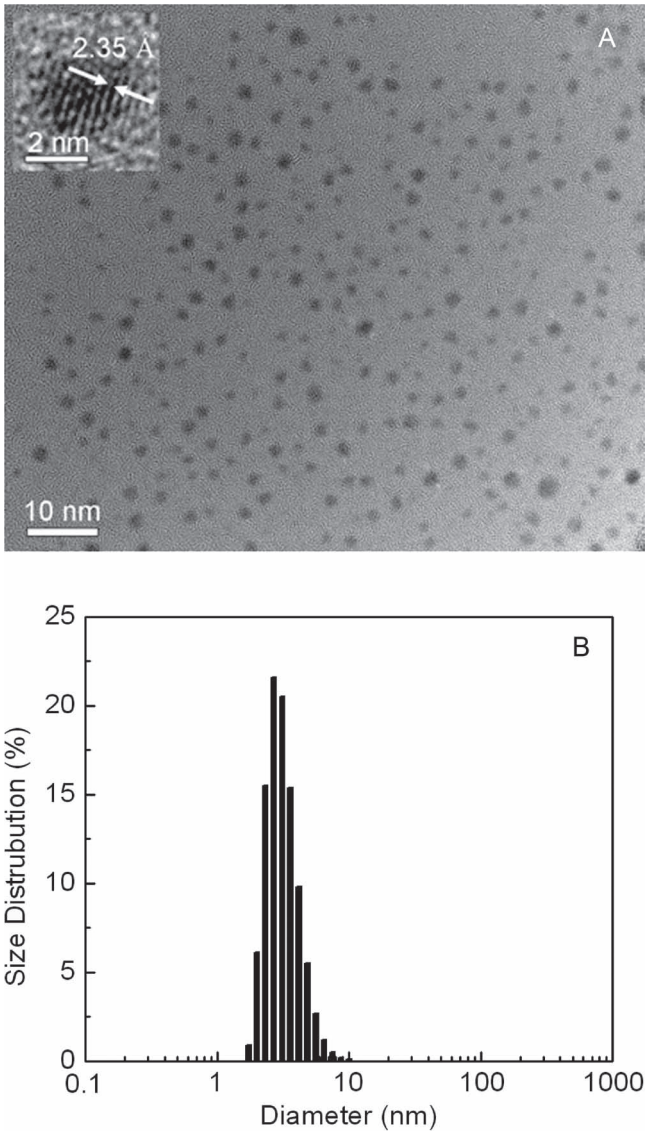

Figure 2. A) Typical TEM image of DHLA–AuNCs. The inset shows a close-up displaying the crystalline structure of an individual nanocluster. B) Size distribution of an aqueous solution of DHLA–AuNCs as determined by DLS.

nanoparticle size in colloidal solution, well below 5 nm, makes DHLA–AuNCs particularly attractive as fluorescence probes, e.g., for labeling biomacromolecules and super-resolution imaging.[23] Their small size and the bidentate chelating effect afforded by the dithiol groups of DHLA suggest an excellent colloidal stability. The zeta potential of the DHLA–AuNCs in phosphate buffer solution (10 mM, pH 7.4) was –(37 ± 3) mV. Typically, zeta potentials below –30 mV are considered an indication of a colloidally stable system.[24] Indeed, we observed that these clusters were stable in buffer solution for over 3 months without any precipitation and the optical properties neither changed significantly over this period (Figure S6 in the SI).

X-ray photoelectron spectroscopy (XPS) measurements were carried out to analyze the valence states of gold and sulfur in our DHLA–AuNCs. The Au 4f XPS spectrum

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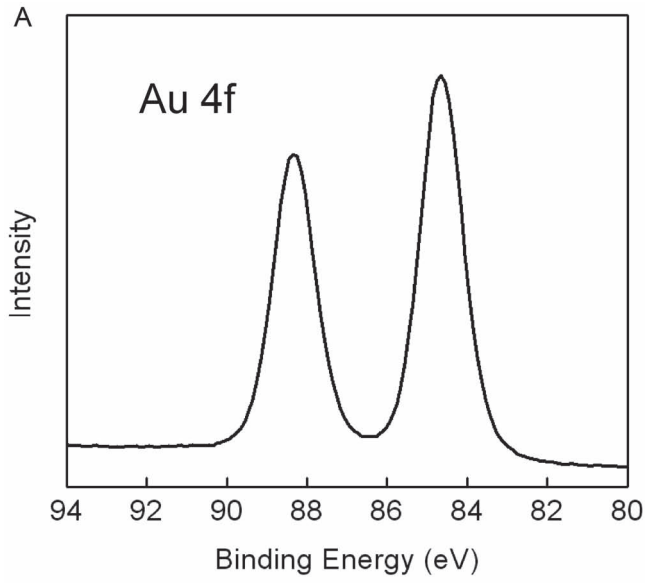

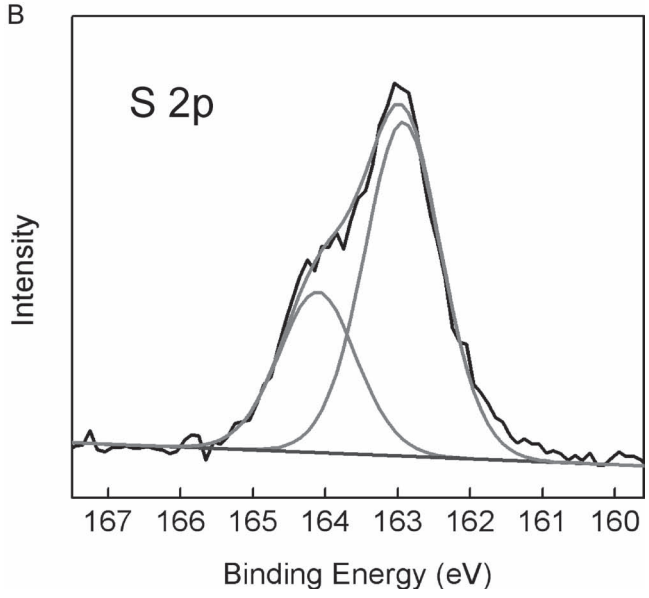

Figure 3. XPS spectra showing the binding energy of Au 4f (A) and S 2p (B) of DHLA–AuNCs. XPS spectrum of S 2p was fitted considering two bands (grey) at 163.0 and 164.1 eV.

(**Figure 3**A) shows the binding energy (BE) of Au $4f_{5/2}$ and Au $4f_{7/2}$ at 88.3 eV and 84.6 eV, respectively. Note that the BE of Au $4f_{7/2}$ falls between the Au(0) BE (84 eV) of a metallic gold film and the Au(I) BE (86 eV) of gold thiolate, suggesting the coexistence of Au(0) and Au(I) in the clusters.[14,16a,21] This observation further supports our hypothesis that a fraction of gold atoms of these luminescent Au NCs exists in the Au(I) oxidation state. Moreover, the XPS spectrum of S 2p reveals an asymmetric band that can be fitted by two peaks (Figure 3B) at 163.0 and 164.1 eV. While the dominant peak at 163.0 eV is assigned to sulfur atoms bound to gold surfaces as thiolate species, the smaller peak at 164.1 eV can be attributed to unbound thiolates, implying that a small fraction of

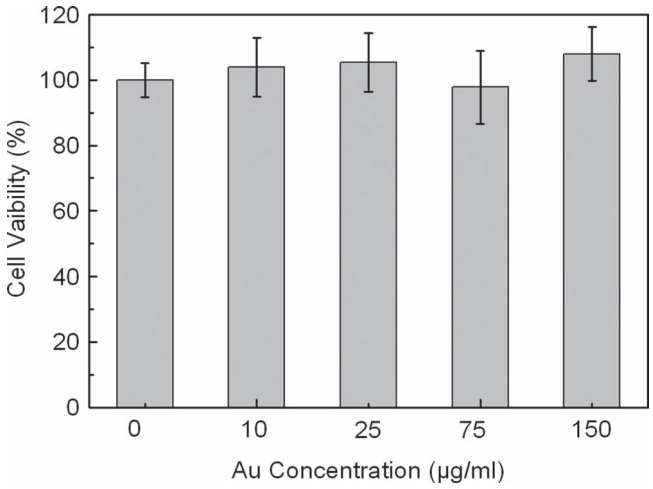

Figure 4. Viability of HeLa cells after 24 h of incubation with different concentrations of DHLA–AuNCs in the cell medium as determined by a MTT assay. The error bars represent variations among four independent measurements.

DHLA may be anchored on the gold surfaces by only one sulfur atom.[11b] Remarkably, the typical band representing the oxidized form of sulfur (at about 167 eV) was absent from the S 2p region of our Au NCs, in contrast to many previous studies of Au cluster,[25] further suggesting an excellent stability of DHLA-capped Au NCs.

The biocompatibility of our clusters was evaluated by testing the viability of live cells upon exposure to DHLA–AuNCs using anthiazolyl blue tetrazolium bromide (MTT) assay.[26] Cell viability was not affected after incubation with DHLA–AuNCs in the concentration range of 0–150 μg/mL (**Figure 4**). Nor did we observe any morphological changes in our microscopic observations that would hint at adverse effects of DHLA–AuNC exposure.

Fluorescence microscopy is a powerful technique for live-cell imaging due to its essentially non-invasive nature.[27] FLIM offers additional advantages because it is sensitive to the local environment of a molecular probe, independent of the fluorescence intensity or local probe concentration. In general, biological tissues exhibit fluorescence from a variety of endogenous fluorophores including aromatic amino acids, pyridinic (NADPH, nicotinamide adenine dinucleotide phosphate) and flavin coenzymes, which can be excited by UV/visible radiation.[28] These fluorophores have characteristic lifetimes in the picosecond to nanosecond regimes[29] whereas our DHLA–AuNCs possess characteristic lifetimes of hundreds of nanoseconds. Consequently, by using lifetime gating in FLIM applications, we can employ our clusters as nanoscale probes to image cells in the complete absence of cellular autofluorescence.

As a first FLIM application, we investigated the internalization of DHLA–AuNCs by live HeLa cells. **Figure 5** shows the intensity (A) and lifetime (B) images of the cells without NCs. Significant autofluorescence is emitted by the cells, with lifetimes ranging from 1.5 to 4 ns. After exposure to Au NCs for 1 h, the interiors of the cells were stained with luminescent emitters exhibiting long fluorescence lifetimes

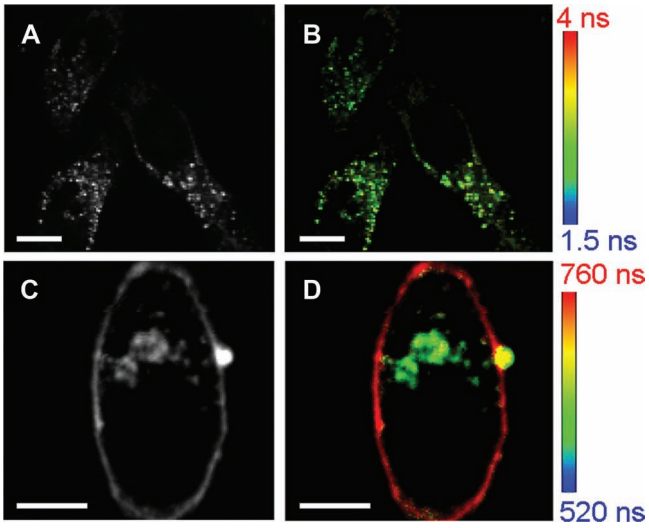

Figure 5. Intensity (A,C) and FLIM (B,D) images of cells only (A,B) and cell incubated with 100 μg/mL DHLA–AuNCs for 1 h (C,D). Scale bar: 10 μm.

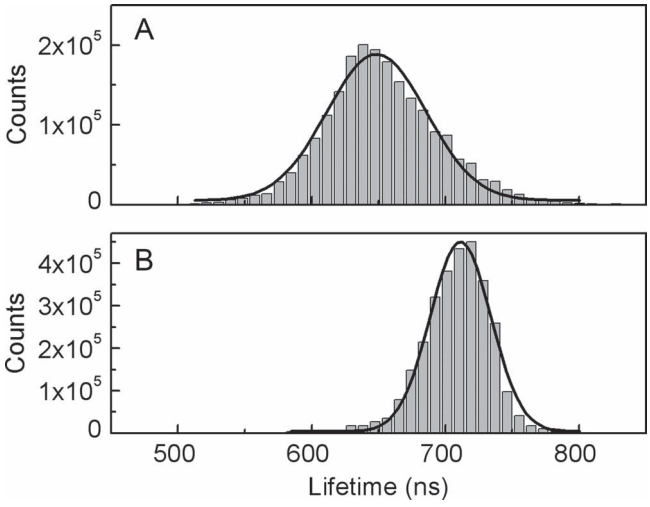

Figure 7. Average lifetime histograms of Au NCs inside the cell (A) and associated with the cell membranes (B), obtained from FLIM images of HeLa cells upon exposure to Au NCs for 1 h.

(500 to 800 ns), as shown in Figure 5D, suggesting that the Au NCs had been internalized by the cells.[30] The fluorescence decay of our prepared DHLA–AuNCs in the cells was found to be slower than that in aqueous solution (**Figure 6**); the intensity-weighted average lifetime of the Au NCs increased from (442 ± 13) to (656 ± 10) ns upon uptake by the cells. This lifetime change may reflect the modified NC environment during the uptake process including the formation of a protein corona around the NCs.[31]

Compared with the corresponding intensity image (Figure 5C), the lifetime image here clearly shows an interesting contrast among Au NCs in different locations, thereby providing additional information. Au NCs near the cell membrane display longer lifetimes than those internalized by the cells (Figure 5D). The analysis of the lifetime distributions for Au NCs in the intracellular (A) and membrane (B) regions (**Figure 7**) also reveal a longer average lifetime for the latter due to the different local environments. The full-width-at-half-maximum (FWHM) of the distribution for intracellular particles, (94 ± 4) ns, is larger than the one in the membrane region, (55 ± 3) ns, reflecting a more heterogeneous environment inside the cells. Thus, FLIM image not only reveals the uptake of Au NCs by the cells but also provides information on their changed local environment.

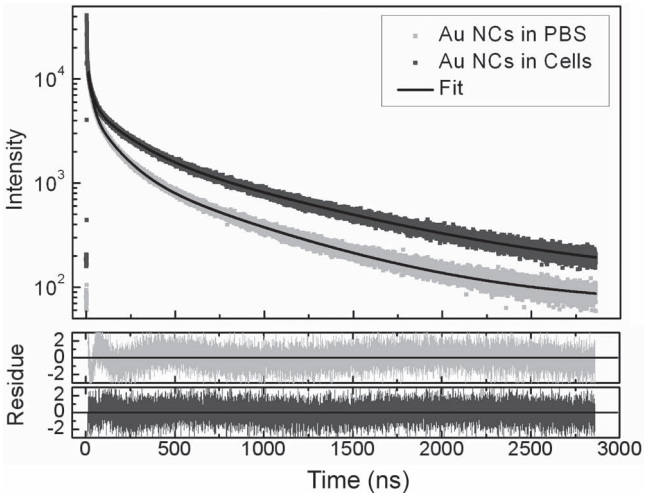

Figure 6. Top: Fluorescence decays for DHLA–AuNCs in aqueous (phosphate-buffered saline, PBS) solution (light grey) and after internalization by cells (dark grey), and the corresponding fitted curves (black) by multi-exponential decay functions. Bottom: Residuals of the fits.

3. Conclusion

In summary, a new, simple and convenient strategy was developed to synthesize water-soluble DHLA-capped luminescent gold clusters. The obtained Au NCs possess high colloidal stability, bright near-infrared fluorescence emission, small hydrodynamic diameter (3.2 nm) and good biocompatibility as verified by a cytotoxicity test, making them promising candidates as novel fluorescent probes for application in biological research. Moreover, the average fluorescence lifetime of DHLA–AuNCs is two orders of magnitude longer than the lifetime of cellular autofluorescence, so that they can easily be imaged by using lifetime gating. A first bioimaging application showed that DHLA–AuNCs are indeed attractive as fluorescence markers, especially for FLIM. We expect that fluorescent Au NCs will find wide application in biomedical research, including intracellular drug delivery, ultrasensitive molecular diagnostics and, possibly, even image-guided therapy.

4. Experimental Section

Reagents: All chemicals used were of analytical grade. LA, gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), Rhodamine 6G, and thiazolyl blue tetrazolium bromide (MTT) were purchased from

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Sigma-Aldrich (Milwaukee, USA). Sodium borohydride ($NaBH_4$) and dimethylsulfoxide (DMSO) were obtained from Carl Roth (Karlsruhe, Germany). Disodium hydrogen phosphate anhydrous (Na_2HPO_4) and sodium dihydrogen phosphate monohydrous ($NaH_2PO_4 \cdot H_2O$) were purchased from Merck (Darmstadt, Germany). Phosphate buffer solution (10 mM) was prepared by mixing different volumes of Na_2HPO_4 (10 mM) and NaH_2PO_4 (10 mM) solution. In all preparations, high-purity deionized water from a Millipore system was used.

Synthesis of DHLA-Stabilized Gold Nanoclusters (DHLA–AuNCs): All glassware was cleaned in a bath of freshly prepared aqua regia ($HCl:HNO_3$, 3:1 by volume) and rinsed thoroughly in Millipore water prior to use. A typical synthesis is described as follows: 1.3 mg lipoic acid was added to 3.9 mL aqueous solution containing 10 µL NaOH (2 M). The mixture was stirred for 15 min, followed by addition of a solution of $HAuCl_4$ (40 µL, 2% by mass). The solution turned from light yellow to colorless upon stirring for another 5 min. Subsequently, an aqueous solution of sodium borohydride (80 µL, 50 mM) was added slowly to the mixture under rapid stirring. The reaction, which turned the colorless solution pale brown, was stopped after stirring overnight. Au NCs were purified by triple centrifugation filtration, using Nanosep filters (Pall Nanosep, Ann Arbor, MI) with a molecular weight cut-off of 10 kDa to remove impurities. The brownish DHLA–AuNCs remained on the filter. They were resuspended in phosphate buffer solution (10 mM, pH 7.4) and stored at 4 °C for later use.

Cell Culture and Cytotoxicity Assay: HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, California), supplemented with 10% fetal bovine serum, 100 U of penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37 °C and 5% CO_2. For the MTT assay to test the toxicity of DHLA–AuNCs on HeLa cells, 12 mM MTT stock solution was prepared by adding 5 mg MTT in 1 mL of sterile phosphate-buffered saline (PBS, contains monobasic potassium, sodium chloride, and dibasic sodium phosphate, Invitrogen), and stored at 4 °C in the dark. Cells were seeded in a 24-well plate in 1 mL of DMEM overnight and, subsequently, incubated with different concentrations of DHLA–AuNCs (0, 10, 25, 75, and 150 µg/mL) in cell medium for 24 h at 37 °C and 5% CO_2. Four replicate samples were prepared for each concentration. Cells were washed twice with PBS followed by addition of 200 µL fresh medium and 10 µL MTT stock solution to each well. They were incubated for 4 h at 37 °C and 5% CO_2. After removing all but 50 µL of medium from the wells, we added 150 µL DMSO to each well, mixed the solution thoroughly with a pipette and incubated at 37 °C for 10 min. The entire content of each well was mixed again before finally measuring the absorbance of the solution at 550 nm.

Imaging Au NCs in Cells with FLIM: HeLa cells were seeded in 2-well LabTek chambers (Nalge Nunc International, New York, USA) and allowed to adhere for 24 h at 37 °C and 5% CO_2. After removing the medium by 3× washing with PBS, cells were incubated with 100 µg/mL DHLA-AuNCs in serum-free DMEM (Invitrogen, Carlsbad, USA) at 37 °C and 5% CO_2 for 1 h. Subsequently, cells were washed with PBS and fixed with 1 mL of 4% (by mass) paraformaldehyde (Applichem GmbH, Darmstadt, Germany) in PBS for 15 min. Afterwards, the cells were washed with PBS and 1 mL of 50 mM ammonium chloride solution to deactivate the paraformaldehyde. Finally, the fixed cells were covered with 1 mL PBS in the chamber and stored at 4 °C for imaging experiments.

FLIM data were collected with the time-resolved confocal fluorescence microscopy system (Microtime 200, PicoQuant, Berlin, Germany) equipped with a water immersion objective (1.2 numerical aperture (NA), 60×) (Olympus, Tokyo, Japan) and a diode laser (LDH-P-C-470, 470 nm, PicoQuant) for picosecond pulsed excitation at a repetition rate of 333 kHz. The fluorescence, after passing through a band-pass filter 690/70 (Chroma Tech, Rockingham, VT), was collected and detected by an avalanche photodiode (Microphoton Devices, Bolzano, Italy). Lifetime maps were calculated on a pixel-by-pixel basis by fitting the lifetime to the intensity decay of each pixel. The fits of the fluorescence decay traces for Au NCs require at least three exponential functions to reach acceptable residuals ($\chi^2 < 1.1$). The intensity-weighted average lifetime (τ_{av}) describing the mean time delay of photon emission after the picosecond laser pulse was calculated according to[32]

$$\tau_{av} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (1)$$

where the α_i represent the fractional weights of the various decay time components τ_i of the multi-exponential fitting.

Nanocluster Characterization: UV–vis absorption spectra were recorded with a Cary 100 spectrophotometer (Varian, Palo Alto, USA); fluorescence spectra were taken on a Fluorolog-3 Spectrofluorometer (HORIBA Jobin Yvon, Edison, USA). HRTEM was performed on a Philips CM 200 FEG ST (FEI, Eindhoven, Netherlands) equipped with a thermally assisted field-emission gun, which was operated at an accelerating voltage of 200 kV. We prepared TEM samples by spraying a dispersion of Au NCs onto a Cu grid covered by a holey carbon film. XPS measurements were carried out on a K-Alpha XPS spectrometer (ThermoFisher, E. Grinstead, UK), using Al Kα X-ray radiation (1486.6 eV) for excitation. All spectra were referenced to the C 1s peak at 285.0 eV. DLS and zeta-potential experiments were carried out on a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) with a 633 nm laser at 25 °C.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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