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TITLE:

Synthesis of Near-Infrared Emitting Gold Nanoclusters for Biological Applications

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SUMMARY:

A reliable and easily reproducible method for preparation of functionalizable, near-infrared emitting photoluminescent gold nanoclusters and their direct detection inside HeLa cells by flow cytometry and confocal laser scanning microscopy is described.

ABSTRACT:

Over the past decade, fluorescent gold nanoclusters (AuNCs) have witnessed growing popularity in biological applications and enormous efforts have been devoted to their development. In this protocol, a recently developed, facile method for preparation of water soluble, biocompatible, and colloidally stable near-infrared emitting AuNCs have been described in detail. This room-temperature, bottom-up chemical synthesis provides easily functionalizable AuNCs capped with thioctic acid and thiol-modified polyethylene glycol in aqueous solution. The synthetic approach requires neither organic solvents or additional ligand exchange nor extensive knowledge of synthetic chemistry to reproduce. The resulting AuNCs offer free surface carboxylic acids, which can be functionalized with various biological molecules bearing a free amine group without adversely affecting the photoluminescent properties of the AuNCs. A quick, reliable procedure

for flow cytometric quantification and confocal microscopic imaging of AuNC uptake by HeLa cells also been described. Due to the large Stokes shift, proper setting of filters in flow cytometry and confocal microscopy is necessary for efficient detection of near-infrared photoluminescence of AuNCs.

INTRODUCTION:

In the past decade, ultrasmall (≤ 2 nm) photoluminescent gold nanoclusters (PL AuNCs) have emerged as promising probes for both fundamental research and practical applications[1–10]. Their many desirable characteristics include high photostability, tunable emission maxima, long emission lifetimes, large Stokes shifts, low toxicity, good biocompatibility, renal clearance and facile bioconjugation. PL AuNCs can provide photoluminescence from the blue to the near-infrared (NIR) spectral region, depending on the number of atoms within the cluster[11] and the nature of the surface ligand[12]. NIR (650-900 nm) emitting AuNCs are particularly promising for long-term in vitro and in vivo imaging of cells and tissues, as they offer high signal-to-noise ratio due to minimum overlap with intrinsic autofluorescence, weaker scattering and absorption, and high tissue penetration of NIR light[13, 14].

In recent years, various approaches that take advantage of Au-S covalent interactions have been developed to prepare NIR-PL AuNCs capped with a variety of thiol-containing ligands[13,15–17]. For biomedical applications, AuNCs must be functionalized with a biological component to facilitate binding interactions. Thus, AuNCs with high colloidal stability that are easily functionalizable in aqueous solvent are highly desirable. The overall goal of the current protocol is to describe a previously reported[18] preparation of AuNCs with a functionalizable carboxylic acid group on the surface by employing thioctic acid and polyethylene glycol (PEG) in an aqueous environment in detail and their conjugation with molecules bearing a primary amine following the acid-amine coupling method. Because of the ease of synthesis and high reproducibility, this protocol can be used and adapted by researchers from non-chemistry backgrounds.

One of the key requisites for applications of AuNCs in biomedical research is the ability to observe and measure AuNCs inside cells. Among the methods available to monitor nanoparticle uptake by cells, flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) offer robust, high-throughput methods which allow fast measurements of internalization of fluorescent nanomaterials in large number of cells[19]. Here, FCM and CLSM method for direct measurement and analysis of PL AuNCs inside cells, without the need for additional dyes, have also been presented.

PROTOCOL:

1. Preparation of near-infrared emitting AuNCs (1)

1.1. Add 1.3 mg (6.3 µmol) thioctic acid (TA) and 10 µL of 2 M NaOH to 3.9 mL of ultrapure water (resistivity 18.2 MΩ.cm at 25 °C) and stir (at least 1,000 rpm) until it dissolves completely (~15-20 min). For faster dissolution of TA, sonicate the mixture. For the synthesis, freshly prepared TA

solution is recommended.

1.2. Add 1.7 µL of $HAuCl_4 \cdot 3H_2O$ (470 mg/mL) of aqueous solution to the solution.

1.3. After 15 min, add 80 µL of $NaBH_4$ (1.9 mg/mL) under vigorous stirring (at least 1,000 rpm) and stir the reaction mixture under the same conditions overnight.

1.3.1. Freshly prepare the $NaBH_4$ solution in ice-cold ultrapure water and add to the reaction mixture immediately after preparation.

NOTE: The synthesis of AuNCs is easily scalable. Up to 2 L of AuNCs was synthesized in a single batch, without any change in the optical properties of the particles.

1.4. (Critical) The next day, purify the solution by applying three cycles of centrifugation/filtration using a membrane filtration device with a molecular weight cut-off of 3 kDa. Without this purification procedure, the following step does not work properly.

1.5. Add thiol-terminated polyethylene glycol (MW 2,000; 2.6 mg; 1.3 µmol) to the solution, adjust the pH to 7-7.5 and stir the mixture overnight to obtain **1**. Purify the dispersion by applying three cycles of centrifugation/filtration using a membrane filtration device with a molecular weight cut-off of 3 kDa.

NOTE: Adjustment of the pH to 7-7.5 is extremely important. Higher pH can result in a blue shift of emission maxima.

2. Conjugation of 3-(aminopropyl)triphenylphosphonium bromide (TPP) on the surface of 1

2.1. Mix the **1** solution (4 mL) prepared in the previous step and 3-(aminopropyl)triphenylphosphonium bromide (2 mg, ~5 µmol). Adjust the pH to 4.5 with 1 M HCl.

NOTE: 3-(Aminopropyl)triphenylphosphonium (TPP) bromide salt was prepared as described in the literature[20].

2.2. Start the reaction by adding an excess of *N*-(3-dimethyl-aminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) (10 mg, 52 µmol). The pH of the solution will increase and should not be allowed to go beyond 6. Monitor the pH of the reaction mixture for the first hour. If the pH increases above 6, reduce it to 4.5–6 by adding 1 M HCl.

2.3. Stir the reaction mixture overnight at room temperature.

2.4. Purify the dispersion by applying three cycles of centrifugation/filtration using a membrane filtration device with a molecular weight cut-off of 3 kDa to obtain **2**. Dilute **2** obtained here with

ultrapure water to the initial volume of 4 mL. The concentration of Au in the solution is 200 µg/mL.

3. Cell culture

3.1. Culture HeLa cells (HPA culture collection) in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37 °C.

3.2. Split and passage the cells when they reach ~80% confluence. To minimize acquisition of new mutants, the number of cell propagations should not exceed 30.

4. AuNC internationalization into HeLa cells

4.1. Seed the cells in a 12-well plate at a density of 20,000 cells/mL (1 mL/well). The goal is to achieve ~50% confluence after 48 h.

4.2. At 48 h post-seeding, aspirate the culture medium and add 400 µL of complete culture medium (for untreated controls) or 500 µg of nanoparticles in 400 µL of complete cultured medium (for treated samples) to each well. Return the cultures to a 37 °C incubator.

NOTE: Addition of high volumes of AuNC solution adversely affects the cell viability. AuNC solutions need to be concentrated. Thus **2** obtained in step 2.4 is concentrated 100 times. 40 mL AuNC was concentrated to 400 µL. A 25 µL aliquot of this concentrated solution was added to 400 µL cell culture media to obtain the desired AuNC concentration.

4.3. After 2 h of internationalization, detach the cells by standard trypsinization according to the manufacturer's protocol.

4.4. Collect the samples in polypropylene microcentrifuge tubes and centrifuge for 5 min at 350 x *g* at 4 °C.

4.5. Prepare the following FCM buffer: pre-chilled phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM NaH_2PO_4, pH 7.4) supplemented with 2% bovine serum albumin at 4 °C.

4.6. Wash the pellets with 1 mL of FCM buffer and centrifuge for 5 min at 350 x *g* at 4 °C.

4.7. Resuspend the pellet in 500 µL of FCM buffer and store samples at 4 °C prior to analysis.

5. Flow cytometry analysis

5.1. Filter all samples using a 5 mL polystyrene round-bottom tube with a cell-strainer cap.

5.2. Before acquiring data using the instrument software, specify the cytometer configuration.

5.3. Format all dot-plots and histograms for 'Acquisitions'.

5.4. Plot a two-parameter dot plot of the forward scatter area (FSC-A) and side scatter area (SSC-A) to show distribution of cells. To exclude doublets, create a two-parameter dot plot of FSC height (FSC-H) vs. FSC-A. To monitor the relative fluorescence intensity in the sample, plot a single-parameter histogram for the fluorescent channel area (FL-A). Use a linear scale to depict FSC and SSC data, and a logarithmic scale for all fluorescent parameters.

5.5. Acquire untreated sample (without nanoparticles) at a low flow rate to minimize coincident events (if allowed by the instrument). During acquisition, adjust the photomultiplier tube (PMT) voltages to get the untreated population on scale on the FSC vs SSC plot. If necessary, adjust PMT voltages for the FL channel to place the unstained population on the left corner of the histogram.

5.6. Select the specific 'gate tab' in the software and draw an appropriate gate around the desired population. Cells inside the gate will move to the next checkpoint.

5.7. Record 10,000 events per sample.

5.8. Record all samples under the same instrument settings.

5.9. Use an appropriate program to analyze the flow cytometry data.

NOTE: Octagon and trigon arrays are located in the left side of the instrument, and up to two trigon arrays are located in the front doors.

5.10. To change filters on a flow cytometer, open the drawer and remove the filter with the detector array in the required position. Replace the filter with a filter of choice. Verify that the filter with the longest wavelength is in position A and the one with the shortest wavelength is in the last position.

5.11. Save and reload the experiment to preserve the instrument settings and gating strategy. A side scatter height (SSC-H) vs. side scatter area (SSC-A) plot can be also used for doublet exclusion. This type of gating can be more sensitive as the FSC detector is not usually a PMT.

6. Internalization of 2 into HeLa cells for confocal laser scanning microscopy (CLSM)

6.1. Seed the cells onto a 4-chamber glass bottom 35 mm dish at a density of 250,000 cells/mL (0.5 mL/chamber). Keep the chamber in a 37 °C incubator with 5% CO_2 atmosphere. The goal is to achieve ~50% confluence after 24 h.

6.2. At 24 h post-seeding, add 100 µg of **2** (or 10 µL from the stock solution of 10 mg/mL) to each

dish chamber containing 0.5 mL of medium with the cells (for treated samples).

6.3. Return the dish to the incubator. Let the cells internalize the AuNCs for 24 h prior to using them for CLSM.

6.4. After the internalization period, discard the medium and wash the cells with pre-warmed fresh medium for 5 min. Repeat the washing step once more. Then fill each chamber with 800 µL of fresh medium.

7. CLSM Imaging of live Hela cells labeled with 2

7.1. For microscopic imaging, use 63x oil (n = 1.518) objective lens (NA = 1.4) in a confocal microscope with Plan-Apochromat.

7.2. Mount the dish on the microscope inverted stage with the chamber warmed to 37 °C and supplied with humidified 5% CO_2 atmosphere.

7.3. To detect internalized AuNC, use a 405 nm laser set at 2% power with an appropriate beam splitter. Set the range of detection wavelengths between 650 and 760 nm.

7.4. Set the resolution of the image to 2048 x 2048 pixels. In the acquisition speed setting, aim for a pixel dwell time around 4 µs. Acquire the image with 2x averaging (line mode, averaging method mean). Set the pinhole to 1 Airy unit (for 405 nm light). For higher sensitivity, use photon-counting mode.

7.5. For correct illumination in transmitted light with differential interference contrast (DIC), use Köhler's setting of the condenser and the field stop. For acquisition of transmitted light, use a 488 nm laser at 0.7% power without any fluorescence detector assigned. Set an appropriate beam splitter for the laser wavelength.

7.6. Acquire two images for each track (red fluorescence and DIC). Track AuNCs by their red fluorescence; cell boundaries are easily determined in the transmitted light with DIC pictures.

REPRESENTATIVE RESULTS:

NIR PL AuNCs were prepared from Au^{3+} in the presence of TA, and then thiol-terminated PEG (MW 2,000) was bound on the AuNC surface to obtain **1** following the workflow shown in **Figure 1.** Amidic coupling between **1** and 3-(aminopropyl) triphenylphosphonium (TPP) bromide provided **2**. As expected, absorption spectra (**Figure 2a**) indicated that AuNCs **1** and **2** do not have a characteristic surface plasmon band and show broad emission from 550 nm to 850 nm (**Figure 2b**). After attachment of TPP to the surface of **1**, the PL increased strongly. Emission from AuNCs was also visible under UV light (365 nm, **Figure 2b inset**). The emission from AuNCs is stable and emission wavelength is independent of excitation wavelength (**Figure 2c**). However, the emission intensity is maximal when excited with UV light.

2 was detected inside HeLa cells by monitoring the PL on a flow cytometer. HeLa cells were incubated for 2 hours with **2** at media concentrations between 0.5 mg/mL and 2 mg/mL. FCM data confirmed uptake of **2** by HeLa cells. NIR fluorescence (>720 nm) was dependent on both time (**Figure 3a**) and concentration of **2** (**Figure 3b**). Maximal intensity was observed with the 780/60 bandpass filter.

AuNCs within cells were imaged non-invasively by using a standard confocal laser scanning microscope. **Figure 4** shows the confocal image of HeLa cells stained with **2** (200 μg/mL). After 24 h of incubation bright red photoluminescence of **2** inside the cells was observed.

FIGURE LEGENDS:

Figure 1: Synthesis of the gold nanoclusters. Workflow of the preparation of **1** and **2**.

Figure 2: Optical properties of the gold nanoclusters. Normalized (**a**) absorption spectra (Inset: Photograph of the aqueous solutions of **1** and **2** under white light) and (**b**) photoluminescent spectra of 200 μg/mL aqueous solutions of **1** and **2** (Inset: photograph of the AuNC solutions under UV light (365 nm)). (**c**) Excitation-emission PL map of **2**. Excitation is shifted by 10 nm steps. The emission peak around 750 nm is very stable (does not shift with excitation) and shows an enormous Stokes shift from excitation. The most efficient excitation occurs around 340 nm.

Figure 3: Detection of gold nanoclusters inside the HeLa cells by flow cytometry. Internalization of **2** into HeLa cells was studied using FCM. The histograms show (**a**) time- and (**b**) concentration-dependent uptake of AuNCs by HeLa cells. In the time-dependent experiment, HeLa cells were untreated (control; 0 h) or treated with 1.28 mg/mL **2** and incubated at 37 °C for the indicated times. The cells were then washed with PBS and analyzed by FCM. In the concentration-dependent experiment, HeLa cells were untreated (control; 0 mg/mL) or treated with the indicated concentrations of **2** and processed in the same way.

Figure 4: CLSM imaging of HeLa cells labelled with the gold nanoclusters. HeLa cells were incubated with **2** (200 μg/mL) for 24 h and imaged with CLSM**.** (**a**) Represents red fluorescence channel (650-760 nm); (b) transmitted light channel (DIC) and (**c**) is overlay of (**a**) and (**b**). Scale bar, 50 μm.

Supplementary Figure 1: Stability test of 1. Photoluminescence spectra of **1** in 1 M NaCl at 0 h and after 72 h. The intensity was normalized to the maxima.

DISCUSSION:

NIR-emitting AuNCs were synthesized using a bottom-up approach in which the gold precursor solution ($HAuCl_4$) was treated with suitable thiol ligands, followed by reduction of Au^{3+}. Reduction of metal ions in aqueous solution tend to aggregate and results in large nanoparticles rather than ultrasmall NCs[21]. To prepare ultrasmall (≤2 nm) PL AuNCs, the synthetic conditions were adjusted to prevent formation of large particles and promote formation of ultrasmall clusters. The nature

of the ligands used to cap the AuNC surface also plays an important role in influencing the structure, electronic and optical properties of the particles[12,22–30]. Therefore, choosing suitable ligands capable of stabilizing ultrasmall clusters is the key to obtain highly fluorescent AuNCs. Thiol-containing ligands are the most commonly used stabilizers in synthesis of AuNCs, owing to the strong covalent bonding between thiols and gold. A previous report[31] indicates that multithiol-based ligands are far superior to monothiol ligands in stabilization of PL AuNCs. Multi-thiol ligands provide enhanced colloidal stability to AuNCs because of the higher number of binding sites between ligand and AuNC surface. Bidentate thiol TA was used for synthesis of NIR PL AuNCs because it provides much improved colloidal stability to AuNCs over a broad range of adverse conditions compared to monothiol ligands[32]. TA also provides aqueous phase growth of nanoparticles with discrete size control, and most importantly, it offers a carboxylic acid group on the surface of the nanoparticles that can be utilized for conjugation of biologically relevant molecules[33,34].

TA stabilizes AuNCs by electrostatic repulsion caused by deprotonated carboxylate groups on the surface[35]. However, in acidic solutions, TA-protected AuNCs become colloidally unstable due to protonation of the carboxylate group. Nanoparticles can be stabilized electrosterically, rather than purely electrostatically. This approach provides colloidal stabilization even in the presence of high salt concentrations and pH changes, which is important for biomedical applications. To confer electrosteric stabilization to the TA-AuNCs, the clusters were subsequently functionalized with thiol-terminated PEG (MW 2,000) at a 5:1 molar ratio of TA:PEG, yielding **1** (**Figure 1**). For successful attachment of thiol-terminated PEG, TA-AuNCs must be purified. Functionalization of AuNC with PEG has improved the aqueous solubility at acidic pH and an increase in colloidal stability in high ionic strength media. It is important that the attachment of thiol-terminated PEG is carried out at pH 7.0-7.5. Higher pH would result in blue shift of the emission maxima. Unbound ligands were removed by centrifugation/filtration using a membrane filtration device with a molecular weight cut-off of 3 kDa. The ligands that are associated with nanoparticles experience significant line broadening in ^{1}H NMR compared to free ligands, which can obscure peak assignments and integration[36]. Significantly broad ^{1}H NMR peak associated with thioctic acid and thiol-modified polyethylene glycol suggests the ligands are bound to the AuNC surface and removal of free ligands[18]. Successful integration of luminescent AuNCs into a biological environment requires stability over conditions, such as high ionic strength because biological media is rich in excess of ions. The stability of **1** was verified by monitoring the photoluminescence in 1 M NaCl over a period of 72 h. No significant change in photoluminescence properties in 1 M NaCl indicates high stability of the AuNCs (**Supplementary Figure 1**). AuNCs were stable in buffered solution for more than a year without any evidence of precipitation (data not shown).

1 offers carboxylic acid on the surface. Carbodiimide based coupling reagents are widely used to covalently link carboxylic acids to amines via formation of amide bond[37]. The most commonly used carbodiimide based coupling reagent in aqueous solution is 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl). EDC·HCl has been used for covalent coupling of TPP bromide with **1** to obtain **2**. One of the major advantages of this protocol

is conjugation of molecules with a primary amine group via amide bond formation without compromising fluorescence and colloidal stability. High-resolution transmission electron microscopy (HRTEM) characterization showed that the AuNCs **1** and **2** both have an average diameter of 1.15 ± 0.2 nm, which indicates the functional coupling do not alter the core size of the AuNCs[18]. Alternatively, the free carboxyl groups can be activated using EDC and Sulfo-NHS[38]. Solutions of **1** and **2** excited with a UV lamp (365 nm) fluoresce bright red (**Figure 1b, inset**), while they appear light yellow under ambient lighting (**Figure 1a, inset**). TPP conjugation increases the AuNC PL due metal-to-ligand charge transfer (MLCT)[18].

Nanoparticles can cause adverse biological effects which can limit their applications in biology. To evaluate the cytotoxicity of **2** on HeLa cells, XTT (sodium 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) cell viability assay was performed. HeLa cells treated with 2 (200 µg/mL) for 48 h showed no loss of cell viability compared to control cells. This observation suggests AuNCs are biocompatible, which makes them promising candidates as fluorescent probes for application in biological research.

When excited with a 405 nm laser, 2 provides a broad emission with a maximum around ~750 nm. The extremely large Stokes shift (~350 nm) allows the emitted light to be reliably distinguished from the exciting light source; however, the FCM filter setting needs to be configured appropriately. For 2, the 780/60 nm bandpass filter is ideal because of the broadness of the filter and the fact that the emission maximum of AuNCs is in the same region. It is very important to use broad bandpass filters at the region of emission maxima for efficient detection of PL[39–42]. The time- and dose-dependent fluorescence signal from cells treated with **2** suggests that FCM can be used to conveniently monitor cell studies using AuNCs. When the incubation time was increased to 24 h, a 40 µg/mL concentration of **2** was sufficient to detect AuNCs by fluorescence in FCM (data not shown). However, for short incubation times (1-2 h), higher concentrations of AuNCs are needed. This method of detecting AuNCs by NIR fluorescence signal with a standard flow cytometer will help further broaden the potential applications of AuNCs in biomedical science. The approach described here could be used to assess rates and mechanisms of cellular uptake[14], relationships between nanoparticle concentration and cellular toxicity, or effects of surface chemistry on nanocluster uptake in a quick and quantitative manner using FCM.

Cellular uptake of **2** by HeLa cells was imaged by CLSM. After 24 h of incubation bright red emission of **2** was detected upon excitation with 405 nm laser. However, a 405 nm laser also excites the intrinsic fluorophores inside the cells. To distinguish the signal of AuNC from autofluorescence, the emission from AuNC was collected above 650 nm. The attractive properties, such as bright near-infrared luminescence, high colloidal stability, good biocompatibility and above results demonstrate that the AuNCs are promising imaging agents for biomedical and cellular imaging applications.

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DISCLOSURES:

Some portions of the methods and results were previously presented in an article by Pramanik et al.[18] Here, these methods have been converted into practical point-by-point protocols. The authors declare no competing financial interests.

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Figure 1

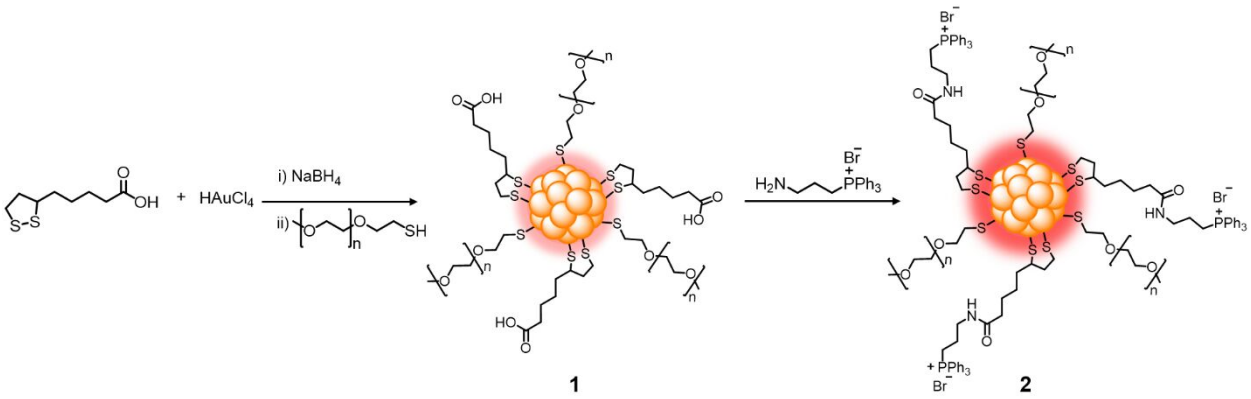

Figure 2

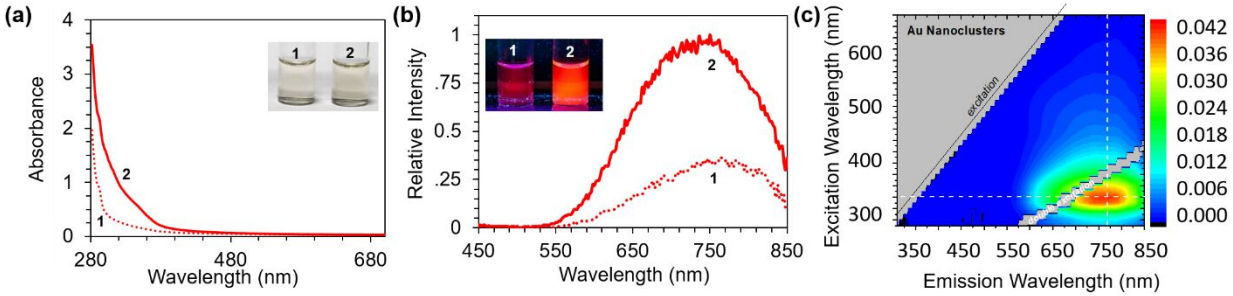

Figure 3

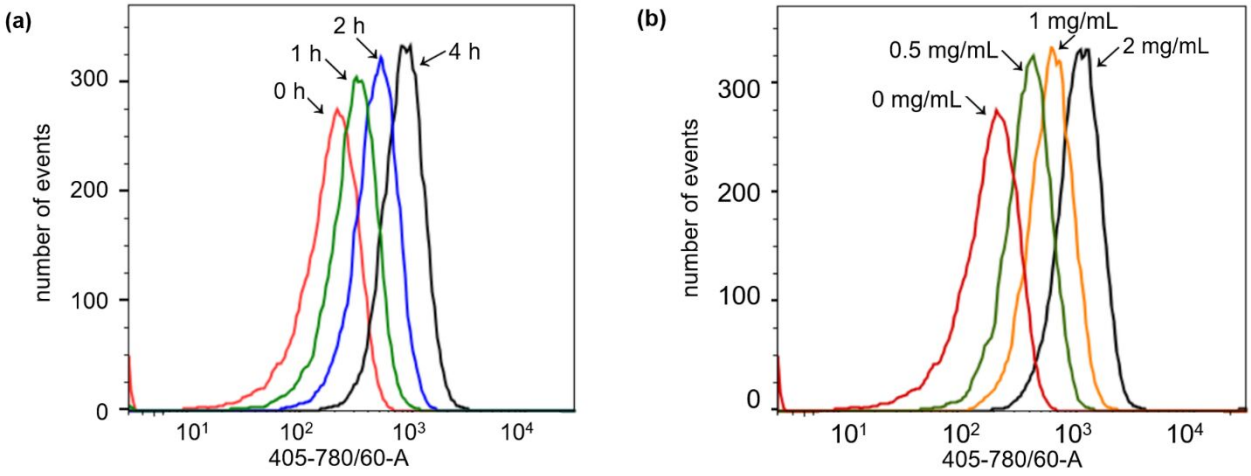

Figure 4

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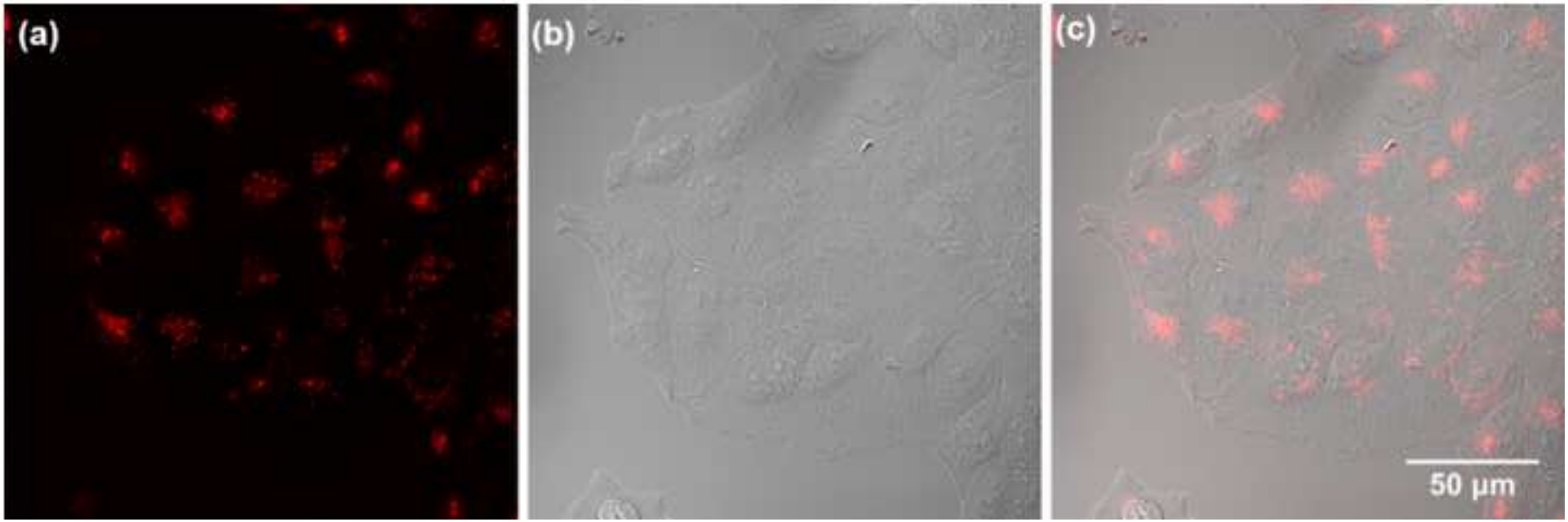

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1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride	TCI Chemicals	D1601
Bovine serum albumin	Sigma-Aldrich	A4161
Disodium hydrogen phosphate dihydrate	PENTA s.r.o.	15130-31000
DL-Thioctic acid, 98%	Alfa Aesar	L04711
Hydrochloric acid 35%	PENTA s.r.o.	19350-11000
Hydrogen tetrachloroaurate(III) trihydrate, ACS, 99.99% (metals basis), Au 49.0% min	Alfa Aesar	36400
O-(2-Mercaptoethyl)-O'-methylpolyethylene glycol 2000	Sigma-Aldrich	743127
Potassium chloride	PENTA s.r.o.	16200-31000
Sodium borohydride	Sigma-Aldrich	452882
Sodium chloride	PENTA s.r.o.	16610-31000
Sodium dihydrogenphosphate dihydrate	PENTA s.r.o.	12330-31000
Sodium hydroxide pellets	PENTA s.r.o.	15740-31000
XTT (sodium 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt)	Thermo Fisher Scientific	X12223

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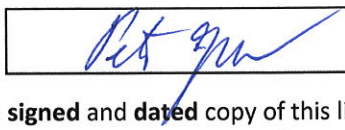
Signature:	
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