Cytosolic Diffusion and Peptide-assisted Nuclear Shuttling of Peptide-substituted circa 102 Gold Atom Nanoclusters in Living Cells

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Abstract. For biological and medical application, a definable nanomaterial and knowledge on its fate after administration are highly recommended if not mandatory. Here, we synthesized a water-soluble gold nanocluster by sodium borohydride reduction of chloroauric acid in presence of 5,5'-dithio-bis(2-nitrobenzoic acid). The resulting gold nanocluster displayed the physical characteristics of a cluster containing an inner core of about 100 gold atoms that was surrounded by an organic monolayer made of about 30 thioaminobenzoic acids (TAB) and 14 anionic thionitrobenzoic acids (TNB). The mixed TAB-,TNB-protected gold nanocluster reacted well in water with thiolated peptides containing a Nuclear Localization Signal (NLS) or a Nuclear Export Signal (NES) mainly by exchange of the TNB, providing gold nanoclusters equipped with 8-9 intracellular active peptides and a remaining ligand coverage consisting mostly of the zwitterion TAB. The behavior of these peptide-gold nanoclusters inside the cytosol and nucleus of cells was then assayed using an electroporation procedure allowing transient plasma membrane permeability. Light and electron microscopy observations demonstrated a consistent inflow and diffusion of the gold nanoclusters into the cytosol. Inside the living cells, the distribution of the gold nanoparticles was specifically driven by the appended signal peptides in a manner similar to the distribution of NLS and NES-bearing proteins, demonstrating diffusion ability, stability and usage of these definable ligand-substituted gold nanoclusters for intracellular applications.

Gold nanoclusters are attracting materials for biological applications because they can be tailored for photoluminescence,¹ for protein inhibition² glutathione detection³ or even sensitizing cancer cells to external radiation sources.^{4,6} The anionic mercaptobenzoic acid (MBA) has been used for producing water-soluble thiolate monolayer protected nanoclusters (AuNCs) of small sizes and even “magic” clusters with precise formula and structures.⁷⁻⁹ The gold cluster stabilized by an MBA-monolayer (AuMBA) could furthermore be conjugated to biological molecules, either by modifying the organic shell¹⁰⁻¹² or by exchange/substitution of the gold-conjugated mercaptobenzoate with an incoming thiolated macromolecule.¹³ Bioconjugates derived from AuMBA have already proven useful for structural analysis of supramolecular assemblies in aqueous solution using cryo-Electron Microscopy (EM) techniques.¹⁰⁻¹² They hold promise as a biomolecule-anchoring platform or as an imaging probe within living cells¹⁴ because the small and defined size of AuMBA should facilitate material characterization and diffusion in dense tissues¹⁵ and inside cells.¹⁶ To fulfill these intracellular application promises, passage across the plasma membrane and knowledge on the chemical and physical behavior of any nanomaterial inside the cytosol or nucleus are major challenging issues that still need to be resolved. First, the nanomaterial needs to be delivered into the cytosol, across the cell plasma membrane without impacting cell viability.^{17,18} In the majority of cases, the exogenous nanomaterial is tailored to bind to the cell surface for entrapment inside intracellular vesicles. When it contains as well elements able to lyse membranes, some leakage into the cytosol may occur but at an extremely poor efficiency.¹⁹⁻²² Second, the chemical and physical behavior of the nanomaterial inside the cell should be compatible with the desired objectives. For performing these investigations, large entrapment of materials within intracellular vesicles severely hampers interpretation and data analysis. Use of an effective delivery method, which bypasses vesicular entrapment for action, should hence bring a substantial benefit in term of data analyses.

Regarding the thiolate monolayer protected AuNCs, the intra-cytosol and intra-nucleus stability of the Au-S bond remains to be clarified. The Au-S bond has not been reported to be enzymatically cleaved but the cytosol contains thiols and particularly reduced glutathione (GSH) in a mM concentration range.²³ Since AuNCs can easily exchange thiolate ligands in solution,^{8,24} it is hence conceivable that the AuNC coupled thiolated ligand (i.e. the attached functional molecule) can suffer exchange with GSH inside living cells. Unstable Au-S bonds were observed for particles that were tailored to get majorly trapped in the intracellular lysosomal vesicles.^{3,6,24,25} However, to our knowledge, no data were reported to clearly demonstrate whether the Au-S bond of thiolate-protected AuNCs inside the cytosol or inside the cell nucleus breaks up or remains steady.

In this report, we develop a novel gold nanocluster allowing synthesis of definable peptide AuNC conjugates and then use an electroporation procedure to investigate the fate and behavior of bioactive peptide-AuNC conjugates inside living cells. First, we synthesized and characterized a new gold nanocluster (AuZ) in which the shell was mostly made of a zwitterionic thioaminobenzoic acid (TAB) because this ligand could stabilize the Au-S bond via a electron-donating substitution,²⁶ and because neutrally charged molecules can shield against unspecific interactions.^{27,28} Second, to probe the ability of peptide-grafted AuNCs to diffuse within the cell and the stability of the Au-S bond inside the cell, we selected peptide domains that selectively assist nuclear import or export of macromolecules through the nuclear pores. The AuZ was reacted with thiolated peptides containing the SV40 Nuclear Localization Signal (NLS), a mutated NLS defective for nuclear shuttling, or the Nuclear Export Signal (NES) of the protein kinase A inhibitor. The intracellular distribution of these peptide-grafted AuNCs was investigated after electroporation-mediated delivery. Light Microscopy and Transmission Electron Microscopy (TEM) imaging showed that the subcellular localization of the gold particles was specifically driven by the bioactive peptide's coverage, demonstrating that these gold particles can diffuse into the cytosol following electroporation to reach a concentration of

more than 4,000 molecules/cell and that the thiolated peptides remain stably coordinated to AuNCs for at least 24h in living HeLa cells.

Results

To obtain gold nanoclusters with zwitterionic ligands at a pH found inside cells, we tried a direct NaBH_4 reduction of HAuCl_4 in presence of 2,2'-dinitro-5,5'-dithiobenzoic acid (the Ellman's reagent DTNB²⁹) (Figure 1A), hoping that the anionic thionitrobenzoic acid (TNB) would be reduced to the zwitterionic TAB during the synthesis.³⁰ The protocol described by Levi-Kalisman and colleagues³¹ was slightly modified and cleanly led to a gold nanocluster (named AuZ) with a narrow mobility when analyzed by polyacrylamide gel electrophoresis (Figure 1B, inset image). The UV/visible absorbance of a 2.6 μM AuZ solution in 0.1 M HEPES, pH 7.4 gradually decreased with an increase in wavelength (Figure 1B). The spectrum did not contain any absorption band at 409 nm characteristic of TNB and did not contain a surface plasmon resonance band at wavelengths above 520 nm. This UV/vis spectrum indicated formation of gold nanoclusters containing less than 140 gold atoms because $\text{Au}_{144}\text{MBA}_{60}$ and AuNCs of higher molecular masses possess a surface plasmon resonance band at 510-530 nm.^{21,32} A routine ^1H Nuclear Magnetic Resonance (NMR) spectrum of the AuZ in D_2O was apparently featureless (Figure 1C). The aromatic peaks of the thiobenzoic acids, normally between 6-8 ppm, were too broad to be visible. The broadening of the signal was attributed to intimate ligand immobilization on the gold particle's surface that decreases the motion and the spin relaxation time of the analyzed atoms. It might also arise from the fact that Au-coordinated ligands have different chemical environment and hence different NMR signals.^{33,34}

A. Synthesis of gold cluster

B. UV-vis spectrum

C. ^1H NMR spectrum

D. Cryo-EM

E. Mass spectrum

Figure 1. Synthesis and analysis of the AuZ nanoclusters. A. Synthesis protocol. B. UV-vis spectrum of the gold nanocluster AuZ (2.6 μM , black line) or thionitrobenzoic acid (18 μM , blue line) in 0.1 M HEPES-Na, pH 7.4. The inset image is a polyacrylamide gel electrophoresis analysis of the AuZ. C. ^1H NMR spectrum of 55 μM AuZ in D_2O . The large peak at 4.7 is the signal of the D_2O and was trimmed. Tertio-butanol (100 μM final concentration) was used as an internal standard. D. Cryo-electron microscopy images of AuNCs in a frozen non-crystalline aqueous solution (96 000 x). The right panel shows a gallery of 99 particles that were cropped from the left image. Statistical analysis on 95 particles gave a mean diameter of 1.42 nm and a standard deviation of 0.16 nm. E. MALDI-TOF mass spectrum of the gold cluster.

To analyze if the AuZ was fully dispersed in water, an AuZ solution was frozen for cryo-EM observation. Images showed mostly a population of individual particles of uniform spherical shape and size (Figure 1D).³⁵ Assuming a spherical morphology for all particles, statistical analysis on 95 particles gave a mean diameter of 1.42 nm with a standard deviation (S.D.) of 0.16 nm. This diameter corresponds to a metallic cluster of 88 gold atoms using the volumetric mass density of Au (19.3 g.cm⁻³). The real diameter of the particle is likely slightly above this mean value because of the applied defocus and delocalized scattering electrons. The MALDI-TOF mass spectrum (MS) of AuZ displayed a population of molecular masses centered at 21,240 Da (Figure 1E). This pattern was strikingly similar to the one published for Au₁₀₂MBA₄₄ (calculated molecular mass of 26.83 kDa) where a broad distribution of masses centered at 21 kDa was obtained, suggesting that the laser-induced desorption and ionization condition promoted in both cases similar sample degradation.³¹

The AuZ was next analyzed by X-ray Photoelectron Spectroscopy (XPS) (Figure 2A). The XPS survey spectrum showed the characteristic peaks of Au 4f, Si 2s, S 2p, C 1s, N 1s and O 1s. The Si 2s peak (around 150 eV) originates from the Si wafer substrate and no other elements are detected. High-resolution analysis of S 2p, and C 1s peaks confirmed that all the aromatic thiolates were fully coordinated to the gold surface (Supporting Information, Figure S1). The N 1s region (inset of Figure 2A) reveals three distinct nitrogen species with peaks at 399.1 eV, 401 eV and 405.9 eV. The peaks at 399.1 and 401 eV can be assigned to an aromatic amine (the TAB) and an ammonium (likely residual ammonium counterions), respectively.³⁶ The N 1s peak at 405.8 eV was ascribed to NO₂-groups³⁷ and originated from the TNB. Integration of the N 1s peaks led to a TAB/TNB ratio of 2.12, suggesting that 68% of the nitro groups were reduced to amines during the nanocluster synthesis. The Au 4f_{7/2} photoelectron peak of the AuZ was shifted by 0.46 eV to higher binding energies relative to the polycrystalline Au(0) (Figure 2B).

Figure 2. XPS spectra of AuZ particles A. survey spectrum with high resolution N 1s core level spectrum included in the inset. B. Au 4f core level peak and C. valence band. The Au 4f and valence band spectra of metallic Au(0) sample are shown for comparison.

In the same time, the spin-orbit splitting E_{so} of AuZ (2.32 eV) was reduced relative to the E_{so} of polycrystalline Au(0) (2.74 eV) (Figure 2C). Using the published relationship between the gold particle size and these values, the AuZ cluster contains on average 103 ± 32 Au atoms.³⁸ The XPS analysis suggested that AuZ is reminiscent of a 102 gold atom cluster, which corresponds to a stable nanocluster according to the closed geometric shell theory.⁷ If AuZ is a 102 gold atom

“magic” cluster, it is surrounded by 44 ligands, here 30 TAB and 14 TNB using the XPS integration ratio, leading to a hypothetical formula of Au₁₀₂TAB₃₀TNB₁₄.

A. Ligand exchange reaction

B. Survey using NMR spectroscopy

AuZ + Glutathione : crude mixture

Figure 3. Survey on the ligand exchange ability of the AuZ gold nanocluster using reduced glutathione (GSH). A. Schematic view of the reaction. The inset image is a PAGE analysis of the AuZ and GS-grafted AuZ (AuGS). B. ¹H NMR spectra of the crude reaction mixture, purified solution containing <10 kDa components and purified resulting gold nanocluster (AuGS). The large peak at 4.7 ppm is D₂O and was trimmed. The peaks between 2-4.7 ppm are assigned to GSH and the ones between 6.5-8.0 ppm correspond to released thionitrobenzoic acid (TNB), thioaminobenzoic acid (TAB) or mixed TNB-, TAB-disulfides. The AuZ (150 μM) and GSH (6000 μM) in D₂O containing *tert*-butanol (100 μM) as an internal standard were incubated for 20h at 20°C. The crude reaction was purified using a dialysis methodology with a 10 kDa cut-off membrane.

A thermogravimetric analysis (TGA) was also performed to determine the percentage of organic materials covering gold clusters since this organic layer decomposes and becomes volatile upon heating at a temperature below the metallic gold (Supporting Information, Figure S2).^{9,31} A total weight loss of 25.5% was obtained and the TGA showed a biphasic profile with a first 6.8% weight loss occurring at 212°C and a second 14.8% weight loss occurring at 420°C. A small discrepancy is observed between the observed 25.5% total weight loss and the calculated 29.2% one for Au₁₀₂TAB₃₀TNB₁₄ but appears within the accuracy range of this method.^{9,31} We suggest that the minor 6.8% weight lost originates from thermal decarboxylation of the 44 ligands (calculated value of 6.9%).³⁹ The ability of the ligands covering the AuZ to be exchanged with incoming water-soluble thiols in aqueous solution was then assayed using reduced glutathione (GSH) (Figure 3A). When the AuZ (150 μ M) was reacted with 40 molar equivalents of GSH, a Glutathione-modified AuZ (AuGS) with an increased electrophoretic mobility in comparison to AuZ was obtained (Figure 3A, Inset image). To further analyze the thiolate ligand-exchange reaction, we performed an experiment using NMR spectrometry because the signal of the Au-bound ligands of the AuZ were hardly detectable using routine NMR settings that are used to analyze small organic molecules (see Figure 1B). If exchange occurs, the aromatic ligands should be released in solution and their NMR signals be hence detected. The AuZ (150 μ M) was reacted with 40 molar equivalents of GSH in D₂O and a NMR spectrum of the crude mixture was taken after 20h incubation at 20°C (Figure 3B). The spectrum exhibited peaks between 6.5–8.0 ppm, which were unseen in the AuZ spectrum. The aromatic NMR signals are attributed to hydrogens of TNB, TAB and mixed disulfides with GSH, suggesting indeed ligand release from the AuZ surface. Integration of the NMR signals indicated that TNB and TAB were released in equal amounts. Using tertio-butanol as an internal standard, the concentration of released ligands was estimated at roughly 1700 μ M or 11.3 ligands/particle with a TNB/TAB ratio of 1. This ratio is higher than the initial TNB/TAB ratio of 0.46 present at the AuZ surface and confirms that ligand exchange can be favored by para-withdrawing groups on

the ligand aromatic ring.³⁶ A dialysis fractionation device with a 10 kDa cut-off was then used to afford solutions containing either the high molecular mass AuGS or <10 kDa species. Both solutions were lyophilized and the residues were analyzed again. The solution containing <10 kDa species presented a slightly different NMR profile in the 6.5-8 ppm range in comparison to the crude solution. This is likely due to slight acidity variation and to complete air-mediated oxidation of thiols to disulfides. The narrow signals (1.8, 2.7, 2.8 and 2.9 ppm) could not be attributed. They likely came from soluble stabilizers present in the dialysis fractionation device. An MS analysis of this solution confirmed disulfide formation and TAB release because an MH^+ of 475.06 corresponding to the MH^+ of GSH-TAB disulfide was detected (Supporting Information, Figure S3). The NMR spectrum of purified AuGS was featureless above 4.7 ppm but displayed broad peaks at 2.2 and 3.7 ppm, attributable to Au-bound glutathiones that have different local chemical environment and restricted mobility in a similar manner than aromatic ligands behaved when they are bound to the AuZ's surface (Figure 1C).⁴⁰

Synthesis of peptide-substituted gold nanoclusters

The AuZ was reacted in HEPES buffer pH 7.4 with increasing amounts of the thiolated peptide HS-NLS which contains an N-terminal Cysteine, followed by the ALNNG sequence that was shown to prevent the metallic gold core from aggregation⁴¹ and then the SV40 NLS sequence (Figure 4).⁴² The reaction products were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining to reveal as well the peptide (Figure 4B). In the reaction mixture at HS-NLS/AuZ ratio of 24, excess peptide (fast moving blue band) was observed, indicating saturation of the substitution sites on the AuZ surface. When the HS-NLS/AuZ ratio was gradually increased from 1 to 24, 8 different bands with gradual retardation were observed. By correlating the HS-NLS/AuZ ratios and the bands, we presumed that NLS peptide-AuNC conjugate at saturation level is surface-grafted with about 9 peptides.

A. Reactivity with thiolated peptides

B. Ligands to peptides substitution assay

C. PAGE analysis

D. MS of purified AuNLS

Figure 4. Reactivity of the AuZ with thiolated peptides. A. Schematic view. B. PAGE analysis of the reaction between AuZ and thiolated Nuclear Localization Signal Peptide (HS-NLS) at increasing molar ratios. *: HS-NLS alone. Last right lane: protein standards. Left numbers point to new gold clusters with different electrophoretic mobilities. C. PAGE analysis of AuZ and purified peptide-substituted gold nanoclusters made at a peptide/AuZ input molar ratio of 32. Cap: CALLNG, NES (Nuclear Export Signal peptide): CALNNGALAKLAGLDINKT_{amide}, NLS (Nuclear Localization Signal): CALNNGAGPKKKRKVED, dNLS (defective NLS): CALNNGAGPKTKRKVED. Gels were stained with Coomassie blue. D. MALDI-TOF spectrum of purified AuNLS made at peptide/AuZ ratio of 32.

Several peptide-gold nanocluster conjugates were similarly prepared by incubating AuZ with respectively the CALLNG peptide alone (Cap), the Cap linked to the protein kinase A inhibitor Nuclear Export Signal peptide (NES),⁴³ the HS-NLS and a defective NLS peptide (dNLS) in which one lysine was replaced by a threonine.⁴² To ensure full substitution, the peptide/AuZ feed ratio was set at 32. The peptide-substituted AuNCs were then purified from the excess of free peptides and released ligands by dialysis. PAGE analysis showed that the Cap-substituted AuNC (AuCap) migrated as a single band with electrophoretic mobility similar to the starting AuZ as expected by the size and net charge of the Cap peptide that matches that of TNB (Figure 4C). The NES- and dNLS-substituted AuNCs (respectively AuNES and AudNLS) were significantly retarded and had an electrophoretic mobility close to NLS-substituted AuNC (AuNLS), suggesting that each gold nanocluster was also covered with 8-9 peptides. The AuNLS displayed a MALDI-TOF mass spectrum containing one major population (maximum at 20.6 kDa) and minor humps at higher molecular weights (Figure 4D). As previously found for AuZ (Figure 1E), the major population can be assigned to an AuNC species devoid of the organic coverage which was presumably fragmented by the laser-induced desorption and ionization.³¹ The novel humps were remarkably equidistant by a value (1.8 kDa) corresponding to the molecular mass of the HS-NLS peptide, suggesting that AuNCs equipped with 1 to 5 NLS peptides partially sustained degradation imposed by the analytical process. The MS of AuCap, AudNLS and AuNES (Supporting Information, Figure S4) exhibited similar molecular weight patterns. The peptide-substituted AuNCs were finally analyzed by electron microscopy (Figure 5). The AuNCs differently stuck to the Carbon film but were homogenous in diameter (average apparent diameters of 1.4 nm) suggesting that these ligand exchange conditions were producing little etching of the gold metallic core.²¹

Figure 5. Transmission Electron Microscopy images of the peptide-modified gold nanoclusters. A. AuNLS. B. AudNLS. C. AudNLS. D. AuNES.

Fate of AuNCs following cytosolic delivery inside living HeLa cells

An electroporation protocol creating transient holes in the plasma membrane to direct influx of proteins inside the cytosol without inducing cellular lethality was described.⁴⁴ The similarity in terms of size between AuNCs and proteins suggests that this electroporation procedure might also promote a direct influx of gold nanoparticles into the cytosol of living cells. This delivery method, which does not rely on any particular design of the particles or passage through vesicular compartments, creates an opportunity to precisely analyze the intracellular behavior of peptide-modified gold nanoclusters and whether they can diffuse and remain stable within the cell cytosol and nucleus.

The effect of the peptide-substituted AuNCs on the viability of native HeLa cells and transiently permeabilized HeLa cells (done using an electroporation device) was explored using a MTT assay (Figure 6).⁴⁵ The experimental conditions were the ones that were optimized for the effective antibody transduction with minimal cell toxicity.⁴⁴ The cell viability was measured 24h after addition of the AuNCs and application of the electric pulses. Addition of AuNCs to HeLa cells with unimpaired cell plasma membrane (no electric pulses; blue bars) did not promote acute cytotoxicity (viability measured at above 75% for all AuNCs). Apparent cytotoxicity was also not observed when the plasma membrane was transiently permeabilized using electric pulses, regardless of the peptide-substituted AuNCs (yellow bars). A similar low cytotoxicity profile was also observed using the non-cancerous human fibroblast HFF-1 cell line (Supporting Information, Figure S5).

Figure 6. Evaluation of the HeLa cell line viability in presence of the peptide-substituted AuNCs (blue bars) and with electric pulses allowing transient permeabilization of the plasma membrane (yellow bars). Viability was measured 24h after addition of the AuNCs. Values are averages and standard deviation of hexaplicates. Final AuNC concentration: 114 nM.

Next, the AuCap, AuNLS and AudNLS were incubated with freshly trypsinized HeLa cells for 30 min without or with an electroporation treatment and the cells were left to adhere and grow for 24 h onto glass lamella. After cell-fixation, the gold particles were revealed using a silver staining procedure (Figure 7). For AuNCs that were incubated with the cells in the absence of the electric-induced membrane-permeability (Figure 7A), gold nanoclusters were mostly observed at distinguishable perinuclear compartments resembling lysosomes. This perinuclear accumulation was not unexpected since any binding to the cell outer-membrane surface can trigger a dynamic entry into intracellular vesicles and sequestration into lysosomes.^{46,47} When the same AuCap, AuNLS, AudNLS nanoclusters were mixed with HeLa cells and then subjected to electric pulses for transient plasma membrane permeabilization (Figures 7B),⁴⁴ the intracellular silver staining was greatly enhanced as compared to non-electric pulsed-cells, indicating that the gold nanoclusters penetrated into cells through the plasma membranes upon the transient electric pulse-induced pores. The AuCaps were dispersed throughout the cytoplasm and the nucleus indicating that they can freely diffuse throughout the cell. The AuNLS strongly accumulated in the cell nuclei. In contrast, the mutated AudNLS carrying a defective NLS was spread throughout the entire cell. Important to mention, most adherent cells containing the different AuNCs had an adherent morphology similar to untreated cells, confirming the MTT assay and that electroporation and cytosolic-delivered nanoclusters do not dramatically interfere with the cellular physiology of this cancer cell line. Observation of a sequence-specific nuclear import of the gold nanocluster clearly demonstrated that the gold-coupled NLS peptides remain coordinated to the gold nanocluster inside the HeLa cells.

A. Incubation of gold nanoclusters with HeLa cells

B. Incubation of gold nanoclusters with membrane-permeabilized HeLa cells

Figure 7. Illustration of each experimental procedure and optical micrographs of HeLa cells incubated for 24h with the indicated peptide-substituted gold nanoclusters (AuNCs). A. The various peptide-AuNCs were added to HeLa cells in suspension. The cells were then added into wells for cell adhesion and monolayer formation. B. The various peptide-AuNCs were added to HeLa cells in suspension and the mixture treated with electric pulses for transient membrane permeabilization. The cells were then added into wells for cell adhesion and monolayer formation. Following the 24h incubation period at 37°C, the cells were fixed with glutaraldehyde (2.5% w/v) and subjected to silver staining for AuNC detection.

To further evaluate the diffusion ability of the AuNC and the stability of the Au-S bond in the nucleus, we evaluated as well a peptide signal-assisted nuclear export using the nuclear export signal peptide-grafted gold nanocluster (AuNES) (Figure 8).

A. AuNES with HeLa cells

HeLa

HeLa + AuNES

B. AuNES with membrane-permeabilized HeLa cells

HeLa + leptomycin + AuNES

HeLa + AuNES

Figure 8. Optical micrographs of HeLa cells incubated for 24h with the AuNES. A. Addition of AuNES to non-permeabilized HeLa cells. B. AuNES and HeLa cells were subjected to electroporation with or without leptomycin, an inhibitor of the NES-dependent nuclear export machinery. Following the 24h incubation period at 37°C, the cells were fixed with glutaraldehyde (2.5% w/v) and subjected to silver staining for AuNC detection.

Addition of the AuNES to native non-permeabilized HeLa cells led to nanocluster accumulation in perinuclear compartments (Figure 8A) in an analogous manner than AuCap, AuNLS and AudNLS did. When the AuNES and the HeLa cells were subjected to electroporation and when the NES-dependent nuclear export was inhibited by leptomycin,⁴⁸ the intracellular AuNES was homogeneously dispersed inside the entire cell, including the nucleus (Figure 8B, left image). In the absence of leptomycin, the nuclear export machinery was fully functional. In this condition, hardly any AuNES was detected in the nuclei (Figure 8B, right image) but rather appeared to localize and aggregate in subcellular organelles. Such redistribution is analogous of that observed with NES-grafted fluorescent proteins.⁴³ Similar specific peptide-mediated nuclear in/out shuttling of AuNCs were obtained using the U2OS cell line and the non-cancerous human fibroblast HFF-1 cell line (Supporting Information, Figures S6 and S7), indicating that the observation was not HeLa-specific and can be extended to another cell lines.

The intracellular distribution of AuCap, AuNLS and AuNES within HeLa cells was further investigated by Transmission Electron Microscopy (TEM) 24h after the electroporation treatment (Figure 9). The gold nanoclusters were grown to 8-13 nm diameter particles by silver-enhancement of specimens before resin-embedding in conditions that do not produce auto-nucleated silver particles (Supporting Information, Figure S8).¹⁴ The electroporation treatment and the presence of gold particles did not lead to any visible histological modification of the HeLa cells and the images showed similar gold nanocluster distribution patterns to those observed in light microscopy. The AuCaps were detected as single particles or in groups, both in the cytoplasm and in the nucleus in a roughly equivalent proportion (Figure 9, left image). The AuNLS were mostly detected as individual dots in the nucleus at areas with low heavy-metal contrast, corresponding probably to areas of the nuclei devoid of condensed chromatin (Figure 9, middle image). In a representative cell section 100 silver-amplified particles were counted in the nucleus (nuclear particle density of 13 AuNLS/ μm^3) and only 14 particles were spotted in the

cytoplasm, including 3 particles that were clearly entrapped inside vesicular bodies. Knowing the volume of the HeLa cell nucleus ($374 \mu\text{m}^3$),⁴⁹ we calculated that the entire nucleus contains about 4,860 AuNLS. The AuNES were barely detected inside the nucleus and were seen to congregate in cytosolic areas (Figure 9, right image).

A. TEM images

B. Illustration of the TEM images

Figure 9. Transmission electron microscopy images of HeLa cells after cytosolic delivery of peptide-substituted gold nanoclusters as indicated. A. TEM images. Individual 8–13 nm diameter dark dots, corresponding to amplified AuNCs, were visualized on the micrographs at high magnification then highlighted in circles; aggregates of AuNCs were featured as columns; cytoplasmic and nuclear localizations were colored in black and red, respectively. B. Illustration of the imaged cells along with the position of AuNCs. The different peptide-AuNCs were delivered by electroporation into the cytosol of HeLa cells. The living cells were incubated for 24 h at 37°C and then processed for electron microscopy observation. The AuNCs were mildly silver-enhanced to ensure AuNC detection and ultrastructure preservation of specimen.

Discussion

Availability of definable nanomaterials for any applications is really helpful and promises reliability. First we demonstrated the feasibility of preparing highly homogeneous gold nanoclusters, which can be further grafted with several thiolate-containing active peptides. A new type of gold nanoclusters of uniform size was synthesized from chloroauric acid and the Ellman's reagent upon reduction with sodium borohydride. Seventy percent of the TNB was reduced to the zwitterionic TAB during the nanocluster formation and was likely assisted by the presence of gold atoms.³⁰ The UV/vis spectrum, cryo-EM image of AuZ in a vitrified aqueous solution, MALDI-TOF MS, XPS, TGA and theoretical calculation on cluster stability³⁰ support the formation of gold clusters nearing the $\text{Au}_{102}(\text{TAB})_{30}(\text{TNB})_{14}$ formula. Although not exclusive, the TNB ligand exchanged more readily than the TAB when reacted with external thiolated molecules, leaving a surrounding shell consisting of grafted peptides and mainly of the zwitterionic TAB ligand. The properties of AuZ and peptide-substituted AuZs to migrate as discrete bands in polyacrylamide gels are highly valuable for materials characterization. PAGE analysis is for instance not suited for characterization of less definable citrate-stabilized 4.5 nm gold nanoparticles (Figure S9). In our hands, AuZ was also substantially easier to produce as a single particle population than the AuMBA as judged by PAGE analysis (Figure S10).

Second, we evaluated the diffusion abilities and the fate of bioactive peptides-substituted AuZs when added to living cells for which the plasma membrane was transiently impaired by electric pulses. The remaining live cells adhering to lamella were seen to contain diffusible AuNCs and peptide-AuNC conjugates that shuttle across the nuclear envelop in a peptide-dependent manner. Altogether, our aggregated data clearly demonstrated that peptides remained grafted onto the gold nanoclusters (i.e.: the Au-S is stable) for at least 24h inside the cytosol and nucleus of cells. These AuNCs were also small enough to pass through electric pulses-mediated holes in the plasma membrane and mobile inside the cytosol and in the nucleus. This observation was in sharp contrast to a similar experiment in where 4.5 nm diameter gold nanoparticles did not homogenously spread inside the cells (Figure S11). Other data on the intracellular fate/behavior

of other types of gold particles and cytosolic/nuclear localization can be found in the literature.

For example, PEGylated 2.4 nm gold particles¹⁶ or tiopronin-protected ca. 3 nm particles,⁵¹ when equipped with Cell Penetrating Peptides (CPP) having an adventitious NLS, were detected in the nucleus 1 to 3 h after addition to living cells but the efficiency of the process was unfortunately not quantified. Gold particles larger than 5.5 nm in diameter were also similarly equipped with CPP but their intracellular distribution appeared heterogeneous and experiment-dependent, ranging from exclusive entrapping onto the cell surface and in cellular vesicles^{16,52} or alternatively penetration into the nucleus.^{19,52-54} Gold particles in the nucleus were in some cases accompanied by a dramatic modification of cell morphology,⁵² indicating that these materials interfered with an important cellular mechanism, leading to cell death. In other cases, the nuclear distribution of gold particles⁵³ and of photoluminescent 2 nm gold nanoclusters⁴⁵ was heterogeneous, suggesting strong non-specific interactions with nuclear components and/or impaired diffusion. Additional insight into the ability of gold nanoparticles to traffic to the cell nuclei were obtained by grafting different and independent bioactive organic species to gold particles of about 15 nm in diameter.^{19,54} However, when quantitative analysis was done, only a small portion and number (<10) of the multifunctionalized particles were detected in the cell nuclei, questioning their nuclear targeting abilities in regard to statistical significance.

In here, the electroporation protocol provided a significant improvement in cytosolic delivery of small and diffusible gold nanoclusters over previously reported methods undergoing a passage into intracellular vesicles. Almost 100% cells contained AuNCs inside the cytosol or into the nucleus and the materials within intracellular vesicles did not overwhelm the ones inside the cytosol (Figures 7 and 8; Supporting Information, Figures S6-7). The delivery efficiency in term of quantity (for instance, >4,000 nuclear particles per cell using the AuNLS) was also appreciated and considerably facilitated data analysis. In our investigation, the electroporation protocol helped demonstrating that peptide-conjugated AuNCs (either the AuNLS or the AuNES), made from the AuZ, can be shuttled across the nuclear pores using cellular machineries

in a protein-like manner. This finding clearly implies a stable Au-S bond between the AuZ and the thiolated active peptide for at least 24h. It opens some intracellular usages of the definable AuZ gold nanoclusters which are amenable to facile derivatization with bioactive peptides or organic ligands and have mobility into the densely packed intracellular environment. The AuMBA has similar properties than the AuZ (Supporting Information, Figure S10). An NLS-grafted AuMBA also accumulated in the cell nuclei using the electroporation treatment suggesting that the intracellular stability of Au-S coordination bond is insensitive to neighboring ligands. Nuclear accumulation of the NLS-grafted AuMBA in the non-cancerous HFF-1 cells appeared nonetheless less intense than that of the NLS-grafted AuZ (Figure S10D), suggesting *in fine* that the nanocluster-bound zwitterionic TABs might diminish undesired association to the HFF-1 cytoskeleton and represent an improved coverage in comparison to the anionic MBAs.

Conclusion

We have synthesized a novel monolayer organothiolate-protected gold nanoparticle by NaBH_4 reduction of a mixture containing chloroauric acid and the Ellman's reagent. The procedure essentially yielded a uniform gold nanocluster, which is easily isolated by precipitation with excess methanol. Analytical data of this gold nanocluster characterized it as close to a superatom cluster containing 102 gold atoms, which are surrounded by an organic monolayer made of about 30 thioaminobenzoic acids and 14 thionitrobenzoic acids. These ligands, preferentially the thionitrobenzoic acids, are readily exchanged with thiolated peptides in aqueous solutions, providing gold nanoclusters that are grafted with ca. 8-9 peptides and mainly coated with remaining zwitterionic TAB ligands. The peptide-gold nanocluster conjugates can furthermore be delivered via an electroporation treatment into the cytosol of living cells wherein the Au-S coordination bond between thiolated peptide and Au(I) at the surface of the Au_{102} nanocluster was stable for at least 24h. Altogether, we have synthesized new definable gold nanoclusters and peptide-AuNC conjugates. We have also demonstrated the usefulness of electroporation for nanocluster cytosolic delivery with low to moderate vesicular entrapment and a 24h-stability of

surface-modified gold AuNCs inside the cytosol and cell nucleus in where they can diffuse and specifically associate to molecular transport machineries. These data opened the usage of precise peptide-functionalized gold nanoclusters for intracellular investigation and application. These AuNCs might be particularly useful for building novel EM probes for high-resolution immunolabeling inside living cells.

Experimental section

General. Materials and additional protocols are described in the supporting information.

Gold nanocluster synthesis. The synthesis was done following a described procedure.³¹ Hydrogen tetrachloroaurate(III) trihydrate (14.2 mg, 36 μ mol), DTNB (24.4 mg, 54 μ mol), NaOH (13 mg, 324 μ mol) and NaBH₄ (4mg, 108 μ mol) were mixed in a methanol/water solution and the mixture was shaken overnight. The gold clusters were then precipitated by addition of a 2 M AcONH₄ solution (2 mL) and methanol (34 mL). The black pellet (gold nanoparticle) was resuspended in water and desalted by a size exclusion chromatography (Sephadex G50). The black fraction was lyophilized resulting in AuZ (4.2 mg, 152 nmol) as a black powder.

Ligand exchange reaction with an increasing ratio of thiolated peptide to gold nanocluster. The thiolated NLS peptide was freshly diluted in water to 1 and 4 mM concentrations. Aliquots (0.8 and 1.6 μ L of the 1 mM NLS peptide solution and 0.8, 1.6, 2.4, 3.2 and 4.8 of the 4 mM NLS peptide solution) were carefully dispensed at the bottom of different 0.5 mL tubes. The AuZ (10 μ L of an 80 μ M solution in 100 mM HEPES pH 7.4, 0.8 nmol) was then rapidly added to each tube containing the peptide (0.8, 1.6, 3.2, 6.4, 9.6, 12.8 and 19.2 nmol). The mixtures were then incubated for 18h and reaction products were analyzed by electrophoresis on a 10% polyacrylamide gel.

Peptide-coated gold nanoclusters. A solution of 80 μ M AuZ (80 μ L in 100 mM HEPES pH 7.4, 6.4 nmol) was rapidly added to a solution containing 10 mM thiolated peptide (20.5 μ L, 205 nmol) and 50 mM TCEP, pH 7.4 (4.3 μ L, 215 nmol) which was previously dispensed into a 0.5 mL polypropylene tube. The mixture was incubated at room temperature for 24h. Each crude

mixture was then diluted with water to 0.5 mL and the gold nanoparticles were purified from low molecular weight components using 0.5 mL Microcon® 30 kDa centrifugal filter device (Merck, Molsheim) and 7 centrifugation cycles (2 x water, 2 x 0.4 M NH_4HCO_3 and 3 x water). The concentration of the nanocluster was determined by measuring absorbance at 520 nm and a ϵ_{520} of $2.7 \times 10^5 \text{ mol}^{-1} \times \text{L} \times \text{cm}^{-1}$. Final concentrations were adjusted to 80 μM in AuNC with water. About 70 to 80% of the gold nanoparticles were recovered. The gold nanoparticles were analyzed using PAGE.

Ligand exchange measured by NMR spectroscopy. The gold nanocluster (3.2 mg, 120 nmol) was dissolved in D_2O (0.5 mL). The concentration was determined by measuring absorbance at 520 nm. The cluster was then diluted in D_2O to a final concentration of 150 μM . A 0.6 mL volume was withdrawn and taken for ^1H NMR analysis at 293 °K. *Tertio*-butanol (final concentration of 0.1 mM) was used as an internal standard. A 0.3 M glutathione, pD 7.4 was freshly prepared in D_2O . An aliquot (12 μL , 3600 nmol) was added to the 150 μM AuNCs (600 μL , 90 nmol). The ^1H NMR spectra were then collected 2 and 6h later. The crude solution was then purified using a 0.5 mL Microcon® 10 kDa centrifugal filter device (5 times water). The flow-through containing the species with molecular masses < 10 kDa and the AuNC solutions were lyophilized, dissolved in D_2O (0.6 mL) and the ^1H NMR spectrum was recorded again.

XPS analyses. A 10 μM nanoparticle solution in water/methanol (9/1)(20 μL) was deposited onto a silicon wafer support. The water/methanol was left to evaporate in air under a fume hood and the sample was dried under high vacuum. The XPS measurements were performed in a UHV setup equipped with a monochromatic Al $K\alpha$ X-ray source and a VSW Class WA hemispherical electron analyzer.⁵⁵ The binding energy scale of the spectrometer was calibrated prior to the measurements and since samples did not have electrostatic charging, the binding energies are presented without any correction. The Shirley method was employed for background subtraction, prior to the fitting procedure. The elemental atomic ratio was calculated by the integrated XPS peak areas of high resolution spectra normalized to the atomic sensitivity factors and by assuming a homogeneous distribution arrangement model.⁵⁶

Electroporation Protocol. Transduction experiments were conducted as previously described using the Neon[®] transfection system and three 10 ms pulses at 1550 V.⁴⁴ For light microscopy imaging, HeLa cells (10^5 cells in 12 μ L of the Neon Buffer R) were mixed with each AuNC (80 pmol, 1 μ L of a 80 μ M solution). For TEM imaging, the electroporation was scaled up 6 fold (6×10^5 cells in 110 μ L Neon Buffer R, 6 μ L AuNCs). After the electric pulses, the cells were diluted into prewarmed cell culture medium without antibiotics (1 mL). The cells were recovered by gentle centrifugation, suspended into the warmed complete cell culture medium without antibiotics and added into wells of 24-wells plates for cell adherence and cell growth into a cell culture incubator.

Cell viability assay. The assay was done according to a published procedure.⁵⁷ The AuNCs and the cells mixtures were treated as described in the electroporation protocol and then diluted for culture in 96-wells plate. Assay was performed 24h after addition of the sample.

Cellular Specimen preparation for Light Microscopy. 24h after the electroporation treatment, the cell monolayers were washed with warmed (37°C) Sorenson's buffer (3 x 1 mL). The cells were then fixed with 2.5% glutaraldehyde diluted in 133 mM Sorenson's buffer (1 mL) for 1h. Unreacted aldehydes were blocked by incubation with a 100 mM Sorenson's buffer, pH 7.4 containing 50 mM glycine (1 mL, 20 min). The plasma membrane was permeabilized using 0.1M Sorenson's buffer pH 7.4 containing 0.05% (w/v) saponin (1 mL, 15 min) before proceeding for silver-mediated amplification of the gold particles according to a modified Danscher protocol⁵⁸ using silver acetate and propyl gallate.⁵⁹ The glass plates were mounted on slides (StarFrost, Knittel Glass, Germany) and samples were observed using a Leica DM5500B microscope equipped with a Leica DFC350FX camera. Image acquisition was made with Leica LAS AF software.

Cellular Specimen preparation for Electron Microscopy. 24h after the electroporation treatment, the adherent cells were washed with warmed (37°C) Sorenson's buffer (3 times 2 mL). The cells were then fixed with 2.5% glutaraldehyde (1 mL) for at least 4 hours. The cells

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were then washed with TEM storage solution (5x, 1 mL). Enhancement of sizes of the gold particles with silver was performed using the SE-EM kit (Aurion, the Netherlands) and according to the manufacturer's protocol. The cells were then contrasted with 0.5% osmium for 20 min followed by 2% uranyl acetate both diluted in water. The specimens were then dehydrated in a solution containing increasing concentrations of ethanol and flat embedded in epon (Ladd Research Industries, Williston, Vermont, USA). The resin-embedded specimens were sectioned into 100 nm thick slices that were deposited on a 200 mesh electron microscopy grid.

ASSOCIATED CONTENT

The supporting file (pdf) is available free of charge The document contains additional experimental procedures; a table summarizing the peptide sequences; XPS spectra and thermogravimetric analysis of AuZ; UV/vis spectra of AuZ after reaction with glutathione; TEM and Mass spectra of peptide-AuNC conjugates; distribution analysis of AuNCs in U2OS and HFF-1 cells; TEM of untreated silver-stained cells; reactivity and delivery assay of peptide-AuMBA conjugates; synthesis and cytosolic delivery of 4.5 nm nanoparticles; fluorescence emission spectra of AuZ and AuGS; HPLC profile and MS of peptides; FT-IR spectra of the AuNCs;; synthesis and cytosolic delivery of Alexa488-labeled AuNLS; and color images of cytosolic delivery of AuNCs in HeLa cells.

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Complement to Materials and Methods

Chemicals

5,5'-dithio-bis(2-nitrobenzoic acid)(DTNB) (Sigma-Aldrich D8130)

Hydrogen tetrachloroaurate(III) trihydrate (Sigma-Aldrich 520918-1G)

Sodium borohydride (NaBH₄) (Aldrich 45,288-2)

Sodium hydroxide (Fluka 71691)

4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Euromedex 10-110)

Trishydroxymethylaminomethane base (Tris) (Euromedex, 26-128-3094B)

Glycine (Gly)(Euromedex 26-128-6405C)

Peptides were purchased from GeneCust (Dudelange, Luxembourg)

Glutathione reduced (GSH) (Sigma G6013-5g)

Methanol (Sigma-Aldrich 179957-5L)

Water Bi-distilled

Tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich BioUltra 75259)

Ammonium hydroxide 28% (EMS cat#10600 EMS group, Hatfield, PA, USA)

Glutaraldehyde 25% solution EM grade (EMS cat#16220, EMS group, Hatfield, PA, USA)

HSPEG (Code PEG1169-0005, MW 2015Da, Iris Biotech, Germany)

Materials. The pH-meter was a Hanna HI2210 instrument combined with a HI 1131B electrode. Centrifugation of 50 mL solutions was performed using an Eppendorf 5810R centrifuge and the rotor A-4-81. Centrifugation of small volumes (0.5 to 2 mL) was performed using a Sigma 1-15K Eppendorf centrifuge with a 12132-H rotor. For gold particle formation, agitation was performed on a rocking platform (Heidolph Rotamax 120). The Nuclear Magnetic Resonance (NMR) data were taken with a Bruker DPX 400 MHz spectrometer. The UV-vis absorbance spectra were recorded using a Varian Cary 100Bio spectrometer. Nanoparticles were purified and concentrated using 0.5 mL Microcon® centrifugal filters (Merck, Molsheim) with cut-off of 10 or 30 kDa according to the manufacturer's specification. The spectrofluorometer was a Xenius Safas (Monaco, France).

Devices

Small volumes of aqueous solutions were dispensed using pipettes (Gilson Pipetman® classic P10, P100, P1000 models). Large volumes were dispensed using serological pipettes (Falcon® 5 10 or 25 mL) and a single channel Omega Pipettor (Argos Technologies Inc, Elgin IL). Reactions were performed unless indicated in polypropylene tubes of different volumes (50 mL, 15 mL, 2.0 mL, 1.5 mL or 0.5 mL).

Preparation of aqueous stock solutions

- Peptides were made at 10 mM concentration in water and stored in aliquots at -20°C.
- Tris(2-carboxyethyl)phosphine (TCEP), 0.1 M solution, pH 7.4 was prepared by incremental addition of 2 M NaOH to a 0.2 M TCEP hydrochloride (286.65 mg in 5 mL H₂O) solution under stirring and by monitoring pH increase with a pH-meter. When the pH reached 7.4, the TCEP solution was set at 0.1 M and stored in aliquots at -20°C.
- 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (23.83 g, 0.1 mol) was dissolved in water (50 mL). The pH was adjusted to pH 7.4 with incremental addition of 1M NaOH and using a pH-meter. The solution was then adjusted to 100 mL and was filtered for sterilization using a 33 mm syringe-driven filter unit (0.22 µm Millex-GP, Millipore, Molsheim France). The 1M HEPES-Na solution pH 7.4 was stored at 4-8°C.
- Hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄ · 3H₂O) (1g, 2.539 mmol) was dissolved in water (6.35 mL) to a final concentration of 0.4 M in aurate. This stock solution was stored at 4-6°C in a brown vial and can be stored for more than 6 months.
- The 2 M ammonium acetate solution was buffered to pH 4.7 with 2 M acetic acid solution using a pH-meter.
- 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (198.2 mg, 50 µmol) was dissolved in 0.3 M aqueous NaOH solution (10 mL) to a final 50 mM DTNB concentration. The orange solution was used within 48h and stored at 4-6°C in the dark when not freshly used.
- Glutathione (GST) solutions were freshly prepared in water and buffered to pH 7.0 using 0.3 M NaOH. For the ESI-MS analysis, the glutathione was buffered to pH 7.8 using NH₄OH.
- The 133 mM Sorenson's buffer, pH 7.4 was prepared by combining 133 mM Na₂HPO₄ (804 mL) with 133 mM KH₂PO₄ (196 mL). After verification of the pH with the pH-meter, the solution was filtered for sterilization using a 33 mm syringe-driven filter unit (0.22 µm Millex-GP, Millipore, Molsheim France).
- The TEM storage solution was a 0.1 M Sodium cacodylate buffer, pH 7.2 containing 2% (w/v) sucrose, 1 mM MgCl₂ and 1 mM CaCl₂. The solution was filtered for sterilization using a 33 mm syringe-driven filter unit (0.22 µm Millex-GP, Millipore, Molsheim France) and stored at 4-6°C.

Estimation of the absorption coefficient of the gold nanocluster. The dried AuZ pellet (4.2 mg) was dissolved in water (350 μL) and the solution pH was adjusted to 7.0 by addition of 50 mM aqueous NH_4OH to a final concentration of $11.28 \text{ g} \times \text{L}^{-1}$. Aliquots (1 μL volume) were withdrawn and diluted with water (final volume of 700 μL) giving a concentration of 16.114 mg/L. An optical density (OD) of 0.153 at 520 nm was measured using a 1 cm-path quartz cuvette. Using the Beer-Lambert equation ($\text{OD} = \epsilon l c$) and an estimated molecular mass of 28382 Da ($\text{Au}_{102}\text{TAB}_{30}\text{TNB}_{14}$), extinction coefficients (ϵ_{520}) of $9.51 \text{ g}^{-1}\text{L} \times \text{cm}^{-1}$ and $2.7 \times 10^5 \text{ mol}^{-1} \times \text{L} \times \text{cm}^{-1}$ were determined.

Preparation of gold nanoparticles. The citrate-stabilized gold nanoparticles were prepared according to a described procedure.¹ After preparation, the particles were purified and concentrated using the 0.5 mL Microcon® centrifugal filters (cut-off: 100 kDa). Particle concentration was estimated at 116 nM by UV/vis spectrometry using the nanoparticle absorption at 509 nm. Next, the gold particles (0.2 nmol) were reacted with the Cap peptide (32 nmol), HSPEG (32 nmol), Cap/HSNLS (9/1) mixture (32 nmol total) and Cap/HSNLS (9/1) mixture (32 nmol total) in 150 mM K_2CO_3 for 24h at room temperature. The particles were then purified and concentrated using the 0.5 mL Microcon® centrifugal filters (cut-off: 100 kDa). Concentrations of the peptide-grafteraqueous solutions were spectrophotometrically estimated and set at 4 μM .

PolyAcrylamide Gel Electrophoresis. 10% Polyacrylamide Gels (1 mm thick) were casted using the BioRad MiniProtean system in 25 mM Tris-186 mM glycine buffer pH 8.3 containing 0.1 % (w/v) Sodium Dodecyl Sulfate (SDS). For electrophoresis, the running buffer was 25 mM Tris-186 mM glycine buffer pH 8.3 containing 0.1 % SDS unless indicated otherwise. The polyacrylamide gel was pre-run for 20 min at 90V. For loading in the wells, 50% (v/v) glycerol in water was diluted to 10% into samples (5-10 μL volume; 40 nmol in AuNC). The electrophoresis was run at room temperature for 45 minutes at 90V. The gold nanoclusters were observed as black-brown bands. Peptides were visualized after Coomassie blue staining.

Thermal Gravimetric Analyses. The analysis was performed on a Q 5000IR apparatus (TA Instruments) under a flow of nitrogen on vacuum-dried particles (0.2990 mg) and at a heating rate of $10^\circ\text{C}/\text{min}$.

Electron Microscopy. Imaging of cells and peptide-modified AuNCs was performed using a FEI G2 F20 Transmission Electron Microscope operating at 200 kV. Image acquisition was performed on a 2048 x 2048 CCD camera (Ultrascan 1000, Gatan Inc., Pleasanton) by collecting 25 adjacent images using the SerialEM software (Boulder, Colorado, USA). The final image was reconstructed by stitching the adjacent images using the Montage plugin package (Jean Daraspe, Université de Lausanne, Switzerland) and the iMod software.²

For standard-EM imaging of isolated nanoparticles, 10 μL of a 5 μM nanocluster solution was

applied onto the ultrathin carbon support film of 100 Mesh EM grids (UMC Utrecht, The Netherlands). After 1 min. adsorption, the excess liquid was removed with a filter paper.

For cryo-EM imaging, samples (3 μ L) of 10 μ M AuNC solution in 10 mM HEPES, pH 7.4 were applied to lacey carbon grids, blotted with filter paper from both sides for half a second in the temperature- and humidity-controlled Vitrobot Mark IV apparatus (FEI, Eindhoven, Netherlands, T = 20°C, humidity 95%, Blot Force 5, Blot time 0.5 sec) and plunge-frozen in liquid ethane pre-cooled by liquid nitrogen to embed the complex in a thin film of vitrified buffer spanning the holes of the carbon film. Images were collected at liquid nitrogen temperature with a FEI Titan Krios EM operating at 300 kV with a dose of 20 electrons per $\text{\AA}^2$ using a FEI Falcon II Camera with pixel size of 0.8936 $\text{\AA}$, integration time 1s, binning 1 and defocus of -0.5 μ m.

MALDI-TOF mass spectrometry. Mass measurements were carried out on an AutoflexTM MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). This instrument was used at a maximum accelerating potential of 20 kV in positive mode and was operated in linear mode at 19 kV. The delay extraction was fixed at 80 ns and the frequency of the laser (nitrogen 337 nm) was set at 5 Hz. The acquisition mass range was set to 5000-30000 m/z with a matrix suppression deflection (cut-off) set to 500 m/z. The equipment was externally calibrated with a standard protein calibration mixture that contained 5 proteins (Bruker Protein Standard II #8207234, Bruker Daltonics GmbH, Bremen, Germany) covering the 10,000-70,000 m/z range. Each raw spectrum was opened with flexAnalysis 2.4 build 11 (Bruker Daltonics GmbH, Bremen, Germany) software. Sample preparation was performed with the dried droplet method using a mixture of 0.5 μ l of sample with 0.5 μ l of matrix solution dried at room temperature. The matrix solution was prepared from a saturated solution of α -cyano-4-hydroxycinnamic acid in water/acetonitrile 50/50 diluted three fold in water/acetonitrile/trifluoroacetic acid 50/49.9/0.1.

Cell culture. The HeLa (ATCC CCL2) and U2OS (ATCC HTB-96) human cancer cell lines and the non-cancer human fibroblast HFF-1 cell line (ATCC SCRC-1041) were maintained in Dulbecco's modified Eagles tissue culture medium (DMEM) supplemented with 10% heat inactivated FCS (Life Technologies), 2 mM L-glutamine and antibiotics. Cells were grown in humidified atmosphere at 37°C and with 5% CO₂. Cells were maintained in culture for less than 12 passages.

Table S1. Sequence of peptides used for preparing Peptide-grafted AuZ gold clusters				
Abbre viation	Activity	Sequence (N->C)	Molecular mass (g.mol ⁻¹)	Reference
GSH	Passivation	γ-ECG (Glutathione)	307.32	^{3,4}
Cap	Protection	CALNNG	590.65	⁵
NLS	Nuclear import	CALNNGAGPKKKRKVED	1828.13	⁶
dNLS	Defective nuclear import	CALNNGAGPKTKRKVED	1801.06	⁶
NES	Nuclear export	CALNNGGLALKLAGLDINKT _{amide}	1941.33	⁷

Figure S1. High resolution XPS spectra of AuZ particles **a)** S 2p and **b)** C 1s. The S2p_{3/2} and S2p_{1/2} peaks are consistent with most sulfur atoms being engaged in a coordination bond with the gold surface atoms⁸ The C 1s components at 284.4, 285.6 and 288.6 eV are assigned to various carbon species C-C, C-O-C, C-N and C=O, O-C=O respectively,⁹ in good agreement with the expected carbon species in the organic coating.

Figure S2. Thermogravimetric curve and derivative weight change curve of the AuZ.

A. Survey of AuZ+GSH reaction using UV/vis absorbance

B. MS of <10 kDa species

Figure S3. Analysis of reaction between AuZ and glutathione (GSH). **A.** UV/vis spectra of the crude reaction (red line), purified GSH-gold nanocluster (AuGS) (blue line) and released low molecular weight components (black line). The red dotted arrow pinpoints a novel absorption band. **B.** ElectroSpray Mass Spectrum of the released species after a 20h reaction of AuZ (45.6 nmol, 200 μ L of a 228 μ M solution) with Glutathione, pH 7.8 (570 nmol, 5.7 μ L of a 0.1 M solution). The glutathione solution was freshly prepared and buffered by addition of NH_4OH . The gold clusters were separated from unreacted glutathione and released species using a 0.5 mL microcon-10 kDa centrifugal filter unit. The flow through contained a thionitrobenzoic acid (TNB) concentration of 848 μ M using the absorption coefficient of $14,150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 412 nm. The mass spectrum was taken 7 days after the reaction.

Comment. The UV/vis spectra of aliquots from the crude reaction, purified AuGS and released low molecular mass species were taken into the NMR spectroscopy survey (Figure 3B). The crude reaction (red line) exhibited now an UV/vis absorption spectrum corresponding to the sum of UV/vis absorption spectra of purified AuGS (blue line) and to released aqueous-soluble ligands (black line). The spectrum of the released species was analogous to the spectrum of TNB (Figure 1B) with the exception of a light shoulder (red dot arrow), indicating release of another ligand from the AuNCs. This absorption band is likely due to TAB. Using an absorption coefficient of $14150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 412 nm for TNB, we estimated the concentration of released

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TNB at 335 μM . This value corresponds to about 50% of the overall concentration of released ligands estimated by NMR and corresponds to a release of a TNB/TAB molecular ratio of 1.

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A. AuGS

B. AudNLS

C. AuCap (CALNG)

D. AuNES

Figure S4. MALDI-TOF Mass spectra of AuGS (A), AudNLS (B), AuCap (C) and AuNES (D).

Figure S5. MTT Cell viability assay using the HFF-1 normal cell line. Freshly trypsinized HFF-1 cells (50,000 cells) in presence of the indicated nanoclusters were added in the Neon transfection device in conditions that were used for electroporation, with the electric pulses (yellow bars) or without electric pulses (blue bars). Cells were then diluted in the cell culture medium and let to growth for 24h. Values indicated the averages and standard deviations of hexaplicates.

Figure S6. Optical microscopy images of U2OS cells incubated for 24h with the indicated peptide-substituted gold nanoclusters (AuNCs). **A, C and E.** The various peptide-AuNCs were

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added to living U2OS cells by dilution in the cell culture medium without an electroporation treatment. **B, D and F.** The U2OS cells were subjected to electroporation in presence of the indicated AuNCs. Following the 24h incubation period at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v) and AuNCs were detected by silver staining. Cell viability, estimated by density of the monolayer, was >80%. Scale bar: 20 µm.

Figure S7. Optical microscopy images of HFF-1 cells incubated for 24h with the indicated peptide-substituted gold nanoclusters (AuNCs). **A, C, E and G.** The various peptide-AuNCs were added to living HFF-1 cells by dilution in the cell culture medium without an electroporation treatment. **B, D, F and H.** The HFF-1 cells were subjected to electroporation in

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presence of the indicated AuNCs. Following the 24h incubation period at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v) and AuNCs were detected by silver staining. Cell viability, estimated by density of the monolayer, was >80%.

Figure S8. Transmission electron microscopy of untreated HeLa cells. After fixation with 2.5% glutaraldehyde, the specimen was treated with the Aurion Silver enhancement solution exactly as the AuNCs-containing specimens. After the silver staining, the HeLa cells were processed for resin-embedding and imaged.

A. Synthesis of functionalized Gold nanoparticles

B. TEM image of AuCi

50 nm

Average diameter: 4.5 nm
Standard Deviation: 0.55 nm (n=220)

C. PAGE analysis

Figure S9. A. Synthesis of peptide-grafted gold nanoparticle from citrate-gold nanoparticle. **B.** TEM image of the starting gold nanoparticle showing a population of particle with average diameter of 4.5 nm. **C.** SDS-denaturing 15% Polyacrylamide gel electrophoresis of the peptide-grafted gold nanoparticles. The gold nanoparticles were red-colored and did not require any staining.

expected from an NLS-driven nuclear import. The AuMBA-NLS appears to be less effectively imported in the HFF-1 nuclei than the AuNLS, suggesting a benefit of the zwitterionic TAB shell. Cell viability, estimated by monolayer density was >80%.

A. Scheme of the experiment

B. Incubation of the nanoparticles with electroporated HeLa cell

AuCi-Cap

AuCi-PEG

AuCi-Cap-NLS

AuCi-Cap-NLS

Figure S11. Evaluation of the intracellular fate of peptide-substituted 4.5 nm nanoparticles. **A.** Scheme of the experiment. **B.** The various gold nanoparticles as indicated were mixed with HeLa cells and the mixtures were subjected to electroporation. Following the 24h incubation period

at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v) and the gold nanoparticles were detected by silver darkening. Results showed heterogeneous distribution. These NLS-equipped nanoparticles did not accumulate in the cell nuclei even when the nanoparticle's surface was equipped with PEG for minimizing unspecific association to cellular component. We did not determine whether the poor cytosolic delivery was due to poor diffusion inside the cytosol or to poor passage across the transient holes. Cell viability, estimated by monolayer density was >80%.

A. Fluorescence emission spectra of the AuZ nanocluster

B. Fluorescence emission spectra of the AuGS nanocluster

Figure S12. Fluorescence emission spectra of the AuZ (A) and AuGS (B) nanoclusters at various excitation wavelengths as indicated. Results indicated that the AuZ and the AuGS have photoluminescent abilities (Ex: 300, Em: 376 nm). The intensity of the photoluminescence was extremely weak and practically useless for *in cellulo* imaging or nanocluster tracking. Condition: 0.6 μM AuNC in 10 mM K_2CO_3 .

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Figure S13. HPLC profile and mass spectrometry analysis of the Cap peptide (CALLNG, $C_{22}H_{38}N_8O_9S_1$; MW : 1801.06). Column: 4.6mmx250mm Diamonsil 5 μ C18. Solvent A: 0.1% TFA in 100% Acetonitrile, Solvent B : 0.1% TFA in water. Gradient: 0 to 25 min, 7 to 32% A.

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Figure S14. HPLC profile and mass spectrometry analysis of the NLS peptide (CALNNGAGPKKKRKVED, $C_{76}H_{134}N_{26}O_{25}S_1$; MW : 1828.13). Column 4.6mmx250mm

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Diamonsil 5 μ C18. Solvent A: 0.1% TFA in 100% Acetonitrile, Solvent B : 0.1% TFA in water. Gradient: 0 to 25 min, 7 to 32% A.

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Figure S15. HPLC profile and mass spectrometry analysis of the dNLS peptide (CALNNGAGPKTKRKVED, $C_{74}H_{129}N_{25}O_{25}S_1$; MW : 1801.06). Column 4.6mmx250mm Inertsil ODS-SP. Solvent A: 0.1% TFA in 100% Acetonitrile, Solvent B: 0.1% TFA in water.

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Gradient: 0 to 25 min, 8 to 33% A.

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Figure S16. HPLC profile and mass spectrometry analysis of the NES peptide (CALNGLALKLAGLDINKT_{-amide}, C₈₄H₁₄₉N₂₅O₂₅S₁; MW: 1941.33). Column

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4.6mmx250mm Inertsil ODS-SP. Solvent A: 0.1% TFA in 100% Acetonitrile, Solvent B: 0.1% TFA in water. Gradient: 0 to 25 min, 8 to 33% A.

Figure S17. FT IR spectrum of the AuZ nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

Figure S18. FT IR spectrum of the AuGS nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

Figure S19. FT IR spectrum of the AuCap nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

Figure S20. FT IR spectrum of the AuNLS nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

Figure S21. FT IR spectrum of the AudNLS nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

Figure S22. FT IR spectrum of the AuNES nanocluster. The spectrum was taken using a Nicolet 380 FT-IR spectrometer and a diamond ATR by ThermoFisher Scientific. Number of scans: 32.

A. Route for labeling AuNLS with Alexa488

B. UV-vis spectrum of Alexa488-labeled AuNLS

Figure S23. Synthesis of Alexa488-labeled AuNLS. **A.** Route for preparing Alexa488-labeled AuNLS. AlexaFluo488 NHS ester (ThermoFisher) (16 nmol) was mixed with the HS-NLS (16 nmol) in 150 mM K_2CO_3 and incubated for 24h for labeling the peptide on the Lysines. The reaction mixture was then treated with TCEP (32 nmol) and then AuZ (8 nmol). After a 24h reaction, unlabeled SH-NLSs (240 nmol) and TCEP (240 nmol) were added and the reaction was incubated for 24h before purification using an ultracentrifugation device with a 30 kDa cut-off. **B.** UV-vis spectrum of the Alexa488-labeled AuNLS (black line). The green line corresponds to the UV/vis spectrum of Alexa488-NHS ester.

A. Scheme of the experiment for characterizing the AuNLSAlexa488

B. PAGE Analysis of AuNLS-alexa488 and TCEP-treated AuNLS-alexa488

C. Fluorescence emission spectra of AuNLS-Alexa488 before and after treatment with TCEP (Excitation wavelength : 480 nm)

Figure S24. Characterization of Alexa488-labeled AuNLS. **A.** Scheme of experiment done for characterizing the AuNLSAlexa488. The nanocluster (0.32 nmol) was treated in water with TCEP, pH 7.0 (500 nmol) in a 6 μ L volume to perform an oxidative dissolution of the Au(0) and to release the ligand from the nanocluster.^{10,11} **B.** PAGE analysis of AuZ, AuNLSAlexa488 and the TCEP-treated corresponding nanoclusters as indicated. After analysis, the gel was visualized under visible light (left image) or under UV (right image). Data showed AuZ and AuNLSAlexa488 to be non-fluorescent (actually they absorb UV light (intense dark band)). TCEP-treated samples showed release of a fluorescent species (the likely released species are indicated in the graph), suggesting that AuNC actually quenches fluorescence. **C.** Fluorescence emission spectra of the AuNLSAlexa488 and of the TCEP-treated AuNLSAlexa488.

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Comment: data indicated that the TCEP-treated AuNLSAlexa488 released fluorescent species whereas AuNLSAlexa488 did not emit the Alexa488 fluorescence. These experiment clearly demonstrated that: -1. AuNLSAlexa488 is grafted with the Alexa488, -2. The AuNC quenches fluorescence of the bound Alexa488 fluorophore, as previously observed.⁴

Figure S25. Analysis of fate of Alexa488-labeled AuNLS after addition to HFF-1 cells. **A.** Images of HFF-1 cells incubated with the AuNLSAlexa488 for 24h. Hoechst 33242-stained cells were observed under bright field, blue channel, for detection of Hoechst 33242-stained nuclei, and green channel for detection of the Alexa488. **B.** Images of HFF-1 cells incubated with the AuNLSAlexa488 for 24h followed by silver staining. **C.** Images of electroporated HFF-1 cells incubated with the AuNLSAlexa488 for 24h. Hoechst 33242-stained cells were observed using phase contrast, under bright field, blue channel (for detection of Hoechst 33242-stained nuclei) and green channel for detection of the Alexa488). **D.** Images of electroporated HFF-1 cells incubated with the AuNLSAlexa488 and for 24h followed by silver staining. Following the 24h incubation period at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v). Data showed absence of Alexa488 fluorescence, suggesting that the Alexa488 remained bound to the

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gold nanocluster (see Figure S26 for experiment showing the AuNC to quench the Alexa488 fluorescence).

A. without gold nanocluster

20 μ m

B. AuCap

20 μ m

C. AuNLS

20 μ m

D. AudNLS

20 μ m

E. AuCap - Electroporation

20 μ m

F. AuNLS - Electroporation

20 μ m

G. AudNLS - Electroporation

20 μ m

Figure S26. Color images of HeLa cells incubated with AuCap, AuNLS and AudNLS. **A.** Untreated cells. **B, C and D.** The various peptide-AuNCs were added to living HeLa cells by dilution in the cell culture medium without an electroporation treatment. **E, F and G.** The HeLa cells were subjected to electroporation in presence of the indicated AuNCs. Following the 24h incubation period at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v) and AuNCs were detected by silver staining.

A. AuNES and leptomycin - Electroporation treatment

B. AuNES - Electroporation treatment

Figure S27. Color images of HeLa cells incubated for 24h with: **A.** AuNES and leptomycin, or **B.** AuNES alone. The AuNES was added to living HeLa cells by dilution in the cell culture medium with an electroporation treatment. Following the 24h incubation period at 37°C, the living cells were fixed with glutaraldehyde (2.5% w/v) and the AuNES was detected by silver staining.

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