Intracellular localization of gold nanoparticles with targeted delivery in MT-4 lymphocytes

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Abstract
The clinical utility of important therapeutic agents is often limited by the poor permeability of biological membranes. Cell penetrating peptides are usually employed to circumvent this challenge. This approach, coupled with gold nanoparticles, are a promising vehicle for drug delivery due to its good biocompatibility profile, negligible toxicity and possibility for multifunctionalization. Here we report the functionalization and intracellular tracking of gold nanoparticles decorated with a TAT cell penetrating peptide and a fluorescein tag in a simple, two step process. Fluorescence microscopy has confirmed the localization of the functionalized nanoparticles to be inside the cells, specifically within, or in close proximity to the nuclei of MT-4 lymphocytes; a HIV-relevant cell line in which this has not been previously demonstrated. The results of this study demonstrate that TAT has been efficiently conjugated to gold nanoparticles to facilitate both cellular and targeted nuclear entry.

Keywords: HIV, gold nanoparticle, MT-4, cell-penetrating peptide, uptake

Classification numbers: 2.05, 5.08

1. Introduction

The impermeable nature of the cell membrane restricts the use of various macromolecules as therapeutic agents [1] and thus poses a challenge for intracellular delivery [2]. The HIV trans-activator of transcription (TAT) peptide is well recognized as a cell-penetrating peptide (CPP), and has been widely used to circumvent this challenge, however, translocation specifically to the nucleus of the cell has not been illustrated sufficiently [1, 3].

Evidence to support nuclear localization is mainly demonstrated by transmission electron microscopy (TEM) showing cell sections that differentiate the nucleus from the cytoplasm [4-6], video-enhanced color and differential interference contrast microscopy [3, 7]. Confocal microscopy studies [8, 9], however, can make use of different fluorescent dyes that stain specific organelles, such as the nucleus, cytoplasm and cell membranes, and thus offers convenient qualitative confirmation on cellular compartmentalization. This is a useful tool to track the localization of gold nanoparticles (AuNPs) with targeted delivery, and can be of special importance in HIV studies where inhibitors are required to act at specific cellular compartments, for example integrase inhibitors where nuclear-targeting is desirable.

There is a lack of research on peptide therapeutics for HIV using targeted delivery, as the majority of research has
been focused on cancer therapeutics [4, 10–13]. Incidentally, the most commonly used cell lines are adherent tumorous cell lines, HeLa cells being the most widely reported choice [3, 6, 14–16]. In contrast to adherent cell lines, suspension cultures are exceptionally challenging to transfect in vitro using conventional methods [17, 18].

MT-4 cells are a human T-cell lymphotropic virus type 1 transformed cell line and a popular choice for investigating the efficacy of anti-HIV agents because they are highly susceptible and permissive to HIV infection [19, 20]. Uptake in this suspension cell line, however, has proven to be difficult since these cells are inherently resistant to transfection [21]. Consequently, there is a paucity of nano-therapeutic research done for HIV in suspension lymphocytes. Bowman et al investigated inhibition of the HIV fusion event using AuNPs in peripheral blood mononuclear cells [22]. To our knowledge, no localization studies have been done in this particular HIV-relevant cell line.

Most AuNP functionalization experiments require that the peptides are cysteine-terminated [3, 7, 23, 24] to provide the thiol sulphydryl (SH) moiety which is necessary for binding to the AuNP surface via thiol-gold affinity interactions [25]. The method described here offers a convenient and alternative approach for using peptides that lack a thiol terminus.

This paper describes the functionalization of spherical AuNPs with a TAT CPP and a fluorescent fluorescein thiocyanate (FITC) label in a simple, two-step process. Confirmation of nuclear localization by fluorescence microscopy is described in MT-4 lymphocytes, which are significant to HIV research.

2. Experimental

2.1. Chemicals and materials

TAT peptide (47–57, Y-G-R-K-K-R-R-Q-R-R-R) [26, 27] and 10 nm spherical AuNPs were purchased from Sigma-Aldrich Co. A mock peptide (A-K-A-A-A-K-A-F-K-F) was synthesized using Fmoc solid phase peptide synthesis and used as a negative control in the cell-uptake experiments. SH-PEG-COOH and SH-PEG-FITC [28] were purchased from Nanocs Inc. (New York). Ethyl (dimethylaminopropyl) carbodiimide (EDC) was purchased from Proteochem and N-hydroxysuccinimide (NHS) was obtained from Purechemistry Inc. 4,6 diamidino-2-phenylindole (DAPI) nuclear stain was purchased from Sigma-Aldrich.

2.2. Preparation of fluorescently labeled gold-PEG-COOH complexes

The concentration of the 10 nm AuNPs as determined by UV–vis spectroscopy was 8 × 10⁻⁵ M. Using a 1 polyethylene-glycol (PEG)/nm² ratio, the final concentration of PEG required for the functionalization experiments was 6.28 × 10⁻⁷ M (using the molar coefficient of extinction for 10 nm AuNPs as reported in literature) [29]. 600 μl of AuNPs was mixed with 40 μl of SH-PEG-COOH and 40 μl SH-PEG-FITC. The solution was mixed for 24 h in the dark and followed by repeated centrifugation at 12 000 rpm for 1 h. The supernatant was carefully removed and the pellet re-suspended in distilled water.

2.3. Functionalization with TAT peptide

TAT peptide was conjugated to the AuNPs via the carbodiimide coupling reaction, which results in the formation of an amide bond between the amine moiety of the TAT peptide and the carboxyl group of the PEG ligands attached to the AuNPs. 18 μl NHS (0.2 M) was added to the AuNP solutions following 24 h of incubation and vortexed for 5 min. This was followed by the addition of 72 μl EDC (0.2 M) and vortexing for 5 min. The TAT peptide (100 μl, 1 mg ml⁻¹) was then added to the reaction mix (at a TAT:COOH molar ratio of 10) and stirred vigorously for 4 h. Purification of the functionalized complex was done by the removal of the supernatant following repeated centrifugation (12 000 rpm for 1 h) and re-suspension of the pellet-containing AuNPs in distilled water. A schematic illustration of the AuNP+PEG+TAT complex is shown in figure 1.

The same procedure was carried out for functionalization of the AuNPs with the mock peptide (instead of TAT) and was used as a negative control for the uptake experiments.

2.4. Characterization experiments

2.4.1. UV–vis absorption spectra. The UV visible spectroscopy was performed on a BioSpec Nano spectrophotometer (Shimadzu, Hyoto, Japan). 2 μl of each sample was used to measure the difference in absorption, indicative of differences in surface modification of the AuNPs.

2.4.2. Agarose gel electrophoresis. Agarose gel electrophoresis was used to determine the difference in mobilities for the different conjugates (AuNP only, AuNP+PEG, AuNP+PEG+TAT). 9 μl of each conjugate was added to 1 μl of 40% (w/v) sucrose and loaded onto a 1% agarose gel. Electrophoresis commenced in 1 X TBE buffer at 120 V for 60 min.

2.4.3. Transmission electron microscopy. Morphological characteristics were further confirmed by TEM. Samples were analyzed using a JEOL JEM-1010 transmission electron microscope operating at an acceleration voltage of 100 kV.

2.5. Cell culture experiments

MT-4 cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. MT-4 from Dr Douglas Richman. Cell cultures were propagated in RPMI 1640 media supplemented with 10% fetal bovine serum (Biochrom, Germany). Cells with a viability of 95% were seeded in 96-well plates at a density of 5 × 10⁴ cells/well. 50 μl of either AuNP+PEG+TAT or AuNP+PEG+mock
were incubated with 200 μl of MT-4 cells for 24 h at 37 °C in a 5% CO2 humidified atmosphere.

2.6. Fluorescence microscopy

After 24 h incubation of AuNP+PEG+TAT and AuNP+PEG +mock complexes in MT-4 cells, cells were fixed on confocal microscopy slides using 4% paraformaldehyde (PFA). Cells were stained with DAPI to visualize the nucleus of the cells. Fluorescence images were captured on a Zeiss LSM 710 Confocal microscope and a FLoidTM Cell Imaging System (Life Technologies, CA, USA).

3. Results

3.1. UV–vis absorption spectra

The absorption spectra for the different AuNP conjugates are shown in figure 2.

Figure 1. Schematic illustration of the formation of the GNP+PEG+TAT conjugate.

Figure 2. UV–vis absorption spectra of the AuNP, AuNP+PEG, AuNP+PEG+TAT and AuNP+PEG+mock layered complexes.

Figure 3. Agarose gel electrophoresis result showing different mobilities of the AuNP (lane 1), AuNP+PEG (lane 2) and AuNP +PEG+TAT (lane 3) conjugates.

3.2. Agarose gel electrophoresis

The agarose gel electrophoresis result is shown in figure 3.

3.3. Transmission electron microscopy

TEM analysis of the AuNPs and AuNP+TAT conjugates are shown in figures 4(a) and (b). Both have a diameter of 10 nm.
3.4. Confocal fluorescence microscopy

The confocal microscopy results of the AuNP-PEG-TAT and AuNP-PEG+mock peptides are shown in figures 5(a) and (b), respectively.

3.5. Epifluorescence microscopy

The results of AuNP+PEG+TAT and AuNP+PEG+mock peptides visualized using epifluorescence microscopy are shown in figures 6(a) and (b), respectively.
4. Discussion

The characterization results demonstrate that TAT-peptide conjugation to the AuNPs was successful. Differences in the absorption can be used to monitor the surface modification patterns of AuNPs [28]. As shown in figure 2, the plasmon absorption spectra for the AuNP, AuNP+PEG, AuNP+PEG+TAT and AuNP+PEG+mock conjugates were different; there was a slight red-shift of the surface plasmon resonance (SPR) peak from 527 to 534 nm when TAT was added to the AuNP. This can be attributed to the change in the refractive index caused by thiol adsorption on the AuNP surface and difference in ligand (size) contributions [30, 31] as an increase in nanoparticle size is associated with a red-shift [32]. There was no significant peak broadening associated with the red-shift, thereby indicating that the dispersity, and therefore integrity, of the AuNPs was maintained upon addition of the PEG and TAT conjugates [31].

This result was confirmed by agarose gel electrophoresis. As shown in figure 3, the AuNPs (lane 1, with a net negative charge) migrated toward the anode. PEGylation of the AuNPs resulted in an overall neutral charge [33] and therefore did not migrate on the gel (lane 2). A protein with an isoelectric point greater than the pH of the buffer used will migrate toward the cathode [34]. In accordance with this, TAT peptide has an isoelectric point of 12.81 and the pH of TBE buffer used is 8.2–8.4 and so migrated toward the cathode. Furthermore, TAT is positively charged [35] and as seen in lane 3, migration of the TAT-conjugate was toward the cathode, confirming successful conjugation of the TAT peptide to the AuNP surface. The distinct difference in mobilities between the different conjugates (with the AuNP migrating much faster than the AuNP+TAT conjugate) is a clear indication that the functionalization was successful.

TEM analysis showed that there was no aggregation of AuNPs observed and rather monodisperse particles were present. Following conjugation of the TAT peptide (figure 4(b)), the dispersity increased as a result of a possible repulsion effect afforded by TAT on the positively charged AuNP surface. The use of PEG as a ligand also results in repulsion due to steric reasons [25]. TEM did not show an obvious increase in the size of the AuNPs following addition of TAT and this is important for delivery of therapeutic agents through the nuclear pore with limited diameters, depending on the cell type and cell cycle [13].

The addition of PEG to the AuNPs increases the stability by preventing aggregation in high ionic strength biological media [16], and also facilitates evasion of the immune system which is important for downstream applications. The half-life of the nanocarrier is also increased by PEGylation [32].

The SH terminus of the PEG molecule is necessary to bind to the AuNP surface via thiol-gold affinity interactions [25]. The choice of PEG-conjugated molecules used in this study was (i) FITC to facilitate fluorescent intracellular tracking and (ii) a COOH moiety, required for attachment to the TAT (and mock) peptide via its amine (NH2) group, respectively. The latter also provides a convenient alternative for AuNP functionalization with peptides that are not cysteine-terminated [16]. The 5000 Dalton PEG spacer arm between the thiol group and the FITC terminus is also
important for decreasing the effect of fluorescence quenching by the AuNPs [36].

Figures 5 and 6 clearly show that TAT is effective in permeating the cell membrane and localizing in and around the cell nuclei, when compared to the mock peptide with no CPP-activity. This sub-cellular compartmentalization is important for directing nanocarriers of drug payloads at specific locations within a cell. The green FITC signal in the confocal images is lower in comparison to the DAPI and this may be due to some degree of fluorescence quenching by the AuNPs [36]. It has been demonstrated that successful nuclear targeting can occur with just 5% of nuclear-localization signal used on PEG-modified AuNPs [6]. The ratio of FITC and TAT can therefore be modified based on the functionalization requirements (size of AuNPs, number of peptides or other biomolecules required).

A limitation in this study was that the orientation of COOH binding to the NH2 moiety of either the peptide terminus or as a side chain in lysine residue(s) present in the TAT peptide, was not determined. Any ambiguity in the binding orientation will be addressed in future work as this is an avenue for further research. We assumed that the peptide linked linearly to the COOH terminus, and that cross-linking via side chains in the peptide would alter the sequence [37] and possibly peptide functionality or efficacy. The results, however, demonstrate that regardless of the binding orientation, TAT functionalized AuNPs can be efficiently delivered into the nucleus of the cells.

5. Conclusion

The development of CPP-conjugated AuNPs for targeted delivery is indeed promising for diseases such as HIV/AIDS, where development of novel therapeutics is essential. Consequently, the development of nanoparticles that can bind, stabilize and deliver therapeutic molecules, and can traverse both the cellular and nuclear membrane is required. We developed and functionalized fluorescent gold nanoparticles, of adequate diameter, containing a nuclear localization signal for targeted delivery. This type of functionalization is important for delivery of agents that require therapeutic action specifically in the nucleus of the cell.

Varying the ratio of SH-PEG-FITC to SH-PEG-COOH will enable multi-functionalization of AuNPs with one or more therapeutic peptides or other biomolecules, and so can be extended to broader biomedical applications such as drug delivery, and this is an avenue for future research.

We have shown for the first time that AuNPs functionalized with a CPP can be introduced into the cellular and the nuclear vicinity of MT-4 lymphocytes.

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