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Enhanced intracellular translocation and cytokine profiling of gold nanoparticles conjugated with human papilloma virus based (HPV-R9) cell-penetrating peptide in cancer cells

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Functionalized gold nanoparticles are emerging as a promising nano carrier for targeted delivery of therapeutic molecules. The present study describes synthesis, characterization and intracellular delivery of gold nanoparticles (AuNPs) functionalized with L1 protein of human papilloma virus peptide (HPV) derived cell-penetrating peptide (CPP) conjugated with R9. HPV-R9 cell penetrating peptide conjugated with AuNPs (fAuNPs) was characterized using UV-visible and Fourier Transform Infrared (FTIR) spectroscopy, transmission electron microscopy, dynamic light scattering and zeta potential. The cellular uptake of fAuNPs was studied in Vero cells using fluorescence microscopy and flow cytometry. The fAuNPs displayed reversible low intracellular aggregation, ~20% cytotoxicity but high efficiency and stability in Vero cells. The cytokines study by real-time PCR was performed in 4T1 cells of the IL-4, IL-12 and IFN-γ, found to be upregulated in comparison to control. The functionalized gold nanoparticles cell penetrating peptide show good intracellular transport to the cellular destinations. Results of this study demonstrate that cell penetrating peptide can be used along with gold nanoparticles for therapeutics application, particularly cancer.

Keywords: Drug delivery, Cellular uptake, Vero cells, UV-visible spectroscopy

To achieve targeted delivery of biomolecules, several delivery vehicles have been developed including nanoparticles[1-4]. Most existing delivery vehicles are toxic to cells, and in most cases the primary uptake into the endosomal vesicle leads to inefficient release of fusion protein into the cytosol. Gold nanoparticles (AuNPs) have been reported to overcome these barriers[5-7] and such AuNPs when functionalized with nucleic acids, drugs, antibodies, PEG[8], siRNA or peptides[8-10] exhibit low cytotoxicity and excellent bio distribution abilities.

Cell-penetrating peptides (CPPs), most commonly used for conjugation with AuNPs, are short peptides with membrane translocation capability and are widely used to deliver therapeutic molecules, nanoparticles, siRNA and nucleic acid[11]. These peptides usually translocate through biological membranes by direct penetration, transient pores and macropinocytosis[12]. The most commonly used CPP is the commercially available HIV TAT (protein trans-activator of transcription) peptide[13] for delivery of macromolecules and/or drugs into the nucleus or cytoplasm. AuNPs functionalized with CPPs translocate to the cytoplasm and the nucleus[14]. Some small molecules and amino acids that contained the reactive group for further modifications, such as cysteine, glutathione to stabilized the AuNPs[15].

Recently, we characterized the novel cell penetrating peptide derived from human papilloma virus (HPV) in nucleic acid delivery (data not shown). The potential CPP is derived from the C-terminus of the L1 protein of HPV. Here, we tried to develop a nano delivery vehicle by conjugating cysteinylated cell penetrating peptide of Human Papilloma virus with AuNPs for delivery of biomolecules with enhanced cellular uptake. AuNPs have been reported to cause changes in the expression of cytokines including IL-4, IL-1β and TNF-alpha[16]. Hence, we did

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cytokine profile of AuNPs and fAuNPs for the IL-4, IL-12 and IFN-γ was studied by means of real-time PCR in mouse mammary tumor cells (4T1). We hypothesized that fAuNPs could be used in the application of targeted drug delivery.

Materials and Methods

Initially, we synthesized the gold nano particle of 10-13 nm size by citrate reduction method and conjugate the FITC labeled HPV-R9 peptide with AuNPs named functionalized gold nano particle (fAuNPs). The AuNPs and fAuNPs were characterized by zeta-sizer, Fourier Transform Infrared (FTIR), UV-visible spectroscopy. The fAUNPs were then transfected in Vero cells and after 24 h the transfection efficiency was measured by fluorescence microscopy. The quantitative intracellular delivery of fAuNPs was measured by flow cytometry and cytotoxicity by MTT assay. Therefore, the CPP stabilized with AUNPs could be an ideal delivery vehicle for the various therapeutic strategies. The fAuNP displayed low intracellular aggregation and toxicity but significant efficiency and stability in Vero cells.

Identification of cell-penetrating peptide

HPV-type 16 L1 protein sequence (region 5703-7307 bp) was analyzed using an *in silico* algorithm. The support vector machine (SVM)–based CPP prediction was performed with a CellPPD web server that predicts efficient cell-penetrating peptides[17]. The SVM + Motif prediction method was selected at an SVM threshold of 0.1 and a motif e-value of 10^6.

Peptide synthesis and analysis

The identified CPP from HPV (TATKRKKRKKC) and its fusion with R9 peptides yields HPV-R9 (TAT KRKKRKKGGGRRRRRRRRRC). It is synthesized by standard solid phase methodology using F-moc chemistry[18]. Precipitated peptides in crude forms are purified by reverse phase chromatography (RP-HPLC) on a C18–semi-preparative column using a UFLC pump system (Shimadzu, Tokyo, Japan) fitted to a photo diode array (PDA) detector. The specific labelling at the N-terminus of both peptides is achieved by conjugating with the FITC before cleavage and deprotection of peptides from the resin. It was analyzed by RP-HPLC, and the molecular masses of HPV and HPV-R9 were 1347.7 and 3027.99 g/mol, respectively.

Cell culture

Vero cells obtained from the National Centre for Cell Sciences (NCCS) in Pune, India and 4T1 (mammary tumor cell line) obtained from ATCC India were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA) and RPMI with 10% Fetal bovine serum, 100 U/mL penicillin, 100μg/mL streptomycin (Gibco, USA) and 10 mM HEPES buffer (Duchefa Biocheme, the Netherlands), respectively. The cells were maintained at 37ºC, 5% CO_2atmosphere and 95% relative humidity.

Synthesis of AuNPs

Gold nanoparticles were synthesized by means of reduction of HAuCl4 by sodium citrate[19]. An aqueous solution of $HAuCl_4$ (250 mL, 1.0 mM) was refluxed for 5-10 min, and a warm aqueous solution of sodium citrate (30 mL, 35 mM) was added. The reflux was continued for about 30 min until a deep red solution developed. The solution was then passed through a 0.45 µm syringe filter to remove any precipitate and stored at 4ºC.

Functionalization of AuNPs with peptides (fAuNPs)

Peptide HPV-R9 (NH2-RRRRRRRRRGGGTATK RKKRKKC-Linker-Cys-CoNH2) wasconjugated to AuNPs by mixing 5 nM of AuNPs with different concentrations of peptide to optimize the peptide amount required for activation and avoid excessive peptide-induced precipitation. The mixture (5 mL AuNPs+10 µg peptide) was incubated for 24 h with gentle stirring and washed three times by centrifugation at 16000 ×g for 20 min, and then the pellet was resuspended in phosphate buffer, pH 7.4. To check for the absence of nonconjugated peptide, 2 mL of the conjugated solution was centrifuged at 16000 ×g for 30 min, evaporated to dryness and finally resuspended in HPLC grade water. Peptide-Cys-AuNPs were first suspended in methanol through sonication to ensure the extraction of representative dispersion volumes.

Characterization of fAuNPs

The functionalization of AuNPs was assessed by UV-visible and FTIR spectroscopy, transmission electron microscopy and zeta potential. For UV-visible spectroscopy, the spectra were collected at wavelengths of 400-700 nm for both AuNPs and fAuNPs separately. FTIR (Nicolet 6700 FTIR, Thermo Fisher) spectra were collected for AuNPs and fAuNPs with over 118 scans per sample at a

resolution of 4 cm^{-1}. Particle size analysis was performed using a Zetatrac instrument (Microtrac). TEM images were taken with a Phillips Biotwin 12 transmission (FEI) electron microscope.

Cellular uptake of fAuNPs

Fluorescence microscopy

The Vero cells were seeded at 2×10^5 cells per well and incubated overnight at 37ºC with 5% CO_2. The cells were transfected with FITC-conjugated AuNPs and fAuNPs for 24 h, fixed in absolute alcohol, stained with DAPI and analyzed by fluorescence microscope (Nikon).

Flow cytometry

The Vero cells were seeded and processed for transfection in a similar way as in fluorescence microscopy. The cells were trypsinized, washed twice with PBS and processed for flow cytometry in an FL1 filter (BD FACS Calibur).

Cytotoxicity assessment of fAuNPs

The cytotoxicity of the fAuNPs was assessed by MTT dye reduction assay. Vero cells were seeded at a density of 1×10^4cells/well in a 96-well plate using minimum essential medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine and 1X penicillin-streptomycin. The cytotoxicity of peptides, AuNPs and fAuNPs was determined by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) dye reduction assay using CellTiter 96®non-radio-active cell proliferation assay (MTT) kit (Promega, Madison, WI, USA). The absorbance was measured at 570 nm using a microplate reader (M5 plate reader).

RT-PCR

The AuNPs and fAuNPs (10 and 20 nm) were transfected in 4T1 cells, and 48 h after transfection the cells were harvested and cell pellet was dissolved in 1.0 mL of TRIzol reagent (Ambion, Life Technologies) and the total RNA was isolated as per manufacturer protocol. The purity and concentration of RNA were checked by a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). One µg of RNA was reverse transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (M/sMBI Fermentas Life Sciences, Maryland, USA) according to the manufacturer's protocol. qRT-PCR was performed using the SYBR Green-I master mix (SYBR Green chemistry), a two-step RT- PCR kit (Brilliant III Ultra-Fast SYBR ® Green QPCR Master Mix" #600882) with 7500 fast instrument (Applied Biosystem). The passive reference dye ROX was used for normalization of the reporter signal (SYBR Green). GAPDH was employed as the internal control, as it has been found to be a suitable endogenous control[20]. A melting curve analysis was performed to determine the specificity of qRT-PCR. The fold change was calculated by 2-ΔΔct as reported earlier[21]..

Results

Synthesis and characterization of fAuNPs

The AuNPs and fAuNPs were characterized by UV-visible spectroscopy and FTIR. The size of fAuNPs was measured by transmission electron microscopy (TEM) (Fig. 1A) The UV-visible spectra of AuNPs displayed a shift from 517 to 525 nm, indicating successful functionalization of the AuNPs (Fig. 1B). To reconfirm the UV-visible spectral shift, the AuNPs and fAuNPs were subjected to FTIR scanning. The size of the AuNPs after conjugation with HPV-R9 increased from 13 to 35.9 nm, indicating successful conjugation (Fig. 2). Once the AuNPs were modified with peptide ligands the colour of AuNPs changed from dark red to blue which indicate the aggregation. In case of HPV-R9 the fAuNPs maintained the red colour that indicative of no aggregation. The FTIR scans displayed a peak

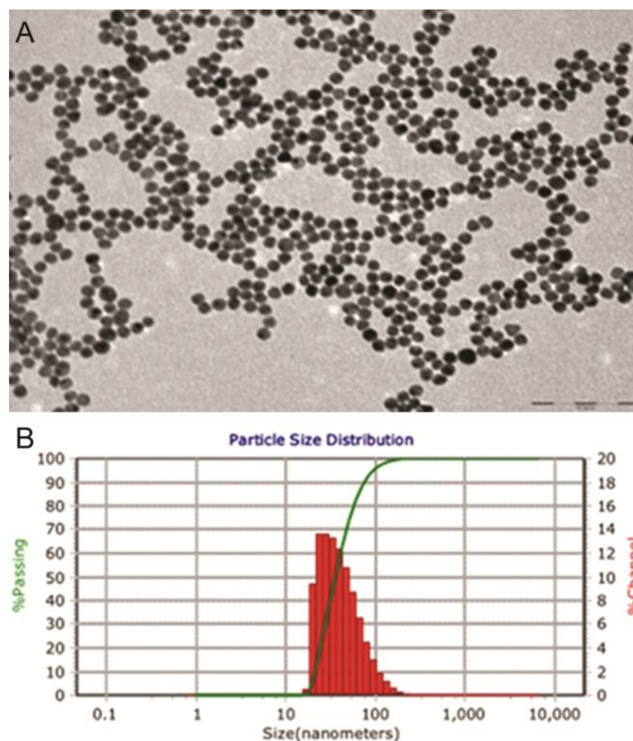

Fig. 1 — (A) Size of fAuNPs by TEM; and (B) Size confirmation of fAuNPs by dynamic light scattering (Zetasizer).

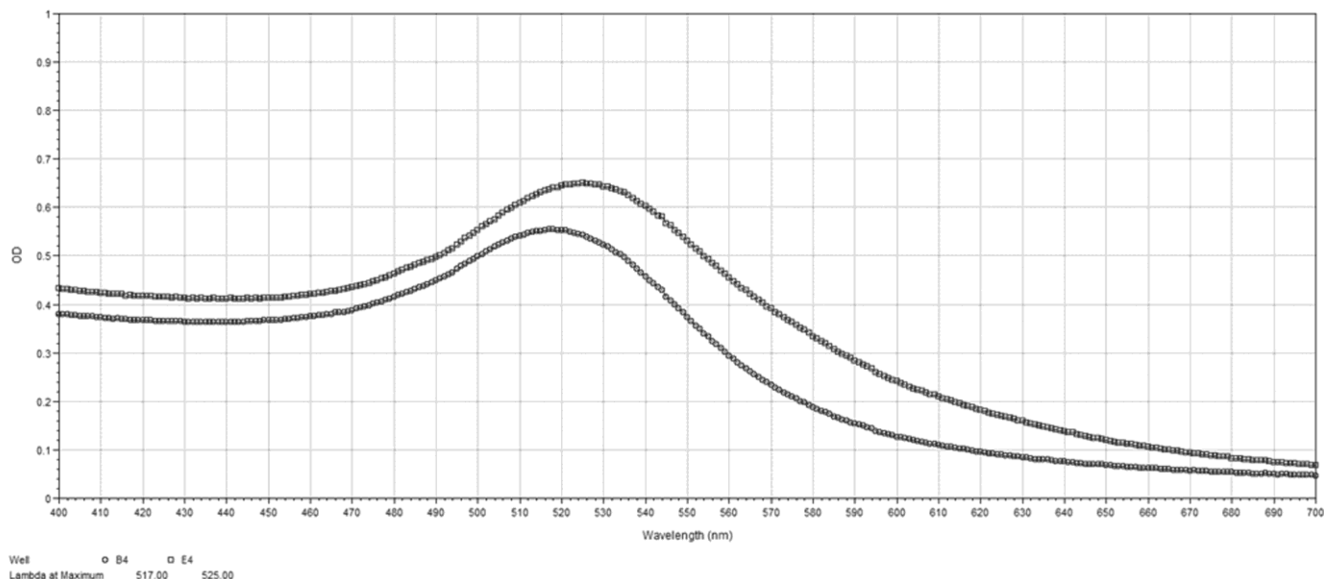

Fig. 2 — Spectral image for characterization of surface functionalized AuNPs by UV-visible spectroscopy.

difference between 1290 and 1120 cm^{-1}, which confirmed the conjugation of peptide to AuNPs (Fig. 3), In addition, the fAuNPs were found to be stable for more than 2 months at 4ºC, with minute aggregates that segregated after gentle vortexing. This result reflects the reversible nature of cluster formation in the nanoparticles.

Cellular uptake of fAuNPs

The cellular uptake of fAuNPs in Vero cells by transfection was assessed and confirmed by fluorescence microscopy after 24 h of transfection (Fig. 4). The green fluorescence indicates the uptake of fAuNPs by the Vero cells and merged image with DAPI shows the localization of fAuNPs in the cells. The qualitative observations of fluorescence microscopy were further reinforced by flow cytometry (Fig. 5). As much as 71.42% of Vero cells were positive in fAuNPs compared to control (AuNPs) in concordance with the fluorescence microscopy results.

Cell cytotoxicity assessment of fAuNPs

The cytotoxicity of fAuNPs was evaluated in Vero cell transfected with fAuNPs and after 24 h p.t. cell viability was evaluated by MTT assay. It was observed that high concentration of fAuNPs (15nM), 80% of the cells remained viable (Fig. 6).

Expression profile of the cytokines

The cytokines expression profiles of IL-4, IL-12 and IFN-Y were studied by real time PCR. The fold change of IL-4 was found to be upregulated in the AuNP control group as well as the 10 and 20 nm fAuNPs (1.70±1.00, 1.13±1.00 and 2.60±1.00), respectively. The fold change of IL-12 was found to be upregulated in 10 and 20 nm fAuNPs as compare to AuNPs control cells (0.385±1.00, 1.064±1.00 and 2.700±1.00), respectively. The fold change of IFN-Y was found to be upregulated in the10 and 20 nm fAuNPs as compared to the AuNPs

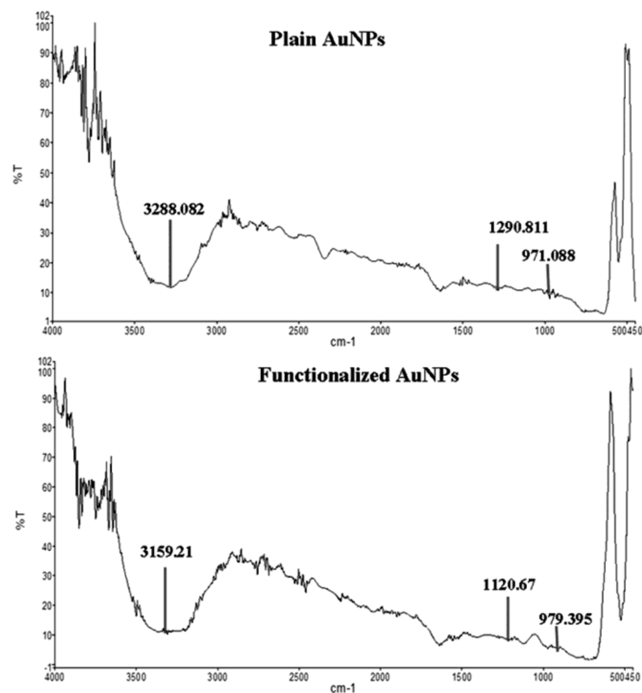

Fig. 3 — Spectra of AuNPs; and functionalized nanoparticles (fAuNPs) characterization for their surface modification by FTIR.

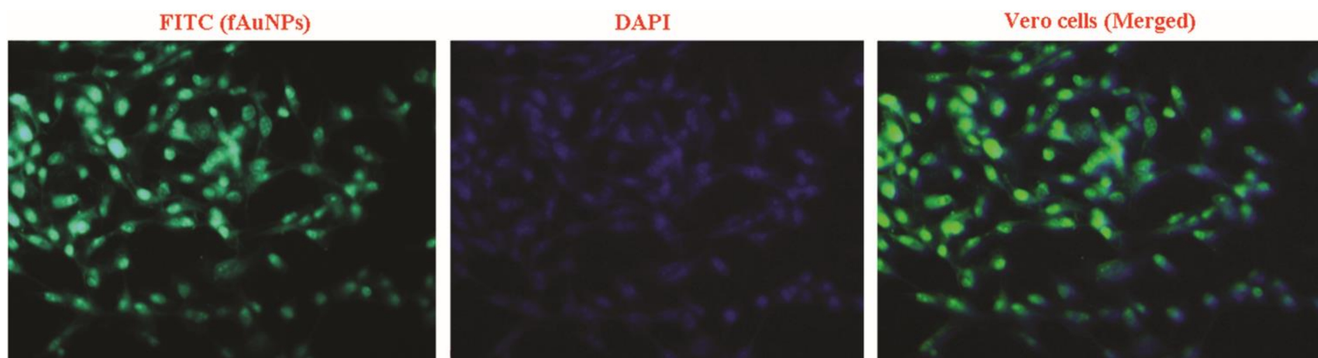

Fig. 4 — Cellular uptake of functionalized nanoparticles by Vero cells by fluorescence microscopy.

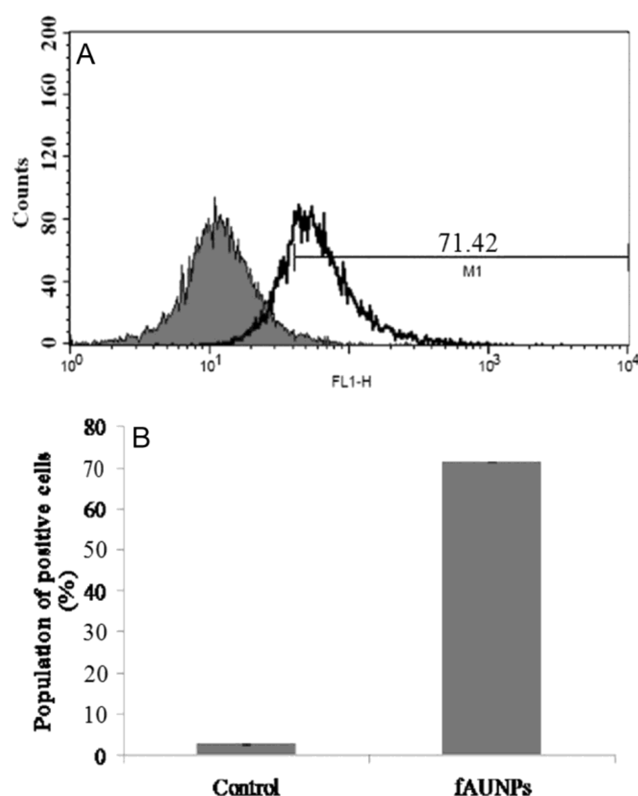

Fig. 5 — Cellular uptake of functionalized nanoparticles by flow cytometry presented in (A) Graphical; and (B) bar diagram

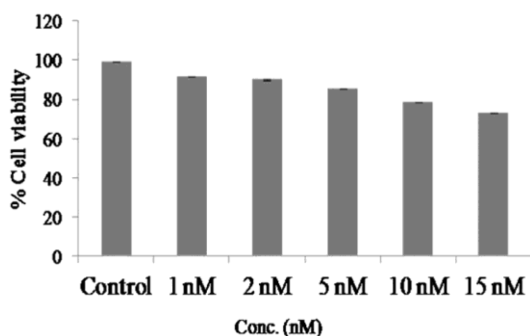

Fig. 6 — Cell viability study after exposure of functionalized nanoparticles at different concentration in *in vitro* and evaluated by MTT assay.

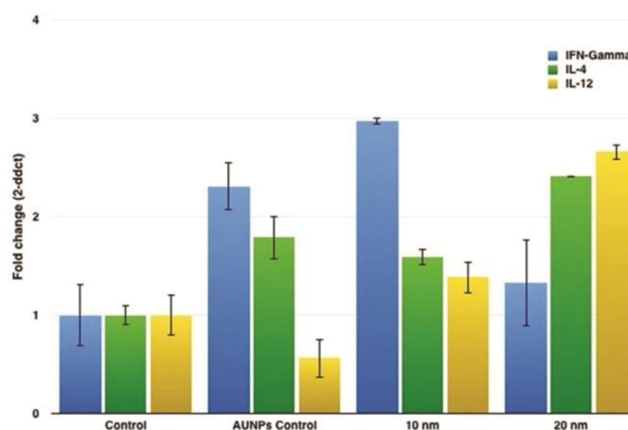

Fig. 7 — The gene expression profile of cytokines (IL-4, IL-12 and IFN-Y) in 4T1 cells by real-time PCR after exposure of fAuNPs with the size 10 and 20 nm

control 4T1 cells (0.918±1.00, 2.566±1.00 and 1.484±1.00, respectively) (Fig. 7).

Discussion

AuNPs are suitable for delivery of drugs to cellular destinations due to ease of synthesis, functionalization and biocompatibility. Peptide-functionalized AuNPs have widely been studied elsewhere for the delivery of drug(s), nucleic acids and siRNAs[22]. To achieve optimum working potential in cellular applications, we have adopted strong methods for a controlled uptake of AuNPs into cells and downstream localization to the cytosol or specific cell organelles. The cationic polymer(s) or coating on AuNPs facilitates their entry into the cells due to the negative charges on the membrane. The cationic, arginine-rich CPPs have been reported to use predominantly direct penetration mechanisms[23]. The amino acid composition significantly alters the internalization of the peptide[24]. The cationic nature of arginine enhances the peptide's internalization by disrupting the membranes of macropinosomes for access into the cytosol[25]. In this study, we designed a novel

CPP HPV-R9 conjugated with 13 nm AuNPs, which facilitate the cellular uptake in Vero cells and its localization in nucleus as well in cytoplasm too. Tiwari *et al*.[26] demonstrated the uptake of VG-21 functionalized GNPs (fGNPs) in eukaryotic cell like HeLa, HEp2 and Vero cells. The conjugation of peptide on the surface of AuNPs was confirmed by UV-visible spectrum, FTIR and zeta-potential. In addition, the fAuNPs displayed very low cytotoxicity to the cell line tested, making them suitable as delivery vehicles in *in vitro* conditions. Several studies like modified amino acid functionalized with AuNPs (pY-AuNPs) suggest them to be potential drug target for cancer therapy with no cytotoxicity in cells[27]. The type-II interferons and interleukins were also found to be upregulated in fAuNPs treated cells showed that the cytokine profiling of the cells after AuNPs exposure. A chemically synthesized anticancer cell penetrating peptide SVS-1 has the ability to promote the rapid uptake of gold nano rods (AuNRs) and AuNPs in HeLa cells and translocated in to cells by non-endocytotic pathway[28].

Furthermore, the nanoparticle diameter and surface coverage of the peptide affected both the internalization of nanoparticles and the sub-cellular target (nucleus or cytoplasm). The uptake of nanoparticles is related to the extent of aggregation, expression of target receptors, endocytosis mechanism and cell phenotypes[29]. The HPV-R9 peptide showed less aggregation within and long-term stability *in vitro*.

Conclusion

We have reported first time the internalization of HPV-R9 peptide conjugated with AuNPs for intracellular delivery in cells. In this study, the fAuNPs conjugated with HPV-R9 peptide shows higher internalization in Vero cells suggesting that this CPP can be used for targeted delivery of therapeutics molecules into the cells. Also, these fAuNPs does not have any cytotoxic effect on the cells. It will enhance availability of therapeutics molecule at the site of diseases by increasing the uptake of modified nanoparticles by cells. Thus, this fAuNPs can be useful for changes in gene expression and might be used as a delivery system.

Conflict of Interest

Authors declare no conflict of interests.

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