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The effect of nanoparticle size and NLS density on nuclear targeting in cancer and normal cells; impaired nuclear import and aberrant nanoparticle intracellular trafficking in glioma

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ABSTRACT

The cell nucleus is an interesting target in many diseases with particular interest in cancer. Previously, nuclear targeted small and large chitosan nanoparticles (S-NPs ≈ 25 nm, and L-NPs ≈ 150 nm respectively), modified with low, intermediate and high densities of NLS (L-NLS, I-NLS and H-NLS) were developed and assessed in L929 fibroblasts. However, to evade apoptosis and stimulate tumor growth cancer cells are capable of manipulating the nuclear-cytoplasmic transport on many levels, making NPs that are capable of nuclear targeting in normal cells incapable of doing so in cancer. For such reason, here, the nuclear delivery efficiency of S-NPs and L-NPs was assessed as a function of their NLS density in cancer and non-cancer cells. For S-NPs, in all cells tested, NLS was unnecessary for nuclear delivery; unmodified S-NPs showed higher nuclear delivery than NLS-S-NPs due to their ability to gain nuclear entry in a passive manner. For L-NPs, L-NLS-L-NPs showed ≈ 8.5, 33, 1.8 and 7.2 fold higher nuclear deliveries than H-NLS-L-NPs in L929 fibroblasts, primary human fibroblasts, HEK 293 and lung cancer cells, respectively. In glioma however, unmodified L-NPs showed highest nuclear delivery, whereas NLS-L-NPs were retained in the cytoplasm. Experiments conducted in the presence of inhibitors of the classical nuclear import pathway indicated that due to overexpression of importin α, classical nuclear import in glioma is impaired leading to aberrant NP intracellular trafficking and nuclear import.

1. Introduction

Nanoparticles (NPs) have developed a reputation for their ability to facilitate drug targeting [1,2]. However, the quest to site specific drug delivery is hurdled on many levels. While it is very difficult to deliver a drug to its target tissue, it also harder to specifically deliver it to its target organelle. Recently, it has become increasingly obvious that cytosolic internalization of a drug does not necessarily imply its interaction with its subcellular target [2,3]. The cytosol is a crowded environment encompassing a network of cytoskeletal filaments that limit the transport of macromolecules and NPs [2], preventing them from realizing their target.

The nucleus holds the roots to several disorders including, neurodegenerative diseases, heart dysfunction and cancer [4,5]. For cancer in particular, the nucleus is a very interesting target. While most low molecular weight chemotherapeutics exert their main action in the nucleus [5], it has been demonstrated that several promising anticancer macromolecular drugs also function primarily in the nucleus [6–8]. However, due to their large size and charge, such molecules have limited cellular entry, and therefore NPs capable of specifically targeting the nucleus in cancer cells would be of great interest.

Under normal conditions, NPs bearing a classical nuclear localization sequence (NLS) are transported to the nucleus via the classical nuclear import pathway. The latter is mediated by importin α and importin β. Importin α binds the NLS utilizing its NLS binding domain and also directly binds to importin β. This complex then docks into the nuclear pore complex (NPC) via direct interaction of importin β with NPC proteins; nucleoporins (Nups) enabling nuclear delivery of the NLS bound cargo [9]. In previous work, small (S-NPs ≈ 25 nm) and large (L-NPs ≈ 150 nm) chitosan NPs modified with different densities of the classical Simian Virus (SV-40) T-antigen NLS for nuclear targeting of proteins were developed [10]. Studies on L929 fibroblasts indicated that for S-NPs an NLS was unnecessary for nuclear targeting, while for L-NPs, a low to intermediate NLS density provided best nuclear localization [10]. However, although strictly regulated, cancer cells are capable

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of manipulating the nuclear-cytoplasmic transport to stimulate tumor growth and evade apoptosis [9,11–13]. Due to the possibility of impairment of the classical nuclear import pathway in cancer cells, it is possible for NPs that show nuclear targeting ability in normal cells to fail to do so in cancer.

In this paper, we study NP intracellular trafficking and nuclear localization ability of chitosan NPs as a function of their size and NLS density in cancer (lung cancer A549 cells and glioma 261) and normal cells (L929 fibroblasts, HEK 293 cells and primary human fibroblasts (hFibs)). Utilizing our previously developed FRET spectroscopy assay [10], NP nuclear delivery (as a function of their NLS density) in the presence and absence of inhibitors of the classic nuclear import pathway was assessed in intact, live cells. Unmodified S-NPs showed higher nuclear delivery in all the cells tested, where the NPs seem to gain nuclear access in a passive manner. Larger NPs however, utilized the classical nuclear import pathway to gain access to the nucleus, with NPs modified with a low-intermediate NLS density providing better nuclear delivery than those with higher one. In glioma however, the latter did not apply, where we prove that due to impaired classical nuclear import, unmodified L-NPs show nuclear targeting ability in glioma whereas NLS L-NPs are retained in the cytoplasm.

2. Materials and methods

2.1. The effect of NP size and NLS density on the nuclear localization efficiency of chitosan NPs in cancer and normal cells

Nuclear localization efficiency was conducted in intact live cells by Förster resonance energy transfer (FRET) spectroscopy according to our previously developed protocol [10]. For FRET, Hoechst (H) (λex:366 nm–λem:486 nm) was used as the donor and fluorescein (λex: 494 nm–λem: 518 nm) as the acceptor. Cells were grown at 37 °C/5% CO_2 in phenol red free RPMI 1640 with L-glutamine and containing 10% fetal calf serum (FCS), 1% penicillin–streptomycin (Lonza, Germany). L929 fibroblasts, hFibs, HEK 293, glioma 261 and A549 lung cancer cells were seeded at a density of 2×10^4 cells per well in a flat bottomed 96 well plate. Bovine serum albumin (ALB, Sigma Aldrich, Germany) was used as a model protein and was modified with fluorescein isothiocynate (FITC; Sigma Aldrich, Germany) according to a protocol detailed elsewhere [14]. ALB-FITC was loaded into small and large chitosan NPs (S-NPs and L-NPs respectively) by the ionotropic gelation method and the NPs were modified with a low, intermediate and high density of NLS (L-NLS, I-NLS and H-NLS) as detailed in earlier work [10]. NP characterization, ALB-FITC loading, NLS tagging and quantification are also detailed in [10].

ALB-FITC loaded S-NPs and L-NPs, and their NLS modified forms were added to cells at a NP concentration of 300 μg/mL and incubated for 24 h. NP containing media were aspirated and Hoechst nuclear staining was performed (1.5 μg/mL). Cells were washed thrice with PBS then analyzed by FRET spectroscopy (FLUOstar Optima, BMG Labtech, UK) [10].

2.2. The effect of classical nuclear import pathway inhibitors on the nuclear localization efficiency of L-NPs

The use of excess NLS [15] and ivermectin [16] has been previously reported to inhibit the importin α/β mediated nuclear import. Accordingly, L929 fibroblasts, hFibs, HEK 293, glioma 261 and A549 lung cancer cells were seeded at a density of 2×10^4 cells per well in a flat bottomed 96 well plate. Prior to NP treatment, cells were treated with 20 μg/mL NLS (Bio Basic Inc., Canada) for 30 min. Following NLS incubation, ALB-FITC loaded L-NPs, L-NLS-L-NPs, I-NLS-L-NPs and H-NLS-L-NPs were added to cells at a concentration of 300 μg/mL and incubated (also in the presence of NLS) for 12 h. L-NP nuclear localization efficiency in the presence of excess NLS was determined by FRET spectroscopy as detailed earlier. Cells that were incubated with L-NPs and NLS NPs in the absence of excess NLS were used as controls.

Similarly, L929 fibroblast and glioma 261 were pretreated with 10 μM ivermectin (Sigma Aldrich, Germany), followed by treatment with 300 μg/mL L-NPs and NLS modified L-NPs (in the presence of ivermectin) for 12 h. Cells that were incubated with L-NPs and NLS L-NPs in the absence of ivermectin were used as controls. L-NP nuclear localization efficiency in the presence of ivermectin was determined by FRET spectroscopy. The experiment was run in triplicates and results were expressed as mean change in FRET efficiency relative to controls and normalized to unmodified L-NPs.

2.3. Nuclear localization of unmodified L-NPs in glioma 261

ALB was modified with RHD as detailed elsewhere [14]. ALB-RHD was loaded into L-NPs by ionotropic gelation [10]. Glioma 261 were seeded on coverslips at a density of 5×10^4 cells per well in a 12 well plate and cultured overnight. ALB-RHD loaded unmodified L-NPs were then added to the cells and incubated for 24 h. After 24 h, NPs were aspirated and cells were washed with PBS. The cells were then subjected to immune-staining of NPC using monoclonal anti-nuclear pore complex proteins mouse IgG (Sigma Aldrich) and FITC-conjugated anti-mouse IgG (Sigma Aldrich). Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (PFA), slides were mounted and studied using confocal laser scanning microscopy (CLSM).

2.4. CLSM assessment to determine the reason behind the change in L-NP nuclear delivery pattern in glioma 261

Glioma 261 were seeded at a density of 5×10^4 cells per well on cover slips in a 12 well plate and cultured overnight. Cells were treated with H-NLS-L-NPs in the presence and absence of excess NLS. Following 24 h incubation, immune-fluorescent staining of importin β was conducted (using monoclonal anti-importin β antibody (I2534 Sigma Aldrich) and a FITC labeled anti-mouse IgG secondary antibody (Sigma Aldrich)). Cells were then studied by CLSM. Immuno-staining of the NPC was also conducted using monoclonal anti-nuclear pore complex proteins mouse IgG (Sigma Aldrich) for control cells (were not treated with NPs or excess NLS) but were also co-stained with Hoechst to confirm correct labeling of the NPC.

2.5. Statistical analysis

Statistical analysis were performed by Graph-Pad InStat software using one way analysis of variance test (ANOVA), different levels of significance were denotes as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. The effect of NP size and NLS density on the nuclear localization efficiency of chitosan NPs

Fig. 1 shows FRET spectroscopy results obtained for S-NPs and L-NPs and their NLS modified forms. For S-NPs, NLS is unnecessary for nuclear delivery, as indicated by the highest FRET efficiency and accordingly highest nuclear localization of S-NPs compared to NLS S-NPs. This trend is conserved across all cell lines tested. For L-NPs a low to intermediate density of NLS seems to provide the highest nuclear localization efficacy in L929 fibroblasts, hFibs, HEK 293 and A549 lung cancer. Surprisingly, in glioma 261 NLS modification of L-NPs seems to decrease FRET efficiency; L-NPs' FRET efficiency are ≈1.5 folds higher than L-NLS-L-NPs and ≈8 folds higher than H-NLS-L-NPs.

In the presence of excess NLS, in L929 fibroblasts, hFibs and A549 lung cancer cells, L-NLS-L-NPs show a significant reduction in FRET efficiency and nuclear delivery, due to saturation of importin α and the classical nuclear import mechanism. The same applies to I-NLS-L-NPs