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ARTICLE TYPE

Pre-miRNA expressing plasmid delivery for anti-cancer therapy

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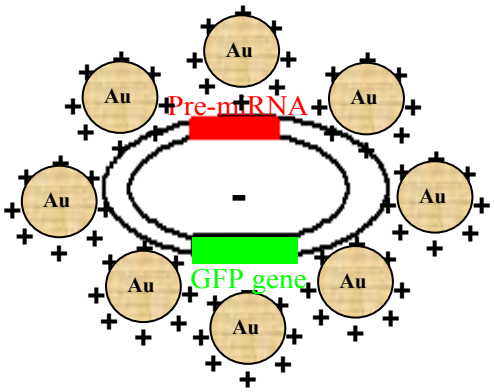
The preparation of Au NPs complex of premiRNA-145/GFP expressing plasmid is reported; the latter is successfully delivered to glioma cells and the transcripted miRNA-145 efficiently decreases the expression of its target gene, connective tissue growth factor (CTGF).

Glioma is the most frequent primary brain tumor, accounting for over 50% of all brain tumors.[1] Glioblastoma, the most malignant form, is characterized by a rapid cell proliferation and invasion of the surrounding normal brain tissue.[2] Current treatment options include surgery, radiation therapy, and chemotherapy. However, because these tumors are resistant to the different forms of chemotherapy and radiotherapy, the prognosis of patients with glioblastoma remains extremely poor, and the average survival time is 14 months.[3] Therefore, the search for novel and more effective alternative therapies against glioma is of outmost importance.

MicroRNAs (miRs) are short non-coding RNAs that regulate diverse cellular functions[4] by silencing gene expression[4,5] on hundreds of targets. In various types of cancers, the abnormal low level of specific miRs have been shown to be correlated with the initiation, progression, migration and invasion of tumor cells.[6] For instance, miR-145 has been reported to be downregulated in various types of cancers.[7] In glioma, miR-145 has been shown to target the connective tissue growth factor (CTGF) gene, and its low expression level is associated with abnormal cell growth and proliferation. We recently reported that premiR-145 transfection (using a virus vector) into glioma cells inhibited cell migration and proliferation,[8,9] suggesting possible new strategies for tumor treatment. Thus, the delivery of miR-145 to glioma cells appears an attractive and challenging therapy. However, the lack of in vivo efficient delivery means, limit their therapeutic use.

Nanoparticles are making significant contributions to the development of new approaches of drug delivery in cancer, and can provide a platform for combined therapeutics with subsequent monitoring of response[10]. In particular, Au-based nanoparticles[11] appear very attractive because of their good biocompatibility,[12], optical properties,[13] high electron density, and ease of conjugation to biomolecules.[14,15] Also, Au nanoparticle-based platforms are considered as effective delivery vehicles for transporting plasmid DNA, small interfering RNA, or anti sense oligonucleotides.[16] Despites these advantages, the direct delivery of miRs into cells remains challenging mainly due to the unstability of RNA as compared to DNA. Several studies of miR delivery are based on its protection by incorporating it into different nanoparticles.[17-20] Herein, we report a novel approach of miRs delivery by transfecting premiR-145 expressing plasmid DNA (instead of unstable mature miRNAs) conjugated with AuNPs. The premiR-145 expressing plasmid DNA was successfully delivered into and expressed by glioma cells, causing efficient increasing of miR-145 level as well as the efficient decrease of the expression of connective tissue growth factor (CTGF) in glioma cells.



Scheme 1. Amine functionalized AuNPs-premiR-145/GFP expressing plasmid DNA (containing green fluorescence protein (GFP) reporter gene) complex.

Results and discussions

The positively charged gold nanoparticles (AuNPs) were prepared as described by the reduction of $HAuCl_4$ with $NaBH_4$ in the presence of *N,N,N*-Trimethyl(11-mercaptoundecyl) ammonium bromide (TMA) in an aqueous solution (molar ratio $Au/NaBH_4$/ TMA of 56/0.1/85).[21] The dark red NPs formed were purified by dialysis and the dispersion solution was found to be stable (for months) at room temperature. HR-TEM image (Figure SI1) of the AuNPs, shows spherical nanoparticles of average size of 35 nm. Moreover, the positive charge of the AuNPs is confirmed by a zeta potential analysis (+ 50 mV; Figure SI2); confirming thus the linkage of the ammonium chains *via* Au-S bond.[21] The UV/vis spectrum of the dispersion of AuNPs in water, displays a Surface Plasmon Resonance (SPR) band at 538 nm (Figure SI3) typical of that expected for 35-40 nm size AuNPs.[22]

The AuNPs were complexed with DNA plasmid encoding for premiR-145 and GFP (designated as premiR-145/GFP; Scheme 1) in 10:1 and 20:1 w/w ratios; during which time the suspension colour changed from brownish-red to light purple. Evidences for electrostatic interaction between the positively charged AuNPs and the negatively charged plasmid are provided by the decrease of the zeta potential from +50 mV to +47 mV(20:1) and +36 mV(10:1) upon increasing amount of the plasmid relative to the NPs. Additionally, the UV/vis of the AuNPs-premiR-145/GFP formed show a significant red shift and broadening of the SPR band (Figure SI4) from 538 to 562 nm (10:1) and 568 nm (20:1); as typically observed for other examples of DNA complexation on gold NPs,[23,24] and attributed to aggregate growth.

For further evidence of complexation we used the EtBr (ethidium bromide) which is a good intercalator to double stranded DNA and is red fluorescent when bound to DNA. As shown in Figure 1, the absence of red fluorescent signal in case of AuNPs + EtBr confirms that EtBr does not bind AuNPs. In the presence of DNA, EtBr is clearly intercalated and exhibits red fluorescence, as shown in images. When EtBr is added to Au-premiR145/GFP NPs (10:1 and 20:1), the merge images show that the red fluorescence is only located on the black spots that are visualized on the DIC pictures corresponding to the AuNPs-premiR145/GFP, clearly confirming the complexation between the plasmid and the Au.

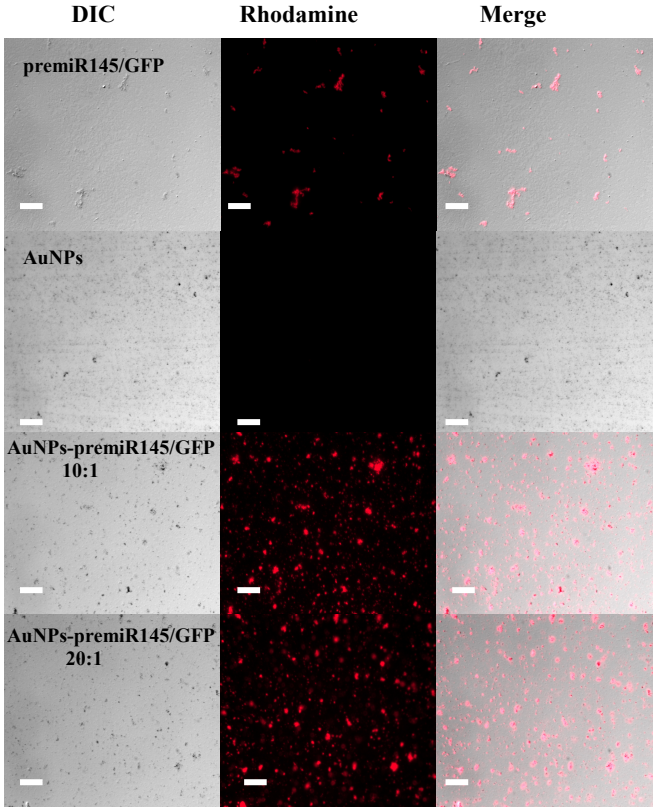


Figure 1. Fluorescent microscope images of premiR-145/GFP + EtBr, AuNPs +EtBr, AuNPs- premiR-145/GFP (10:1) + EtBr, AuNPs-premiR-145/GFP (20:1) + EtBr. Left: bright-field images (DIC); middle: red fluorescence (Rhodamine mode: λ_{exc} 550 nm, λ_{em} 573 nm), right: merge. Scale bar 10 µm.

Since the premiR-145 expressing plasmid contains the green fluorescence protein (GFP) reporter gene, the transfection of the plasmid into cells can be visualized by fluorescence microscopy. A172 cells incubated with the AuNPs-premiR145/GFP complex were examined by the visualization of the GFP expression by fluorescence microscopy after 72 h of transfection (Figure 2).

As shown in Figure 2, no green fluorescence was observed either in the untreated A172 cells, nor in presence of the plasmid; clearly indicating that plasmid transfection does not occur spontaneously. However, when the cells were incubated with AuNPs-premiR-145/GFP (10:1 and 20:1), green fluorescence (of GFP) is clearly observed inside the cells (Figure 2B and C), demonstrating high uptake of the Au-plasmid assembly as well as expression of the GFP gene by the cells. Based on the fluorescent data about 40-50% of the cells were GFP positive.

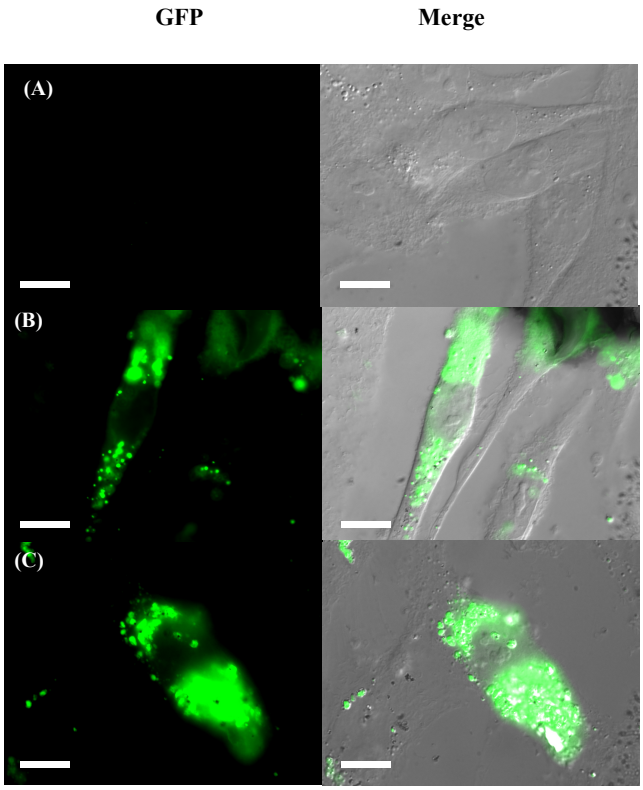


Figure 2. Fluorescent microscope images of untreated A172 cells (A) and A172 cells treated with Au-premiR-145/GFP 10:1 (B) and 20:1 (C). Green fluorescence mode (λ_{exc} 489 nm, λ_{em} 509 nm filter =green. The cells were incubated with AuNP-premiR145/GFP complexes for 72 h; after which time the cells were washed with PBS, fixed with 4% paraformaldehyde and analyzed by fluorescent microscopy. Scale bar 10 µm.

To further demonstrate the ability of the AuNPs to deliver premiR-145 expressing plasmid into the cells, we measured the levels of mature miR-145 in the transfected cells using real-time PCR analysis (Figure 3). We also examined the effect of the AuNPs-premiR-145/GFP complex delivery on the expression of the miR-145 target gene, connective tissue growth factor (CTGF).

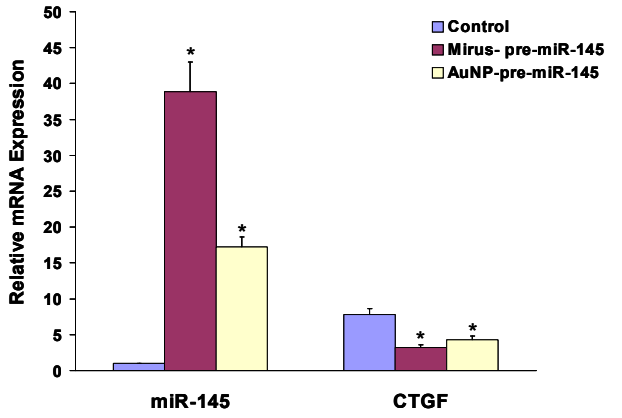


Figure 3. Real time polymerase chain reaction measurements of miR-145 and CTGF mRNA expression. The measurements were performed using A172 cells untreated (blue) and treated with (purple) premiR-145 in the presence of Mirus transfection reagent; or with (yellow) AuNPs- premiR-145/GFP complex (AuNPs-premiR145/GFP ratio was 10:1). *P‹ 0.001. The measurements were performed 72h post transfection.

As demonstrated in Figure 3, the real-time PCR analysis shows increased expression of miR-145 in the AuNPs-premiR-145/GFP treated cells compared to control untreated cells. The highest level of miR-145 was found in cells treated with AuNPs/premiR-145 in a 10:1 w/w ratio. We recently identified CTGF as a novel target gene of miR145 that mediates the effect of this miRNA on glioma cell migration. As presented in Figure 3, the AuNPs-premiR-145/GFP decreased the expression of CTGF by about 45% compared to its expression in the control cells. Therefore, our results indicate that the AuNPs were able to efficiently deliver premiR-145 into the glioma cells and to target this important target gene in these cells. Although transfection with the Mirus reagent appeared to be more efficient in increasing the levels of mature miR-145 in the cells, the difference between the two approaches is only about 2 fold, in contrast to the large increase in miR-145 induced by the AuNPs compared to the control cells. This difference can be due to different kinetics or different levels of AuNPs that may be used. Importantly, the degree of CTGF expression was similar for the Mirus and AuNPs transfection suggesting a similar functional effects.

Conclusions

In the current work we demonstrate the successful delivery and expression of premiR-145 expressing plasmid conjugated with AuNPs in A172 glioma cells, by inducing a significant increase in miR-145 level as well as the efficient decrease in the expression of the miR-145 target gene, connective tissue growth factor (CTGF) in glioma cells. We propose that AuNPs can serve as an efficient route to introduce functional mature miRNA into glioma cells by complexing with a DNA plasmid of pre-miRNA.

Notes and references

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† Electronic Supplementary Information (ESI) available:

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Graphical Abstract

The premiR145/GFP expressing plasmid DNA was delivered into glioma cells and the transcripted miRNA145 efficiently decreases the expression of CTGF