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An optofluidic approach for Gold nanoprobe based-cancer theranostics

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ABSTRACT

Suppression of overexpressed gene mutations in cancer cells through RNA interference (RNAi) technique is a therapeutically effective modality for oncogene silencing. In general, transfection agent is needed for siRNA delivery. Also, it is a tedious and time consuming process to analyze the gene transfection using current conventional flow cytometry systems and commercially available transfection kits. Therefore, there are two urgent challenges that we need to address for understanding and real time monitoring the delivery of siRNA to cancer cells more effectively. One, non-toxic, biocompatible and stable non-viral transfection agents need to be developed and investigated for gene delivery in cancer cells. Two, new, portable optofluidic methods need to be engineered for determining the transfection efficiency of the nanoformulation in real time. First, we demonstrate the feasibility of using gold nanorods (AuNRs) as nanoprobe for the delivery of Interleukin-8 (IL-8) siRNA in a pancreatic cancer cell line- MiaPaCa-2. An optimum ratio of 10:1 for the AuNRs-siRNA nanoformulation required for efficient loading has been experimentally determined. Promising transfection rates ($\approx 88\%$) of the nanoprobe-assisted gene delivery are quantified by flow cytometry and fluorescence imaging, which are higher than the commercial control, Oligofectamine. The excellent gene knockdown performance (over 81%) of the proposed model support *in vivo* trials for RNAi-based cancer theranostics. In addition to cancer theranostics, our nanoprobe combination can be also applied for disease outbreak monitoring like MERS. Second, we present an optical fiber-integrated microfluidic chip that utilizes simple hydrodynamic and optical setups for miniaturized on-chip flow cytometry. The chip provides a powerful and convenient tool to quantitatively determine the siRNA transfection into cancer cells without using bulky flow cytometer. These studies outline the role of AuNRs as potential non-viral gene delivery vehicles, and their suitability for microfluidics-based lab-on-chip flow cytometry applications.

Keywords: gold nanorods, flow cytometry, inertial microfluidics, gene knockdown, RNA interference, microfluidic chip, cancer theranostics, pancreatic cancer

1. INTRODUCTION

Poor prognosis and high mortality rates in pancreatic cancer cases are a consequence of intrinsic as well as extrinsic drug resistance.¹ Besides the conventional radiative and chemotherapeutic approaches, adjuvant therapy,² targeted therapy³⁻⁵ and stromal therapy⁶ are few of the recent modalities proposed for pancreatic cancer therapy. RNAi-based gene therapy is one of the potential therapeutic techniques which catalyzes suppression of overexpressed gene mutations in cancer cells.⁷ RNAi involves administration of small double-stranded RNAs (siRNAs) to cells, which can induce targeted intracellular degradation upon complementary messenger RNA (mRNA) encounter. RNAi-based therapies have shown promising potential for their therapeutic effect in cancers such as breast, liver, and lung cancer.⁸⁻¹² Particularly, pancreatic cancer cells such as MiaPaCa-2 exhibit high IL-8 mRNA expression which require targeted siRNA signaling within the tumor microenvironment to promote cancer cell apoptosis.¹³

In general, transfection agent is needed for siRNA delivery because naked siRNAs are unstable and too small to permeate the cell membrane. Also, they are negatively charged and possess very short half-lives to survive nuclease mediated degradation. Therefore, non-toxic, biocompatible and stable non-viral transfection agents need to be developed and investigated for gene delivery in cancer cells. Perhaps transfection agents could be both viral and non-viral, however, non-viral agents are a preferred choice due to their simple synthesis techniques, biodegradability, low toxicity and low immune response. In this paper, AuNRs are probed as non-viral transfection agents for the delivery of IL-8

siRNA to MiPaCa-2 pancreatic cancer cell line, and their cytotoxicity, loading capacity and transfection efficiency is characterized. Figure 1 is an illustrative schematic of the proposed nanostrategy to transfect MiPaCa-2 cancer cells with AuNRs–siRNA nanoprobe to bring about IL-8 gene knockdown by RNAi. The performance of AuNRs as effective non-viral gene delivery vehicles is compared with a standard control, Oligofectamine. Measurement of transfection efficiency is done through fluorescence imaging and conventional flow cytometry technique. Nonetheless, it is tedious to analyze the gene transfection using current conventional flow cytometry systems. Therefore, new, portable optofluidic methods need to be engineered for determining the transfection efficiency of the nanoformulation in real time. In the following part, we propose a low-cost, optical fiber-integrated microfluidic chip for miniaturized on-chip flow cytometry which can provide a fair estimate of transfection efficiency. This technology aims for affordable diagnostic and therapeutic healthcare in low-end clinical settings.

Figure 1. Illustration of the RNAi phenomenon for IL-8 mRNA suppression in MiaPaCa-2 pancreatic cancer cells. AuNRs act as nanocarriers for transfecting IL-8 siRNA inside the cell. Upon successful delivery into the cytoplasm, siRNA forms an assembly with RISC and gets dissociated with its antisense strand. Thus, degradation of IL-8 mRNA results when it encounters the sense strand of siRNA.

2. MATERIALS AND METHODS

2.1 Synthesis of AuNRs and characterization of nanoprobe

Seed-mediated growth method was used to synthesize AuNRs in a cetyl trimethylammonium bromide (CTAB) surfactant solution as described before.¹⁴ The cytotoxicity of CTAB-coated AuNRs was reduced by stepwise binding of poly(sodium styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) polymers. Thus, positively charged AuNRs/PSS/PAH (read as AuNR in rest of the text) were synthesized. For transfection, various concentrations of IL-8 siRNA were mixed with AuNRs, and allowed to rest for 30 min. To confirm the formation of stable AuNRs–siRNA nanoprobe, particle size and zeta potential measurement was done using 90-Plus particle size analyzer (Brookhaven Instrument Corp.) at room temperature. Particle size measurements were also performed at 4 °C and 37 °C to study the stability of the AuNRs and AuNRs-siRNA nanoprobe. Also, agarose gel electrophoresis was performed to confirm the effective binding of the siRNA with the AuNRs on a UV transilluminator (Bio-Rad).

2.2 MiaPaCa-2 Cell culture

Human pancreatic cancer cell line, MiaPaCa-2 (CRL-1420) (American Type Culture Collection), was cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone), 10% fetal bovine serum (FBS, Hyclone), penicillin (Gibco, 100 $\mu\text{g mL}^{-1}$) and streptomycin (Gibco, 100 $\mu\text{g mL}^{-1}$) in a UV incubator at 37 °C in a humidified atmosphere with 5% CO_2 .

2.3 Cytotoxicity measurement of AuNRs–siRNA nanoprobe

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay kit was used to determine the cytotoxicity effect of AuNRs–siRNA nanoprobe on the live cells. As per protocol described previously,¹⁵ MiaPaCa-2 cells were seeded into 96-well plates and allowed to adhere for 24 h. Post this, the medium of the 96-well plate was changed and incubated for 20 min. The cells were then treated with different formulations of AuNRs–siRNA nanoprobe for 24 and 48 h. MTT (5 mg mL^{-1} , 20 μL in PBS) was added to the cells and left for incubation for 4 h at 37 °C with 5% CO_2 . Dimethylsulfoxide (DMSO, 150 μL , Sigma) was then added to dissolve the precipitate with 5 min gentle shaking. Absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad). The absorbance of the sample well relative to the control well (non-treated cells - 100%) yielded the cell viability.

2.4 Transfection of cancer cells with IL-8 siRNA

The MiaPaCa-2 cells (1×10^4) were seeded into 6-well plates in DMEM medium to attain 30–50% confluence for performing transfection. Prior to transfection, the medium of the cultured cells was changed to OPTI-MEM (Invitrogen), and incubated for about 20 min. FAM-tagged IL-8 siRNA (siRNA^{FAM} - 20 μL , 10 μM - Sense: 5'-AMGUUGGAGCUUGGCGUAGUU-3'; Antisense: 5'-CUACGCCACAAGCUCCAACUU-3') or negative control siRNA (NC-siRNA^{FAM} -20 μL , 10 μM - Sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense: 5'-TTAAGAGGCUUGCACAGUGCA-3') were gently mixed with 1 mg mL^{-1} AuNRs each and incubated for 30 min. Different concentrations of the abovementioned AuNRs–siRNA^{FAM} mixture were added to the cells in the 6-well plates and cultured continuously for four hours. Thus, the various test ratios of nanoprobe obtained for transfection were (i) only AuNRs, (i) only siRNA^{FAM}, (ii) 0.625:1 AuNRs–siRNA^{FAM}, (iii) 1.25:1 AuNRs–siRNA^{FAM}, (iv) 2.5:1 AuNRs–siRNA^{FAM}, (v) 5:1 AuNRs–siRNA^{FAM}, (vi) 10:1 AuNRs–siRNA^{FAM} and (vii) 20:1 AuNRs–siRNA^{FAM} nanoprobe. Commercial transfection reagent Oligofectamine™ (Invitrogen) was mixed with siRNA^{FAM} (Oligo–siRNA^{FAM}) and used as positive control. Four hours post transfection, cells were separated for fluorescence imaging and flow cytometry for the determination of transfection efficiency.

2.5 Fluorescence imaging

To determine the qualitative estimation of AuNRs assisted-siRNA transfection into the cancer cells, *in vitro* fluorescence microscopy was performed using a fluorescence microscope (Eclipse-Ti, Nikon). The incubated cells were washed with PBS twice and fixed with 4% formaldehyde. They were then stained with DAPI (Sigma) for nucleus fluorescence imaging. Thereafter, two filter sets-one for imaging the cell nucleus (DAPI 359/461 nm) and second for imaging the siRNA (FITC 492/518) were applied for fluorescence imaging.

2.6 Flow cytometry

To determine the quantitative estimation of AuNRs assisted-siRNA transfection into the cancer cells, a conventional table-top flow cytometer (FACSCalibur, Becton Dickinson, Mississauga, CA) was used. The transfected MiaPaCa-2 cells were washed with PBS twice and treated with trypsin. A filter set for measuring siRNA fluorescence (FITC 492/518) was applied for determining the transfection efficiency. Transfected cells were classified based on the FITC-fluorescence signal, and a statistical evaluation was done.

2.7 Gene Expression Analysis

To investigate the effect of AuNRs nanovehicles in transfecting the MiaPaCa-2 cancer cells with IL-8 siRNA and bringing about gene knockdown of IL-8 mRNA levels, semi-quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted.¹⁶ Briefly, the total amount of RNA extracted from MiaPaCa-2 cells 48 hours after transfection using TRIzol reagent (Invitrogen) was quantitated by a spectrophotometer (Nano-Drop ND-1000). Then, cDNA was synthesized from 2 μg of total RNA using the reverse transcriptase kit (Promega). The relative IL-8 mRNA expression level was determined by normalizing it to the expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as control. The primer sequences for different genes were:¹⁷ IL-8, forwards 5'-CTTCTAGGACAAGAGCCAGGAAGAAACCAC-3' and reverse 5'-

GTCCAGACAGAGCTGTCTTCCATCAGAAAG-3'; GAPDH, forwards 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCT-GTTGCTGTA-3'.

2.8 Design of microfluidic chip for on-chip flow cytometry

A miniaturized and portable flow cytometry device consisting of an optical fiber-integrated microfluidic chip is proposed. The device utilizes a simple hydrodynamic setup to align randomly fed cells/particles at the input reservoir of the chip into a focused stream of single cells/particles at the outlet part of the chip. The microfluidic chip can focus cells/particles using self-generated inertial forces¹⁸⁻²⁰ based on the sample flow's hydrodynamics in the designed curved microchannel without the need of additional sheath flow. Thereby, the focused stream of single cells is illuminated by a laser diode and their photo-signals such as fluorescence, forward-scattering and side-scattering are detected by photomultiplier tubes (PMTs). In our device, the illumination and photo-detection are achieved by the integration of tapered optical fibers into the microfluidic system. The output voltage signals from the PMTs are processed, and cell/particle count of the desired sample is performed. The chip is designed on SU-8 photoresist coated-Silicon wafers using the well-known soft lithography technique, and is thereafter fabricated by PDMS casting. The as-obtained PDMS moulds are then irreversibly sealed on glass substrates using plasma oxygen treatment.

3. RESULTS AND DISCUSSION

Firstly, the synthesized AuNRs and AuNRs-siRNA nanoprobe (see section 2.1) were subjected to hydrodynamic size and zeta potential measurement for investigating the effect of different ratio of siRNA bound on the surface of AuNRs. Prior to this, the synthesized AuNRs were characterized on TEM, yielding an average width and length of 22.34 ± 1.57 nm and 47.26 ± 2.35 nm, respectively (Figure 2b). It was interestingly observed that the size of the nanoprobe grew as the ratio of AuNRs-siRNA nanoprobe increased, but this trend continued only until 10:1 AuNRs-siRNA ratio after which the size dramatically reduced for the 20:1 AuNRs-siRNA ratio (Figure 2c). This confirms the successful binding of siRNA on AuNRs and gives a limit on the AuNRs-siRNA ratio beyond which the siRNA binding reaches a saturation level for the given quantity of siRNA. Also, naked-AuNRs have a zeta potential of 35 ± 5 mV, and the zeta potential values for AuNRs-siRNA nanoprobe reduce and even become negative (-25 ± 2 mV for 0.625:1 AuNRs-siRNA ratio) as the siRNA concentration is increased (Figure 2c). Thus, positive zeta potential of naked AuNRs support the binding of negatively charged siRNA to form stable AuNRs-siRNA nanoprobe. The gel electrophoresis images of the naked AuNRs, free siRNA and the different ratio AuNRs-siRNA nanoprobe also confirm the successful binding of siRNA on the AuNR surface. High-contrast images for AuNRs-siRNA nanoprobe are clearly visible for the 5:1, 10:1 and 20:1 nanoprobe while high-contrast images for free siRNA are dominant for the 2.5:1, 1.25:1 and 0.625:1 nanoprobe (Figure 2a). These observations highlight the fact that a suitable AuNR-siRNA ratio must be chosen for maximum siRNA transfection into cancer cells. Based on these set of results, we determined the optimum ratio of AuNRs-siRNA nanoprobe for achieving high transfection efficiency into cells. First and foremost, the zeta potential of the AuNRs-siRNA nanoprobe must be positive in order to invade the negatively charged cell membrane. Hence, only the 5:1, 10:1 and 20:1 nanoprobe out of all the ratios could be chosen. Second, the gel electrophoresis images possess similar contrast for the 5:1, 10:1 and 20:1 nanoprobe. Third, the hydrodynamic size measurements reveal weak binding for the 20:1 nanoprobe. Thus, to achieve highest transfection while maintaining the stability of the synthesized nanoprobe, the 10:1 ratio was chosen and demonstrated as the optimum ratio of AuNRs-siRNA nanoprobe in the next set of experiments.

The size of the synthesized AuNRs and AuNRs-siRNA nanoprobe showed negligible change as they were subjected to temperatures lower (4°C) and or higher (37°C) than the room temperature (25°C), indicating that AuNRs are stable with respect to temperature variations (Figure 3a). To investigate the toxicity of the non-viral AuNRs to be used as gene delivery nanovehicles, an MTT assay was performed on MiaPaCa-2 cells treated with different concentrations of AuNRs. An average of over 90% cells were found viable subjected to a high AuNR dosage range (1.28 o.d.mL^{-1}) even 48 hours post treatment (Figure 3b), suggesting that AuNRs are highly biocompatible and non-toxic. Thereafter, the suitability of these non-viral AuNRs as nanovehicles for siRNA delivery in MiaPaCa-2 cells *in vitro* was determined.

Four hours post-transfection, the qualitative and quantitative estimation of transfection was obtained using fluorescence microscopy and flow cytometry, respectively. While the color-coded images of the AuNRs-siRNA transfected-cells showed the presence of FAM labeled-siRNA with different intensities in accordance with the ratio of the AuNRs-siRNA nanoprobe, no FAM signal was however observed in cells transfected with siRNA only (Figure 4). Moreover, the 10:1 and 20:1 nanoprobe showed similar transfection which was also comparable with the commercial transfection agent,

Oligofectamine. These results clearly outline the need of a nanocarrier for unstable, negatively charged siRNA delivery into the cancer cells, and the ability of AuNRs as suitable nanovehicles for siRNA transfection.

Figure 2. a) Agarose gel electrophoresis of synthesized AuNRs-siRNA nanoplexes. b) TEM images of the synthesized AuNRs. c) The variation in the hydrodynamic size and zeta potential with different ratios of AuNRs-siRNA nanoplexes. The o.d. dose of siRNA molecules in the nanoplexes was 0.008 o.d.

Figure 5a-j shows the fluorescence intensity flow cytometric plots of MiaPaCa-2 cells transfected with free siRNA, only-AuNRs, different ratios of AuNRs-siRNA nanoplexes. To calculate the transfection efficiency, the entire intensity plot was divided into two populations: P2-expressing low fluorescent signals representing autofluorescence, and P3-expressing strong fluorescence from the FAM label representing the successfully transfected cells. The transfection efficiency was determined by $P3/(P2+P3)$.

Figure 3. a) Stability of AuNRs and AuNRs-siRNA nanoprobe with temperature in terms of effective hydrodynamic size. b) MTT assay results showing the cell viability of MiaPaCa-2 cells treated with AuNRs at different concentrations for 24 and 48 hours. Results are represented as mean $\pm$ SD, n = 6.

Figure 4. Fluorescence microscopy images of MiaPaCa-2 cells treated with different formulations. Cell nuclei are stained with DAPI (blue) and the siRNA is stained with FAM (green). The results are compared with Oligofectamine (Oligo-siRNA^{FAM}).

The results show negligible transfection in cases of free siRNA (0.22 ± 0.10 %), naked-AuNRs (0.16 ± 0.05 %), and blank cell groups (0.19 ± 0.08 %) whereas high and similar transfection performance with 10:1 (88.40 ± 2.14 %) and 20:1 (87.10 ± 2.23 %) AuNRs-siRNA nanoprobe (Figure 5k). Also, the average fluorescent intensity per cell count increases with increasing ratio of AuNRs-siRNA nanoprobe (Figure 5l). Interestingly, it is quite similar among the 10:1, 20:1 AuNRs-siRNA nanoprobe and the Oligo-siRNA control group, suggesting that the loading capacity of AuNRs gets saturated around the 10:1 ratio for a given quantity of siRNA. Hence, the choice of the 10:1 AuNRs-siRNA nanoprobe for achieving maximum transfection efficiency in MiaPaCa-2 cells is thus justified.

Figure 5. Flow cytometry results showing the transfection efficiency of AuNRs-assisted siRNA into MiaPaCa-2 cells. (a)-(j) show distribution of cells treated with PBS, AuNRs, siRNA^{FAM}, Oligo-siRNA^{FAM}, and AuNRs-siRNA^{FAM} (0.625:1, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1). The o.d. dose of siRNA was fixed at 0.032 o.d. per well. (k)-(l) are the quantitative evaluation of the results from (a)-(j) showing the transfection efficiency and average fluorescent intensity of each group respectively. Average fluorescence intensity presents the FAM intensity in the cells. The transfection efficiency is determined by P3/(P2+P3) as described in text. The relative values shown are mean $\pm$ SD, n = 5; *, P<0.01 compared to Oligo-siRNA group.

Figure 6. The relative IL-8 mRNA expression in MiaPaCa-2 cells for the various treatment groups detected by quantitative real time RT-PCR. The relative values shown are mean $\pm$ SD, n = 4; *, P<0.01 compared to Blank group.

The real-time RT-PCR results (Figure 6) show significant decrease in IL-8 mRNA level in MiaPaCa-2 cells post transfection with AuNRs-siRNA nanoprobe and Oligo-siRNA while negligible reduction in gene suppression in blank

cell groups, free siRNA, only-AuNRs. AuNRs as nanocarriers of the IL-8 siRNA demonstrate superior performance (81.02 ± 10.14 %) than the control, Oligofectamine (76.04 ± 6.03 %). These results show that AuNRs can be used as an effective non-viral vector for siRNA delivery to cancer cells and bring about gene knockdown of the target mRNA through RNAi mechanism.

It is convincingly clear from the results that a flow cytometer plays an inevitable role in measuring disease diagnosis and therapeutics quantitatively. However, its high-cost, bulkiness and complex instrumentation restrict its use to high-end clinical settings and research laboratories. In this paper, we also present an optical fiber-integrated microfluidic chip that utilizes simple hydrodynamic and optical setups for miniaturized on-chip flow cytometry (Figure 7). With its novel and sheathless hydrodynamic design, the microfluidic channel is able to align cells/particles (10-20 μm) to a single line of focused cells. Post transfection with AuNRs-siRNA nanoprobe, the 14-16 μm MiaPaCa-2 cells can be readily focused in our engineered portable flow cytometry set-up, and the transfection efficiency can be determined by measuring the output fluorescent signal. The lab-on-chip flow cytometry device offers distinct advantages when compared with the conventional bulky flow cytometer: lower volume of samples is needed, fewer sample preparation steps, cartridge-type one-off use chip, easier handling and operation, and low cost. The chip provides a powerful and convenient tool to quantitatively determine the siRNA transfection into cancer cells without using bulky flow cytometer.

Figure 7. Illustration of a lab-on-chip flow cytometry device for theranostics applications. a) Schematic of the sheathless microfluidic chip for focusing cells/particles as they traverse the length of the channel, with the associated fluidic (inlet and outlet) and optical instrumentation (laser diode, optical fibers, PMTs and DSO). AuNR-siRNA transfected-cells can be fed into the inlet, and the transfection efficiency can be determined by detecting the siRNA fluorescence (FAM) and the total cell count (DAPI) from the output signals of the respective PMTs. b) Microscopic image of the optical fibers integrated with the PDMS microfluidic chip near the outlet region. The detection of fluorescent signals through the flowing cells is a measure of siRNA delivery with respect to the total number of cells. (Size of cells/particles not to scale)

4. CONCLUSION

In this paper, we proposed and investigated the feasibility of using AuNRs as nanovehicles for siRNA transfection into pancreatic cancer cells. The synthesized AuNRs-siRNA nanoprobe show excellent transfection efficiency ($\approx 88\%$), estimated by flow cytometry and fluorescence, comparable to the standard control, Oligofectamine. AuNRs are biocompatible and have low cytotoxic effect on the cells, and hence are potential non-viral candidates for RNAi-based therapeutic interventions to be used in clinical settings in future. Also, successful siRNA transfection induces significant IL-8 gene knockdown (over 81%) in MiaPaCa-2 cells. Furthermore, we present a simple, low-cost and sheathless method of on-chip flow cytometry based on inertial focusing of cells/particles. Without using sheath flows or other external instruments for sample focusing, the system is greatly simplified. However, the figure-of-merit (FoM) of the proposed miniaturized lab-on-chip device in its current form is quite lower than the conventional table-top flow cytometer. This can be primarily attributed to the limitation on the inlet flow rate (which is currently 600 $\mu\text{l}/\text{min}$) for sustaining a focused flow within the PDMS fabricated-microfluidic channel. A possible solution for a higher FoM could be to explore other transparent polymeric materials that can withstand high-pressure microfluidic flows. Also, the present version is limited to two-parameter sensing as compared with multi-parameter capability of the conventional counterpart. Alternative labeling strategies, for example, using quantum dots, could be explored for this. The proposed miniaturized and portable flow cytometry system is targeted for a larger footprint in low-end semi-urban settings for increased diagnostic and therapeutic interventions.

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