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Combination of Nanogel Polyethylene glycol-Polyethylenimine and 6 (hydroxymethyl)-1,4-anthracenedione as an Anticancer Nanomedicine

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Abstract

Nanoparticles have many potential applications. Polyethylene glycol-polyethylenimine (PEG-PEI) nanogels have been used to deliver nucleic acids and oligonucleotides into cells. We first synthesized various nanogels with altering ratios of CH₂O:CH₂N (methylene protons) in PEG-PEI and tested their cytotoxicity using a rodent pancreatic adenocarcinoma cell line (Pan 02). Results indicate that the nanogel PEG-PEI with lower methylene protons ratio (4:1) exhibited a strong cytotoxicity over the higher methylene protons ratio (~6.8:1). We then incorporated a newly synthesized anti-cancer drug, 6-(hydroxymethyl)-1,4-anthracenedione (AQ) analogue (AQ10) into nanogel PEG-PEI with higher methylene protons ratio (~6.8:1) and tested the effect of AQ10 encapsulated nanogel PEG-PEI (AQ10-nanogel PEG-PEI) and AQ10 dissolved in DMSO on Pan 02 cell growth. Our results showed that, Pan 02 cells treated with AQ10-nanogel PEG-PEI was three to four times more effective in cell growth attenuation than the cells treated with AQ10 alone. Nanogel PEG-PEI apparently acts as a drug delivery agent by penetrating the cell membrane and carrying the anti-cancer drug AQ10 into the cells, so that they become more susceptible to the drug even at a lower dose. These results suggest that AQ10-nanogel PEG-PEI is a potentially effective anti-cancer agent in pancreatic cancer therapy.

Keywords

Nanogel PEG-PEI; AQ10; Pan 02 cells

1. INTRODUCTION

Nanotechnology is a rapidly emerging drug-delivery system that makes possible the controlled release of many small molecules.^{1–4} PEI, a polyamine polymer in combination with PEG has been used as a nonviral gene delivery agent.^{3, 5–7} The polyamine polymer chains can be cross-linked to form a nanogel, which increases the stability of the complex.^{2, 4, 5, 8, 9} PEI alone is very toxic to cells;^{5, 8, 10} in order to reduce the cytotoxicity, PEI is coupled with PEG which makes the compound more water soluble and less toxic.^{4, 5, 9, 11, 12} Here we first synthesized different nanogels with altered ratio's of methylene protons in PEG-PEI and studied the cytotoxicity on the mouse pancreatic adenocarcinoma (Pan 02) cell line.

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The AQ analogue, AQ10 (first synthesized and characterized by Hua et. al. 2006) was shown to have an anti-cancer effect by initially triggering early and late markers of apoptosis and later causing internucleosomal DNA fragmentation.^{13–15} We tested several nanogels with methylene protons ratio in PEG-PEI ranging from ~6.8:1 for CH₂O:CH₂N (methylene protons) to 4:1 and less. We incorporated AQ10 into the less toxic nanogel PEG-PEI with CH₂O:CH₂N (~6.8:1), and tested the effect on Pan 02 cell growth using a cell proliferation assay. Our results showed that AQ10-encapsulated nanogel PEG-PEI is significantly more effective in altering the growth of Pan 02 cells compared to AQ10 or nanogel PEG-PEI alone.

2. MATERIALS AND METHODS

2.1 Materials

PEI (~25 kDa), PEG (8 kDa), 1,1'-carbonyldiimidazole, *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, and 1-hydroxybenzotriazole (HOBT) were purchased from Aldrich Chemical Co. AQ10 was prepared as described.^{14, 15} Nanogel PEG-PEI was prepared by following a reported method¹⁶ with modification, and spectral characterization of the produced nanogel PEG-PEI was studied (*vide infra*). 6-Carboxytetramethylrhodamine (TAMRA) was purchased from Invitrogen and was activated with DCC, HOBT, and *N*-hydroxysuccinimide to product TAMRA-SE. Sephacryl S200 and membranes were purchased from Fisher Scientific. ¹H NMR spectra were recorded on 200 and 400 MHz Varian UNITYplus instruments. Atomic force microscopy (AFM) images were obtained from a Nanoscope IIIa SPM atomic force microscope (Digital Instruments, Inc. Santa Barbara, CA). A HP 8543 UV-Vis Spectrophotometer was used for obtaining UV-vis spectra. The murine Pan 02 cell line was purchased from DCTD Tumor Repository (NCI).

2.2. AFM experiments

AFM images were collected using a tapping mode with a high aspect ratio tip (Veeco Nanoprobe TM tips, Model TESP-HAR). A solution of nanogel PEG-PEI -AQ10 was prepared as followed for AFM studies. A solution of 2.7 mg of nanogel PEG-PEI-AQ10 was dissolved in 500 µL of deionized water and diluted with 240 µL of DMSO. A small aliquot (20 µL) of each sample was removed and placed onto freshly cleaved mica, washed with deionized water twice, and dried with N₂. AFM images on different locations of the mica were then obtained from a Nanoscope IIIa SPM instrument.

2.3. Separation of PEI

A Sephacryl S200 (30 g) chromatographic column was prepared with deionized water. To it 7.0 g (0.28 mmol) of PEI (MW ~25 kDa; contaminated with lower and higher MW materials) in 20 mL of deionized water was loaded. Deionized water was used as eluant. The middle fractions (based on weight distribution) were collected and lyophilized to give 3.64 g (0.146 mmol) of PEI (MW ~25 kDa). ¹H NMR (D₂O) δ 2.72 (bs, CH₂N), 2.68 (bs, CH₂N); the above two signals are overlapped and the number of hydrogens cannot be determined from integration.

2.4. Activation of PEG

To a solution of 2.0 g (0.25 mmol) of PEG (MW 8 kDa) in 7 mL of dry acetonitrile under argon was added 0.41 g (2.5 mmol) of 1,1'-carbonyldiimidazole, and the solution was stirred at 40° C for 2 hours. The crude product was purified by dialysis using a MWCO 2kDa membrane twice with 800 mL of 10% ethanol in deionized water at 4°C for 4 hours. The solution was lyophilized to give 1.84 g of activated PEG. ¹H NMR (CDCl₃) δ 7.69 (s, 1 H, ArH), 7.11 (s, 2 H, ArH), 3.62 (s, 190 H, CH₂O).

2.5. Preparation of nanogel PEG-PEI

Nanogel PEG-PEI was prepared by following a similar micellar method [16]; the molecular weight of PEG is ~8 kDa and that of PEI is ~25 kDa. Synthesis started from activated PEG (activation with 1,1'-carbonyldiimidazole; ~63 μmol were used) and PEI (~40 μmol were used) (Scheme 1). To a solution of 1.0 g (40 μmol) of PEI (MW ~25 kDa) in 300 mL of deionized water was added dropwise a solution of 0.50 g (62.5 μmol) of activated PEG (MW ~8 kDa) in 2 mL of dichloromethane. The reaction solution was sonicated in a water bath for 10 minutes, and the organic solvent was removed on a rotary evaporator resulting in a transparent solution. The solution was dialyzed with a 12K – 14K MWCO membrane in 800 mL of 10% ethanol in deionized water for 1 day at 25°C and lyophilized to give nanogel PEG-PEI. This nanogel PEG-PEI was again treated with 1.0g (125 μmol) of activated PEG in 2 ml of dichloromethane and worked up as mentioned above to give 1.32 g of nanogel PEG-PEI. ^1H NMR (D_2O) δ 3.70 (s, area 44, CH_2O), 3.40 – 2.60 (m, area 6.5, CH_2N). Based on the weight of the product, we estimate that the molecular weight of the nanogel PEG-PEI is ~33 KDa (for each mole of PEI, one mole of PEG is added). The initial treatment of PEI with activated PEG provided a partial cross-linkage of PEG, in which for each mole of PEI, there is ~ 0.5 mole of PEG attached; this partial linking of PEG-PEI is toxic to cancer cells.

2.6. Synthesis of nanogel PEG-PEI-rhodamine

A mixture of 15 mg (32 μmol) of 6-Carboxytetramethylrhodamine (TAMRA), 9.9 mg (48 μmol) of DCC, 6.52 mg (48 μmol) of HOBt, and 4.44 mg (39 μmol) of *N*-hydroxysuccinimide was dried under vacuum and maintained under argon. To it, 1 mL of dry DMF was added via syringe. The resulting solution was stirred at 50–55°C for 2.5 hours, cooled to room temperature, and added a solution of 200 mg of nanogel PEG-PEI in 1mL of acetonitrile. The solution was stirred at 40°C for 12 hours, cooled to 25°C, dialyzed with a 12k - 14k MWCO membrane in 10% ethanol in deionized water at room temperature for 1 day, and lyophilized to give 186 mg of PEG-PEI-rhodamine. ^1H NMR (D_2O) δ 8.50 (s, area 0.03), 8.10 (m, area 0.015), 7.90 (m, area 0.015), 7.73 (m, area 0.015), 7.37 (m, area 0.06), 3.70 (s, area 100, CH_2O), 3.20 – 2.60 (m, area 14.7, CH_2N). UV-vis (H_2O), $\lambda_{\text{max}} = 557 \text{ nm}$ and $\epsilon_{\text{max}} = 1.57 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (assuming the MW ~33 KDa).

2.7. Inclusion of AQ10 (1%) in nanogel PEG-PEI-rhodamine

To a solution of 50 mg of PEG-PEI-rhodamine in 5 mL of deionized water, was added 0.5 mg (2.1 μmol) of AQ10 in 1 mL of acetonitrile. The resulting solution was lyophilized to give 50.5 mg of nanogel PEG-PEI-rhodamine-AQ10.

2.8. Cell culture

Pan 02 cells were maintained in medium containing RPMI 1640 (Invitrogen), 10% fetal bovine serum (FBS, Atlanta Biologicals), and 1x pen/strep (Invitrogen) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

2.9. Loading of nanogel PEG-PEI into Pan 02 cells

Pan 02 cells were seeded at 3×10^4 in a 12-well plate. At ~70% confluency, nanoparticles were added at 0.05 mg/well and incubated for 12 hrs. Following incubation, excess nanoparticles were removed by washing wells with 1X PBS, and fresh media was added. The loading of nanoparticles into cancer cells was visualized using a Nikon Eclipse epifluorescent microscope and images were captured using a Roper Cool Snap ES camera and Metamorph 7 image analysis system.

2.10. Cell proliferation evaluation

The number of viable cells was evaluated by the MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Roche Diagnostics GmbH, Germany) as well as hemocytometer and trypan blue exclusion analysis. Pan 02 cells were seeded in a 96 well plate. After reaching ~70% confluency, the media was replaced with fresh medium containing DMSO or AQ10+DMSO, and nanogel PEG-PEI or AQ10-nanogel PEG-PEI at different concentrations. After incubation for 48 hrs, the media was replaced with fresh medium containing 0.5 mg of MTT reagent per ml of medium and incubated for four hours at 37°C. Then, 100 μ L of solubilization reagent was added to each well and incubated overnight at 37°C. Absorbance was measured in an ELISA plate reader at 550 nm, with the absorbance at 690 nm to correct for background, and viability was expressed as the percentage of untreated controls. As an alternative method to validate the MTT assay, trypan blue exclusion and hemocytometer counting assay was performed in parallel. All experiments were done in duplicate and were performed three times.

2.11. Statistical analysis

All values are expressed as means $\pm$ SE. Values of untreated cells were taken as 0%. Statistical analysis of all Pan 02 cell proliferation assays were analyzed using repeated measures of ANOVA. When a significant *F*-ratio was demonstrated by the ANOVA-R, post hoc tests (Bonferroni) were applied to describe significant Pan 02 cell proliferation and dose effect of Nanogel PEG-PEI-AQ10 or AQ10 or DMSO. Statistical significance was considered if *P* < 0.05.

3. RESULTS AND DISCUSSION

3.1. Synthesis of nanogel PEG-PEI and rhodamine-attached nanogel PEG-PEI

Based on the nanogel synthesis method, the putative nanogel PEG-PEI structure is shown in Scheme 1. However, this structure has not been confirmed; therefore, ¹H NMR spectrum was performed, which revealed a ratio of ~6.8:1 for ¹H NMR (methylene protons). Based on the weight of the product from the coupling reaction of PEG and PEI, the ratio of PEG: PEI is ~1:1; this preparation of nanogel PEG-PEI was found to be non-toxic to cancer cells. However, when the methylene proton ratio was decreased to 4:1, the nanogel PEG-PEI was highly toxic to cancer cells (Figure 2). This 4:1 ratio of nanogel PEG-PEI along with other different ratios of CH₂O to CH₂N in PEG-PEI was prepared from different treatments of activated PEG with PEI (see methods). Thus, an important finding was that the methylene protons ratio is critical and should be kept $\geq$ ~6.8:1 to avoid significant solubility issues or undesirable bioactivities, such as cytotoxicity. This is important when the effects of small molecule drugs such as AQ10 are studied after incorporation into the nanogel PEG-PEI, in order to distinguish anti-cancer effects due to the small molecule drug versus possible cytotoxic effects of the carrier. The attachment of rhodamine helps to study the incorporation of nanogel PEG-PEI into cells. ¹H NMR spectrum revealed that only a small amount of rhodamine molecules were attached to PEG-PEI complex. UV-vis spectrum of nanogel PEG-PEI-rhodamine showed the presence of rhodamine in the nanogel PEG-PEI.

3.2. Studies on the sizes of nanogel PEG-PEI-AQ10 particles by AFM

The sizes of nanogel PEG-PEI-AQ10 particles were measured using an AFM instrument with tapping mode. Several samples were prepared and they exhibited similar images (two representative images are shown in the top panel of Figure 3). The zoom-in image of one particle is shown in the middle left panel of Figure 3. Some of the small nanogel PEG-PEI particles aggregate to form short fibril-like materials; one of these aggregates was depicted in

the bottom left panel of Figure 3. Overall, the nanogel PEG-PEI-AQ10 particles were rather evenly sized small round particles with diameter of ~23 nm and height of 1 nm.

3.3. AQ10 inhibits Pan 02 Cell Viability in a dose dependent manner

Previous studies showed that AQ10 can significantly decrease HL-60 and LL/2 cancer cell growth.^{14–16} The results from the cell proliferation assay showed that AQ10 significantly attenuated Pan 02 cell growth beginning at 4.2 μ M compared to un-treated and DMSO treated cells (Figure 4).

3.4. Uptake of nanogel PEG-PEI by Pan 02 cells

We tested the potential uptake of nanogel PEG-PEI by Pan 02 cells. The AQ10-nanogel PEG-PEI was covalently tagged with rhodamine to visualize whether AQ10-nanogel PEG-PEI was internalized by Pan 02 cells. Figure 5 shows that the majority of red-fluorescent labeled nanoparticles were distributed in the cytoplasm of Pan 02 cells over a period of 12 hours.

3.5. AQ10-nanogel PEG-PEI inhibited Pan 02 cell proliferation in a dose-dependent manner

The results from the MTT assay (Figure 6A) showed that nanogel PEG-PEI (~6.8:1, methyl protons ratio) by itself had no significant effect on the Pan 02 cell viability compared to control or untreated cells. In contrast, incubation with 1% AQ10-nanogel PEG-PEI significantly decreased cell proliferation at 0.06, 0.08 and 0.1 mg dose per ml of medium, compared to cells incubated with nanogel PEG-PEI alone treated and untreated cells. Figure 6B shows the results from hemocytometer-trypan blue exclusion assays. A similar effect was noted: cells that were incubated with 1% AQ10-nanogel PEG-PEI showed a significant decrease in the total number of viable cells at 0.06, 0.08, 0.1 mg dose per ml of medium compared to nanogel PEG-PEI alone. Importantly, AQ10 alone was three to four times less effective in growth attenuation of Pan 02 cells than AQ10 incorporated into nanogel PEG-PEI. This is presumably due to low water solubility of AQ10. Poor solubility of AQ10 in aqueous solution appears to make the drug less efficient; when incorporated into nanogel PEG-PEI, the AQ10 is more efficiently taken up by the cells, thereby decreasing the effective dose.

4. CONCLUSION

The current study provided experimental support for five new findings. First, the methylene protons ratio of PEG-PEI in the nanogel alters the toxicity of the nanogel PEG-PEI. Second, nanogel PEG-PEI can be loaded with and release a therapeutic anticancer drug, AQ10. Third, rhodamine (TAMRA) dye molecule can be incorporated into nanogel PEG-PEI to study the localization of nanogel PEG-PEI in cells. Fourth, AQ 10 dissolved in DMSO inhibits Pan 02 cell proliferation. Fifth, when AQ10 is incorporated into nanogel PEG-PEI, it causes a significant reduction in viable cell numbers of Pan 02 cells compared to AQ10 alone. These results suggest the possibility of using nanogel PEG-PEI as an efficient drug delivery vehicle by encapsulating toxic anti-cancer compounds such as AQ10.

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References

1. Cegnar M, Kristl J, Kos J. Nanoscale polymer carriers to deliver chemotherapeutic agents to tumours. *Expert Opinion on Biological Therapy* 2005;5:1557–1569. [PubMed: 16318420]

2. Vinogradov SV. Colloidal microgels in drug delivery applications. *Curr Pharm Des* 2006;12:4703–4712. [PubMed: 17168773]
3. Vinogradov SV, Batrakova EV, Kabanov AV. Nanogel PEG-PEIs for oligonucleotide delivery to the brain. *Bioconjug Chem* 2004;15:50–60. [PubMed: 14733583]
4. Vinogradov SV, Bronich TK, Kabanov AV. Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells. *Adv Drug Deliv Rev* 2002;54:135–147. [PubMed: 11755709]
5. Sung SJ, Min SH, Cho KY, Lee S, Min YJ, Yeom YI, Park JK. Effect of polyethylene glycol on gene delivery of polyethylenimine. *Biol Pharm Bull* 2003;26:492–500. [PubMed: 12673031]
6. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr J. A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in vivo: Polyethylenimine. *PNAS* 1995;92:7297–7301. [PubMed: 7638184]
7. Goula D, Benoist C, Mantero S, Merlo G, Levi G, Demeneix BA. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther* 1998;5:1291–1295. [PubMed: 9930332]
8. Vinogradov SV, Zeman AD, Batrakova EV, Kabanov AV. Polyplex Nanogel PEG-PEI formulations for drug delivery of cytotoxic nucleoside analogs. *J Control Release* 2005;107:143–157. [PubMed: 16039001]
9. Vinogradov SV, Batrakova EV, Li S, Kabanov AV. Mixed polymer micelles of amphiphilic and cationic copolymers for delivery of antisense oligonucleotides. *J Drug Target* 2004;12:517–526. [PubMed: 15621677]
10. Dong W, Jin GH, Li SF, Sun QM, Ma DY, Hua ZC. Cross-linked polyethylenimine as potential DNA vector for gene delivery with high efficiency and low cytotoxicity. *Acta Biochim Biophys Sin*, (Shanghai) 2006;38:780–787. [PubMed: 17091195]
11. Vinogradov SV, Bronich TK, Kabanov AV. Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. *Bioconjug Chem* 1998;9:805–812. [PubMed: 9815175]
12. Erbacher P, Bettinger T, Belguise-Valladier P, Zou S, Coll JL, Behr JP, Remy JS. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). *J Gene Med* 1999;1:210–222. [PubMed: 10738569]
13. Wang Y, Perchellet EM, Ward MM, Lou K, Hua DH, Perchellet JP. Rapid collapse of mitochondrial transmembrane potential in HL-60 cells and isolated mitochondria treated with anti-tumor 1,4-anthracenediones. *Anticancer Drugs* 2005;16:953–967. [PubMed: 16162972]
14. Perchellet EM, Wang Y, Weber RL, Sperflage BJ, Lou K, Crossland J, Hua DH, Perchellet JP. Synthetic 1,4-anthracenedione analogs induce cytochrome c release, caspase-9, -3, and -8 activities, poly(ADP-ribose) polymerase-1 cleavage and internucleosomal DNA fragmentation in HL-60 cells by a mechanism which involves caspase-2 activation but not Fas signaling 2. *Biochem Pharmacol* 2004;67:523–537. [PubMed: 15037204]
15. Hua DH, Lou K, Battina SK, Zhao H, Perchellet EM, Wang Y, Perchellet JP. Syntheses, molecular targets and antitumor activities of novel triptycene bisquinones and 1,4-anthracenedione analogs. *Anticancer Agents Med Chem* 2006;6:303–318. [PubMed: 16842233]
16. Vinogradov SV, Kohli E, Zeman AD. Comparison of Nanogel PEG-PEI Drug Carriers and their Formulations with Nucleoside 5'-Triphosphates. *Pharm Res.* 2006

Figure 1.
Structure of AQ10

Figure 2.

The effect of altered methylene protons ratio in PEG-PEI on Pan 02 cell viability. * Significantly different from untreated cells, †significantly different from nanogel PEG:PEI (4:1).

Figure 3.

Top panel: AFM images of two different locations of the nanogel PEG-PEI AQ10 (1%) sample on mica. Middle left panel: a zoom-in AFM image of a nanogel PEG-PEI particle, and its height and width are shown in the middle right panel. Bottom left panel: a zoom-in AFM image of an aggregated nanogel PEG-PEI particle, and its height and width are shown in bottom right panel.

Figure 4.

Dose effect of AQ10 on Pan 02 cell viability: Cell proliferation assay showed that AQ10 significantly decreased the Pan 02 cell viability compared to untreated Pan 02 cells. * Significantly different from untreated cells, [†]significantly different from DMSO treated cells.

Figure 5. Uptake of rhodamine-labeled AQ10-nanogel PEG-PEI particles by Pan 02 cells after incubation for 12 hours. The rhodamine-labeled AQ10-nanogel PEG-PEI particles are seen in the cytoplasm (arrows). 40x magnification.

Figure 6.

Dose effect of nanogel PEG-PEI and 1% AQ10-nanogel PEG-PEI on Pan 02 cell viability based on MTT assay (panel A) and the hemocytometer-trypan blue exclusion (panel B). * Significantly different from untreated cells, [†]significantly different from nanogel PEG-PEI alone treated cells.

Scheme 1.

Synthesis and putative structure of rhodamine-labeled nanogel PEG-PEI.