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Collagen synthesis promoting pullulan–PEI–ascorbic acid conjugate as an efficient anti-cancer gene delivery vector

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ABSTRACT

Cationized pullulan (pullulan–PEI; PP) was synthesized and further modified with an anti-oxidant molecule, ascorbic acid (PPAA) at various ratios. The nanoplexes formed at an optimum ratio of 4:1 was within a size of 150 nm and had a zeta potential of 9–14 mV. The nanoplexes at this ratio was used for further investigations. The cell internalization and transfection efficiency of these nanoplexes were determined in presence of serum. The internalization and transfection efficiency were found to be unaffected by the presence of fetal bovine serum. Another interesting observation was that this polymer was found to have collagen synthesis promoting property. The collagen synthesis effect of these polymers was quantified and observed that PPAA3 promoted the highest. Transfection efficiency was evaluated by assessing the p53 gene expression in C6 rat glioma cells and cell death was quantified to be 96% by flow cytometry, thus establishing the high efficacy of this polymer.

1. Introduction

Cancer is one of the most debilitating diseases known to mankind and is the second most leading cause of death globally. Currently gene therapy is being considered as a promising strategy for treating cancer. Cationic DNA condensing polymers are exploited for improved cellular uptake. Cationized pullulan with polyethyleneimine (PEI) has been reported as excellent gene carrier from this lab earlier (Rekha & Sharma, 2011). While designing a carrier for gene delivery in this study, a polymer that can tackle cancer metastasizes by improving host response against cancer invasion was considered. For a cancer to metastasize from primary sites to other parts of the body, it has to penetrate the physiological barrier put forth by extracellular matrix and basal membrane. Collagen forms primary constituent of these barriers. Cancer metastasis starts with collagen degradation. Collagen degradation is assisted by matrix metalloproteinase's (MMPs). The degradation products are said to activate monocytes into macrophages which in turn release more MMPs (Jones, Sanes, & Herrington, 2003; Ray & Stetler-Stevenson, 1994). Hence, a system which can improve the collagen density and inhibit MMPs may act as antagonists for cancer metastasize.

Anticancer property of ascorbic acid has been investigated since 1970s (Cameron, Pauling & Leibovitz, 1979, Padayatty et al., 2006). There is a host response toward malignant invasion and the factors involved can be at stromal level or the systemic level (Cameron et al., 1979). Cameron et al. (1979) proposed that the ascorbic acid can play a role in various host resistance mechanisms at stromal and systemic level. One of these host defense mechanisms includes improving the first line defense against invasive growth by reinforcing the matrix integrity by collagen. Collagen synthesis is assisted by ascorbic acid and hence may be used to improve the collagen density. Again, cellular invasion and metastasis in cancer is through extracellular matrix remodeling by MMP2 and MMP9. Ascorbic acid is also shown to have inhibitory effect on MMP-2 and 9 (Emara & Cheung, 2006) and also reported to have a stimulating effect on tissue inhibitor of metalloproteinase1 (TIMP1) which is a natural inhibitor of MMP's (Nusgens et al., 2001). Hence ascorbic acid can be an ideal choice to modify a gene delivery vector which can impart a collagen synthesizing property and MMP inhibition which will be beneficial toward solid tumor therapy. Here, our objective was to develop a nucleic acid delivery system capable of promoting collagen synthesis. Cationized pullulan with PEI was used as parent material for synthesizing gene delivery vector. L-ascorbic acid was used to further modify PEI conjugated pullulan. Ascorbic acid was selected by the authors for its anticancer activity and anti metastasize activity. The suitability of the gene delivery system developed for CNS is not dealt with in this study.

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2. Materials and methods

2.1. Materials

Pullulan (Sigma–Aldrich), CDI, polyethyleneimine (10 kda Polysciences), ascorbic acid (Polysciences), 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, Minimum essential medium, trypsin, ethylenediaminetetraacetic acid (EDTA), DNase I (Sigma–Aldrich Chemicals Co., USA.), Deoxyribonucleic acid sodium salt from calf thymus (ctDNA) (Worthington Biochemical-Corp), YOYO iodide and Hoechst 33342 (Invitrogen), fetal bovine serum (FBS) (GIBCO, USA). All other reagents were of analytical grade from Merck, India.

2.2. Methods

2.2.1. Synthesis of polymers

Pullulan was cationized with PEI. Briefly, pullulan was dissolved in dimethyl sulfoxide and was activated by CDI. To this activated pullulan, PEI dissolved in 20 mM borax was added and was stirred overnight. To the obtained solution acetone was added and the precipitate was washed thrice with acetone. The precipitate was then dissolved and dialysed against distilled water; the product was coded as PP.

Ascorbic acid was conjugated to PP using CDI chemistry. Ascorbic acid at quantities 30 mg (11.36 mM), 60 mg (22.71 mM) and 100 mg (37.85 mM) were used For this, CDI was added to ascorbic acid dissolved in DMSO containing 1% acetonitrile and was incubated under dark for one and half hour. After incubation period, PP was added and kept under overnight stirring. Cold acetone was added to the solution which precipitated ascorbic acid conjugated PP, named as PPAA. PPAA were dissolved in distilled water and dialysed. Polymers were coded as PPAA1 (11.36 mM), PPAA2 (22.71 mM) and PPAA3 (37.85 mM).

2.3. Characterization of PPAAs

2.3.1. ^1H NMR and FTIR

^1H NMR spectra of PPAA was measured in D_2O using a 500 MHz spectrometer (Bruker Avance NMR Spectrometer). δH (500 MHz; D_2O; Me4 Si) by water suppression.

FTIR analysis of pullulan and conjugates were done in a Nicolet Impact 410 FTIR spectrophotometer with a scan range 500–4000 cm^{-1}.

2.3.2. Determination of ascorbic acid

Ascorbic acid was estimated by spectrophotometric analysis using potassium permanganate. In short, standards/samples were treated with potassium permanganate in sulfuric acid solution and were read at 525 nm (Supplementary Table 1).

2.3.3. Preparation of nanoplexes with PPAA and calf thymus DNA (ctDNA)

Nanoplexes were prepared as previously described (Rekha & Sharma, 2011). Briefly, polymer (1 mg/ml) solution was prepared. Polyplexes were prepared by mixing polymer of desired amount to DNA by vortexing. Five micrograms of polymer in 1 ml distilled water was rapidly added to 10 μg of ctDNA in 10 μl of distilled water and was vortexed at high speed to form nanoplex at ratio 0.5:1. Similarly 10, 20, 30, 40 and 50 μg of polymer was mixed with 10 μg of ctDNA in 10 μl to prepare nanoplexes at ratio 1:1, 2:1, 3:1, 4:1 and 5:1 respectively.

2.3.4. Size and zeta potential of nanoplexes

The size and zeta of the nanoplexes prepared as described above were determined using dynamic light scattering measurement using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a temperature of 25 °C.

2.4. Determination of buffering capacity

Buffering capability of the polymer plays a key role in endosomal escape mechanisms. The buffering capacity of PEI, PP and PPAA, saline and ascorbic acid were evaluated by acid base titration over a pH range of 10–4. The pH of 1 mg/ml solution of polymer in saline was adjusted to 10 using 0.2 N NaOH then titrated against 0.01 N HCl, the pH being noted after each addition of 50 μl of acid. A graph was plotted with pH against the volume of HCl (Supplementary Fig. 1).

2.5. Agarose gel electrophoresis

2.5.1. Gel retardation assay

Self-assembling property of polymers with DNA was checked by gel retardation assay. Nanoplexes with ctDNA at w/w ratio from 0.5:1 to 5:1 were prepared by earlier mentioned method. Electrophoresis was then done with naked ctDNA as control.

2.5.2. Nanoplex stability assay: protection against plasma and DNAse

Nanoplexes formed were incubated with 20 μl of plasma for 30 min to assess their stability in presence of plasma. DNase I protection assay was also performed to investigate the ability of polymers to protect ctDNA from endonuclease degradation. The polyplexes were then incubated with DNase of concentration 853 U/ml at 37 °C for 30 min. For 2 μgm of DNA, 2.28 μl of DNase was used. Naked ctDNA with and without DNase 1 treatment were positive and negative control respectively. The reaction was stopped with termination buffer and electrophoresis was carried out. For heparin competitive assay, heparin (5 μl at concentration 1000 U/ml) was added to the remaining mixture to loosen up DNA that might be protected by polymer within nanoplexes. Electrophoresis with heparin treated mixture was then done.

All electrophoresis was carried out in 1× TAE buffer, with 2 μl of 10 mg/ml ethidium bromide as stain (Bio-Rad laboratories, CA, USA) at 75 V for 45 min. The DNA bands were visualized and photographed using Image Analyser (LAS 4000, Fuji).

2.6. Polyacrylamide gel electrophoresis

Interaction of polymer with plasma proteins was assessed by native PAGE analysis. The polymers of interest (1 mg/mgl) were incubated with 20 μl of plasma for 30 min. PEI and saline treated plasma were considered as positive and negative controls respectively. After a 30 min incubation time, supernatant was collected by centrifuging at 8000 rpm for 10 min. Supernatant was mixed with sample buffer in 2:1 ratio. Electrophoresis was carried out in a Mini-PROTEAN II electrophoresis cell (Bio-RAD, CA, USA). 0.2% Coomassie brilliant blue R 250 was used to stain the gel and later destained using buffer containing acetic acid, methanol and distilled water. The gel was then photographed using an image analyzer (LAS 4000, Fuji).

2.7. Evaluation of in vitro cytotoxicity

Cytotoxicity of polymers was studied by MTT assay using C6 rat glioma cells. A cell density of 1×10^3 cells/well was seeded on a 96 well plate and was incubated for 24 h in an incubator at 37 °C under 5% CO_2 atmosphere. Polymers at a concentration 25,