



Short communication

Development of a method to quantify the DNA content in cationic peptide–DNA nanoparticles



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ABSTRACT

Gene therapy has the potential to provide safe and targeted therapies for a variety of diseases. A range of intracellular gene delivery vehicles have been proposed for this purpose. Non-viral vectors are a particularly attractive option and among them cationic peptides have emerged as promising candidates. For the pharmaceutical formulation and application to clinical studies it is necessary to quantify the amount of pDNA condensed with the delivery system. There is a severe deficiency in this area, thus far no methods have been reported specifically for pDNA condensed with cationic peptide to form nanoparticles. The current study seeks to address this and describes the evaluation of a range of disruption agents to extract DNA from nanoparticles formed by condensation with cationic fusogenic peptides RALA and KALA. Only proteinase K exhibited efficient and reproducible results and compatibility with the PicoGreen reagent based quantification assay. Thus we report for the first time a simple and reliable method that can quantify the pDNA content in pDNA cationic peptide nanoparticles.

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1. Introduction

To exhibit a therapeutic response genetic materials must be delivered intracellularly, which requires the pDNA to be condensed into a suitable vehicle that can enable its entry into the cells [1]. Furthermore, such vehicles can provide stability to the DNA in the extra-cellular matrix, and in the presence of phagocytes, opsonins and DNAses. Once inside the cell extra measures are required to make sure that the DNA can escape the endosome to reach the nucleus for its desired action [1–3]. A variety of viral and non-viral vectors have been developed and are being explored for this purpose [4,5]. Non-viral gene delivery systems have been developed and have proven their efficiency for safe delivery of the gene inside the cells [6]. Cationic peptides are able to efficiently condense DNA and penetrate the cell membrane for the delivery of the cargo.

Abbreviations: EtBr, ethidium bromide; dsDNA, double stranded DNA; pDNA, plasmid DNA; SDS, sodium dodecyl sulphate; DMAPS, (3-(*N,N*-dimethylmyristylammonio) propanesulfonate); N:P, nitrogen:phosphorus ratio; TAE, tris acetate–EDTA.

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Furthermore, their simple structure and versatile nature allow their application for variety of treatment strategies [7–10]. For pharmaceutical processing and further clinical applications a method is required to accurately quantify the amount of DNA present in such delivery systems. Direct estimation of the amount of DNA present in non-viral gene delivery systems has been a challenge and researchers have had to rely on either indirect methods of estimation where the untrapped amount of DNA was quantified or avoided quantification at all. While there is a range of methods available for DNA quantification (such as ethidium bromide (EtBr), Hoechst bis-benzimide dyes or the PicoGreen® reagent) the challenge has been in extracting DNA out of the delivery system in such a way that does not damage the DNA nor interfere with the subsequent quantification method.

The PicoGreen reagent was selected for the assay development as it is specific to dsDNA, is simple to use and can quantify efficiently concentrations as low as 25 pg/ml dsDNA concentration in the presence of RNA and single stranded DNA (ssDNA) [11,12]. To our knowledge there is no direct method for the quantification of pDNA condensed in the form of peptide nanoparticles, which urged us to explore the ability of a range of disrupting agents to extract the DNA from cationic peptide nanoparticles and be compatible with quantification of DNA with the PicoGreen reagent.

2. Materials and methods

2.1. Materials

Quant-iT™ PicoGreen® dsDNA assay kit and Maxi-prep PureLink™ HiPure plasmid purification Kit and DNAase/RNAase free distilled water (USP water for injection, GIBCO) were procured from Invitrogen Life technologies, UK. EtBr, sodium dodecyl sulphate (SDS), DMAPS (3-(*N,N*-dimethylmyristylammonio) propanesulfonate), Triton X-100, guanidine hydrochloride, proteinase K (BioUltra, ≥ 30 units/mg protein) were purchased from Sigma–Aldrich, UK. The cationic peptides KALA [13] and RALA [14] were purchased from Biomatik, USA. They were synthesized by solid phase synthesis and characterized by high performance liquid chromatography (HPLC) and mass spectroscopy and supplied as lyophilized powders. The plasmid used was pEGFP-N1. It was cloned and purified using a Maxi-prep PureLink™ HiPure plasmid purification Kit (Invitrogen Life technologies, UK) according to the manufacturer's instructions, then re-suspended in distilled water (Invitrogen), aliquoted and stored at -20°C . HPLC grade water was freshly collected from PURELAB Prima and PURELAB Maxima HPLC (ELGA LabWater).

2.2. Formulation of cationic peptide–DNA nanoparticles

Cationic peptide–DNA nanoparticles were prepared with a charge ratio of 10:1 (N:P, nitrogen:phosphorous; the molar ratio of positively charged nitrogen atoms in the peptide to negatively charged phosphates in the pDNA backbone) by adding an appropriate volume of cationic peptide solution to $1\ \mu\text{g}$ pDNA with a final volume of $50\ \mu\text{l}$. The complexes were then incubated at room temperature for 20 min before further experimental analysis or characterization.

2.3. Characterization of nanoparticles for particle size and zeta potential

Particle size and zeta potential analysis were performed by dynamic light scattering (DLS) using a Malvern zetasizer (Nano ZS, Malvern Instruments, UK) at 20°C . For size determination the average of 5 readings (at least 10 run each) was taken of each sample, data is presented as mean \pm S.D. For zeta potential measurement samples were diluted to 1 ml with HPLC grade fresh water before analysis, results are also presented as mean \pm S.D. ($n = 10$).

2.4. Disruption of the nanoparticles

A range of disrupting agents were evaluated; the strong anionic surfactant sodium dodecyl sulphate; zwitterionic surfactant DMAPS; non-ionic surfactant triton X-100, denaturing agent guanidine hydrochloride and an enzyme proteinase K. Their efficacy was evaluated by agarose gel electrophoresis. Samples were prepared by mixing $10\ \mu\text{l}$ of nanoparticles (equivalent to $0.2\ \mu\text{g}$ of pDNA) with $10\ \mu\text{l}$ of $2\times$ double strength working stock of the disrupting agent.

2.5. Gel retardation assay

Samples were electrophoresed through a 0.8% agarose gel containing EtBr with Tris acetate–EDTA (TAE) running buffer at 80V for 1 h and analyzed using a gel imaging system (Biochemi® Multi-spectrum imaging system, UVP, UK). Images are representative of a minimum of three independent studies.

2.6. PicoGreen assay

The PicoGreen assay was performed using black flat bottom 96-well microtitre plates (Sterilin Ltd., Thermo Scientific, UK). Each well contained $50\ \mu\text{l}$ of sample with $50\ \mu\text{l}$ of disrupting agent. A range of temperatures and incubation times were investigated. After incubation $50\ \mu\text{l}$ of PicoGreen reagent (diluted 200-fold with $1\times$ TAE buffer) was added to each well. The plates were further incubated for 60 min in the dark and were then read using a Synergy 2 Multi-Mode microplate reader (BioTek Instrument Inc., UK) using excitation at $485/20\ \text{nm}$ and the fluorescence emission filter $528/20\ \text{nm}$. Calibration plots were prepared in the range of 50 – $1000\ \text{ng/ml}$ for both pDNA alone and cationic peptide–DNA nanoparticles at equivalent DNA concentrations in Tris buffer (pH 8.0, $20\ \text{mM}$).

2.7. Statistical analysis

Statistical analysis was performed using Graphpad prism 6 and Graphpad InStat 3 (Graphpad software Inc., La Jolla, USA). One-way ANOVA followed by Dunnet post hoc test was performed to compare the data set (more than three groups). A p value less than 0.05 was considered to indicate statistically significant differences between groups.

3. Results and discussion

3.1. Size and zeta potential of cationic peptide–DNA nanoparticles

Cationic nanoparticles were synthesized by direct electrostatic interaction where cationic charge of the peptide interacts with the negatively charged phosphate backbone of the DNA and causes its collapse in the form of nanoparticles. The particle size of these nanoparticles was determined to be $70.6 \pm 7.5\ \text{nm}$ (z -average diameter) with a polydispersity index (PDI) of 0.24 ± 0.05 ($n = 10$) and zeta potential was determined to be $+34.47 \pm 3.19\ \text{mV}$ ($n = 10$).

3.2. Quantification of DNA in cationic peptide nanoparticles

As the DNA is condensed with the cationic peptide it becomes buried in the core of the formed nanoparticles, as evidenced by their net positive zeta potential. This phenomenon makes the DNA inaccessible to DNA quantification assays based on UV and fluorescence spectroscopy of complexes formed with the pDNA. For the same reason they are not visible in the agarose gel electrophoresis (both phenomena can be seen in Fig. 1). This indicates that a disrupting agent is required to free the DNA to allow its accurate quantification.

3.2.1. Disruption of the nanoparticles with SDS

Sodium dodecyl sulphate is a commonly used anionic surfactant for a variety of applications in the biological science protocols. As shown in Fig. 1A SDS successfully disrupted the cationic nanoparticles at concentrations $\geq 0.1\%$ w/v. 1 kb plus ladder and pDNA only samples were loaded for comparative purpose and as positive control. No fluorescence was observed in the very first lane that contained native nanoparticles only indicating there was no free DNA available to enter the gel and interact with EtBr as observed previously for both KALA and RALA nanoparticles prepared at this charge ratio [13,14]. The lane containing the 0.001% w/v SDS showed some fluorescence in the well indicating that this lower concentration somewhat disrupted the nanoparticles but was insufficient to free it completely to allow migration down the gel, while the higher concentrations demonstrated acceptable migration.

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