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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.

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 DNAases. 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. 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 unentrapped 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.

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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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 μg pDNA with a final volume of 50 μl. 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 ± 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 ± 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 μl of nanoparticles (equivalent to 0.2 μg of pDNA) with 10 μl of 2× double strength working stock of the disrupting agent.

2.5. Gel retardation assay

Samples were electrophoresed though a 0.8% agarose gel containing EtBr with Tris acetate–EDTA (TAE) running buffer at 80 V for 1 h and analyzed using a gel imaging system (Biochemi® Multispectrum 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 μl of sample with 50 μl of disrupting agent. A range of temperatures and incubation times were investigated. After incubation 50 μl of PicoGreen reagent (diluted 200-fold with 1× 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 nm and the fluorescence emission filter 528/20 nm. Calibration plots were prepared in the range of 50–1000 ng/ml for both pDNA alone and cationic peptide–DNA nanoparticles at equivalent DNA concentrations in Tris buffer (pH 8.0, 20 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 ± 7.5 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 ± 3.19 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 ≥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.

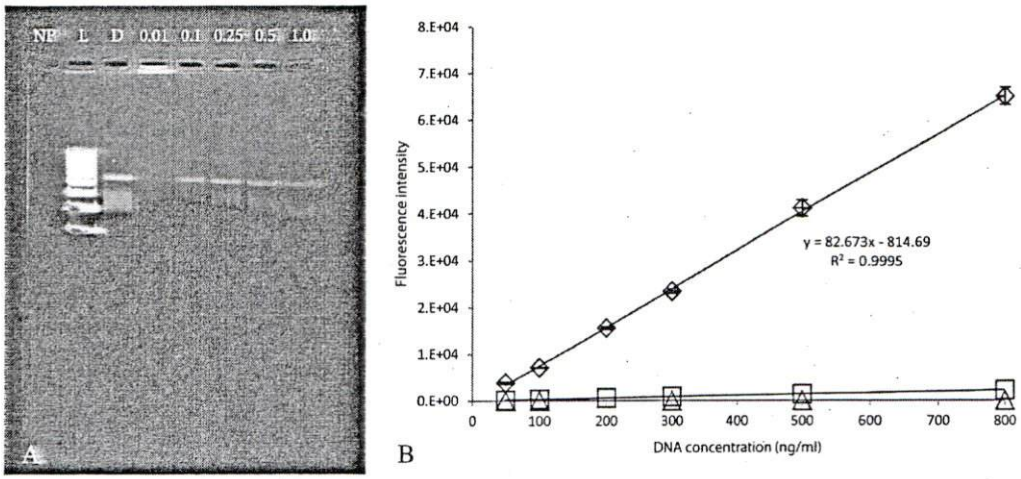

Fig. 1. Efficacy of SDS as a disrupting agent. 'A': Agarose gel electrophoresis of nanoparticles digested with different concentration of SDS indicated in each lane. NP: native nanoparticles; L: 1 kb plus DNA ladder; D: native DNA; Numbers in subsequent lanes denote SDS concentration (%w/v). 'B': PicoGreen assay performed to quantify the DNA in nanoparticles with and without SDS disruption. Diamonds: DNA alone; Squares: Nanoparticles; Triangles: Nanoparticles disrupted with SDS 1% w/v. Data are means of 5 replicates. Error bars represent standard deviation.

The same concentration of SDS was used to disrupt nanoparticles before attempting to quantify the pDNA using the PicoGreen assay; the results are shown in Fig. 1B. The PicoGreen assay was able to accurately quantify the pDNA alone (r^2 value of 0.999); but the fluorescence exhibited by both native and disrupted nanoparticles was scarcely detectable in the presence of SDS. The lack of fluorescence exhibited by the nanoparticles supports the assertion that pDNA is buried within the nanoparticles. However, these results indicate that while SDS disrupts the nanoparticles it also quenches the fluorescence of the PicoGreen: pDNA complex. These results are in line with previous findings where SDS concentrations >0.1% w/v were reported to decrease the fluorescence to base values [11,15].

3.2.2. Selection of digestion agent

Fig. 2A shows the disruption of the nanoparticles by DMAPS. Various concentrations of the surfactant was employed at room temperature, 37 °C and 60 °C; none of the lanes show pDNA migration indicating that DMAPS failed to disrupt the nanoparticles. Triton X 100 was tested in the concentration range 0.1–100 mM, only 1 mM showed a weak band in the gel (Fig. 2B). To explore this phenomenon a narrower range was studied (0.5–4 mM); these always gave faint bands but the behaviour was inconsistent and these results were therefore deemed unacceptable. Guanidine hydrochloride (1–5 M) freed pDNA to allow migration but the fluorescence intensity was low and no improvement was observed even after raising the temperature to 60 °C (Fig. 2C). Proteinase K was investigated in the concentration range of 0.1–2 mg/ml at room temperature, 37 °C and 60 °C. The results (Fig. 2D) indicate that the proteinase K successfully disrupted the nanoparticles in all temperature conditions and concentrations examined except when 0.1 mg/ml was incubated at room temperature for just 15 min. These encouraging results lead to further assessment of its employability with the PicoGreen assay.

3.3. Development of proteinase K assisted PicoGreen assay

From the findings of the gel retardation assay a concentration of 0.5 mg/ml proteinase K was selected as it was the lowest concentration that worked in all tested conditions. This concentration was used to digest the nanoparticles prior to performing the PicoGreen assay and compared with pDNA only and undigested nanoparticles (Fig. 3A). As shown in the previous experiment (Fig. 1B) the curve produced from assay of the nanoparticles alone showed very low fluorescence levels. Curves of nanoparticles and native pDNA that had both been incubated with proteinase K showed similar fluorescence with no significant difference ($p > 0.05$) at any concentration. Whereas, the calibration curve from of native pDNA (without proteinase K) showed significantly higher fluorescence indicating that the addition of proteinase K quenches some fluorescence and that the process variables must to be optimized.

3.3.1. Optimization of method variables

As shown in the preliminary experiment the application of 0.5 mg/ml enzyme concentration showed a decrease in the fluorescence intensity, thus a range of enzyme concentrations were studied in order to find the minimum concentration of proteinase K required for the cationic nanoparticle digestion which does not interfere with the fluorescence measurements in the PicoGreen assay fluorescence. As can be seen in Fig. 3B at concentration >100 μg/ml proteinase K starts interfering with the fluorescence. A significant decrease in florescence intensity was observed with 500 and 1000 μg/ml ($p < 0.01$) when compared with fluorescence from nanoparticles treated with 100 μg/ml proteinase K concentration. Whereas, fluorescence shown by samples treated with ≤100 μg/ml proteinase K concentration showed no significant difference than pDNA alone. Thus 100 μg/ml was selected as the highest concentration of proteinase K to be used for the assay.

Two other process variables: incubation temperature and duration of incubation were also studied and results are shown in Fig. 3B. When the incubation was performed at 37 °C all enzyme concentrations 10–100 μg/ml showed complete digestion after just 30 min that was similar to incubation of 60 and 90 min. Whereas, at room temperature the enzyme activity was found to be low; as in 30 min of incubation only 100 μg/ml enzyme could digest the nanoparticles, after 60 min of incubation 50 and 100 μg/ml enzyme could digest the nanoparticles and after 90 min all three concentrations worked effectively. Thus, on the basis of these results for future experiments 37 °C was selected as incubation temperature with 60 min of incubation time. Also, the intensity of the digested nanoparticles dilutions were similar to the fluorescence obtained from pDNA alone, indicating complete digestion of nanoparticles and no depression in the fluorescence intensity due to presence of the proteinase K in these concentrations. Thus, the higher concentration of 100 μg/ml was selected for the assay.

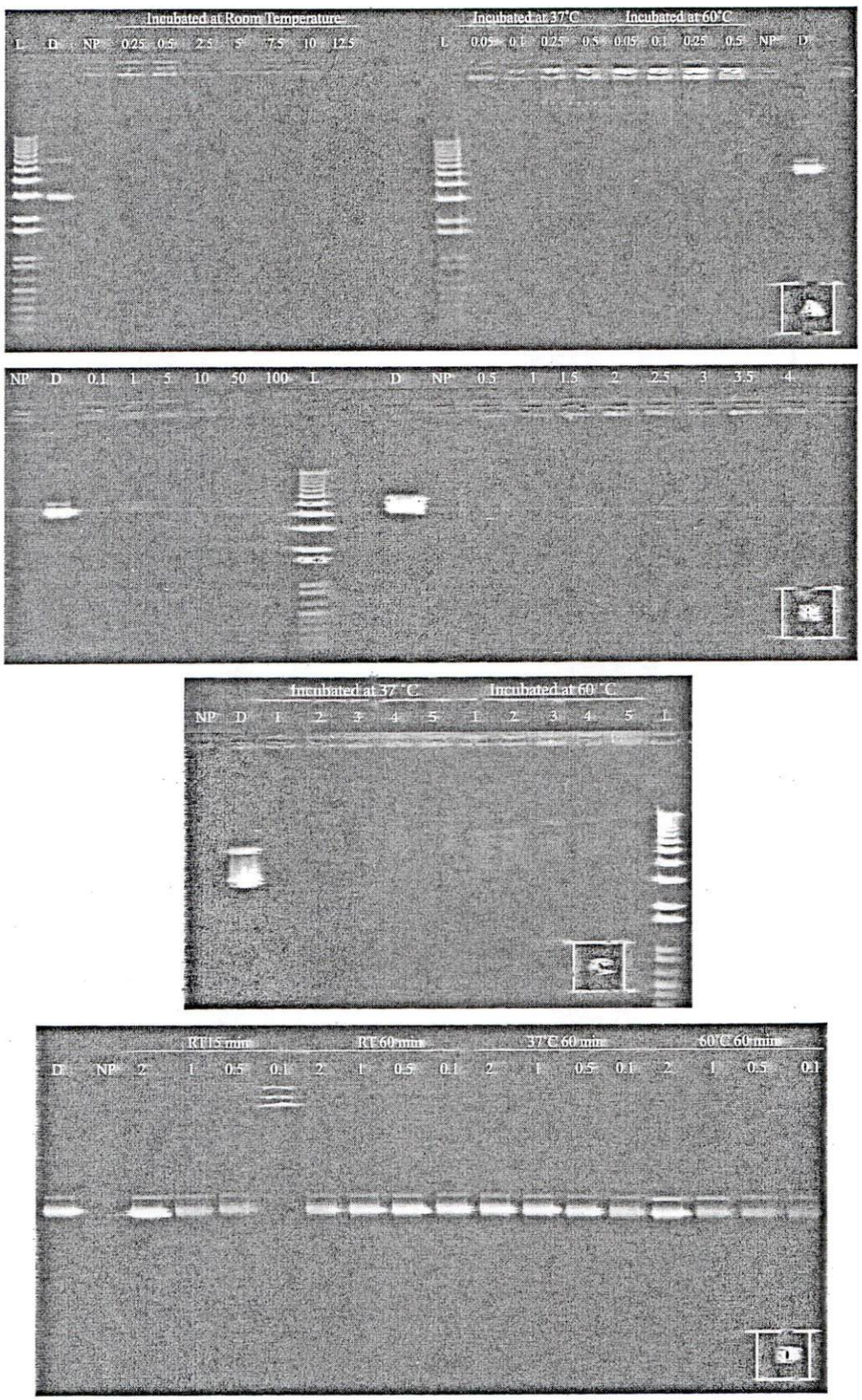

Fig. 2. Gel retardation assay to assess the disrupting ability of various agents. For all lanes 'L' denotes 1 kB plus ladder; 'D' denotes native DNA; 'NP' denotes nanoparticles only and numbers above lanes denote the concentration of disrupting agent added to the nanoparticles. 'A': Disruption of nanoparticles by DMAPS at room temperature, 37 °C and 60 °C, digits denote concentration in % w/v; untreated pDNA and nanoparticles were also loaded as control and denoted as D and NP in the figure; 'B': Disruption of nanoparticles by Triton X-100, digits denote concentration in mmol; 'C': Disruption of nanoparticles by guanidine at 37 °C and 60 °C, digits denote concentration in mol. 'D': Disruption of nanoparticles by proteinase K at room temperature, 37 °C and 60 °C, digits denote concentration in mg/ml.

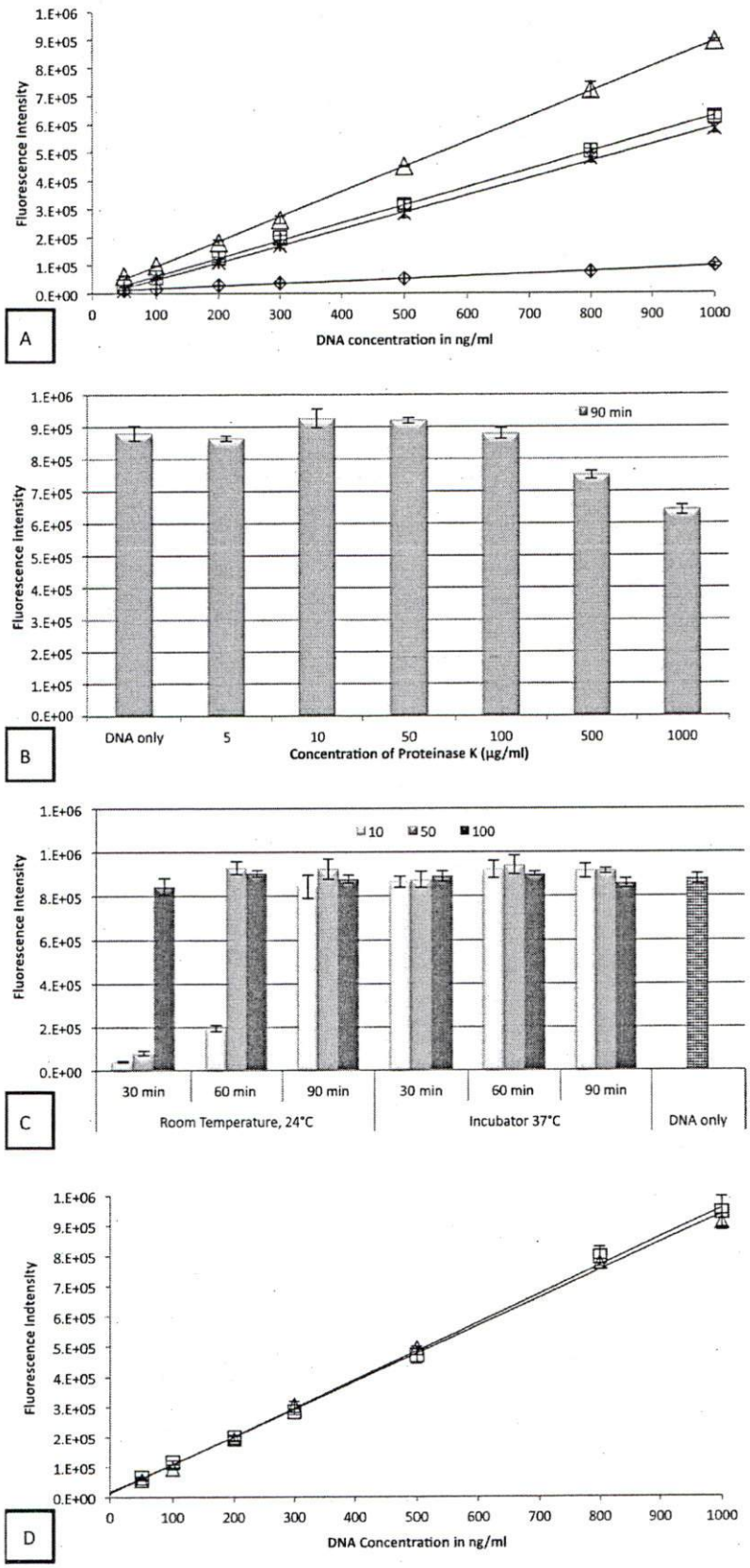

Fig. 3. Development of the proteinase K assisted PicoGreen assay. 'A': Quantification of nanoparticles alone and disrupted with proteinase K; pDNA concentration was 1 µg/ml. Triangles: pDNA only ($r^2 = 0.999$); Cross: pDNA with proteinase K ($r^2 = 0.999$); Diamonds: Nanoparticles alone; Squares: Nanoparticles disrupted with proteinase K ($r^2 = 0.997$). B: Effect of enzyme concentration on fluorescence. C: Effect of incubation duration, incubation temperature and concentration of proteinase K; the columns at each time point represent enzyme concentrations of 10, 50 and 100 µg/ml, respectively. D: Calibration plot showing Squares: Nanoparticles disrupted with proteinase K using the optimized protocol ($r^2 = 0.998$) Triangles: pDNA only ($r^2 = 0.997$).

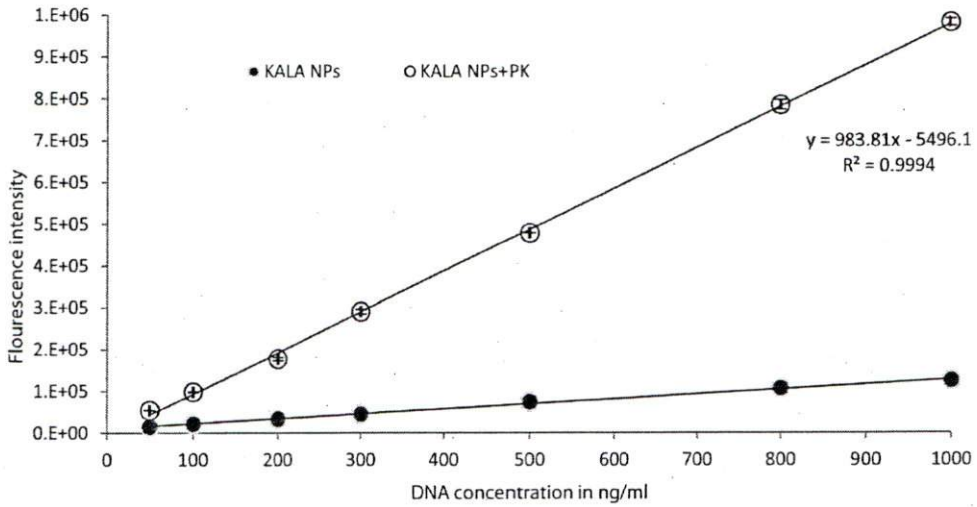

Fig. 4. Quantification of the DNA condensed with the optimized protocol. Filled circles: KALA nanoparticles alone; open circles: KALA nanoparticles digested with proteinase K ($r^2 = 0.999$).

3.4. Optimized protocol

The optimized protocol involved application of 100 μg/ml proteinase K in Tris buffer (pH 8.0, 20 mM) with incubation for 60 min at 37 °C followed by addition of PicoGreen reagent. A calibration plot using the optimized process, is presented in Fig. 3C. Calibration curves for native pDNA and for nanoparticles showed similar fluorescence values at each data point, while r^2 values are noted to be 0.997 and 0.998, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to ICH Q2-R1 guidelines [16] and ICH Topic Q2B [17] using the slope (S) of the calibration curve and the residual standard deviation, σ, of the regression line by the following:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S} \quad (1)$$

$$\text{LOQ} = 10 \times \frac{\sigma}{S} \quad (2)$$

and were found to be 2 ng/ml and 6 ng/ml, respectively.

3.5. Quantification of DNA condensed with KALA

KALA nanoparticles were prepared as described by Wyman et al. [13]. The amount of DNA present was quantified with the optimized protocol and the calibration curve is depicted in Fig. 4. The KALA nanoparticles showed minimal fluorescence when undigested, while pre-digestion with proteinase K gave a straight line ($r^2 = 0.999$). The LOD and LOQ for the KALA nanoparticles is calculated to be 2.5 ng/ml and 7.6 ng/ml, respectively. These data demonstrate the applicability of the developed protocol to peptide condensed DNA nanoparticles.

4. Conclusion

The ability to accurately quantify the amount of nucleic acid packed with/within a delivery system has been a major issue in the development of novel non-viral gene delivery systems. All currently available DNA quantification assays require accessibility of the reagent to the DNA, which is a major obstacle in the quantification of condensed DNA. We have developed and optimized a proteinase K based modified PicoGreen assay to quantify the amount of DNA inside cationic peptide nanoparticles. Proteinase K was found to successfully disrupt the nanoparticles and did not interfere with the PicoGreen assay. Optimum incubation conditions for proteinase K to digest RALA cationic peptide nanoparticles were evaluated to give an optimized PicoGreen assay protocol that was also able to quantify DNA loading in KALA:DNA nanoparticles.

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