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Research Article

CATIONIC DENDRIPLEXES FOR GENE THERAPY REQUIRES AT LEAST 5h TO EQUILIBRATE

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ABSTRACT

The task of developing a non-viral vector system for efficient delivery of drugs and genes has been a major concern to molecular scientists. Several efforts at formulating DNA-polycation complexes that show relatively consistent particle size and shape has not yielded perfect outcome. Some of these formulation variables can be exploited for optimum achievement towards development of DNA-polmer complexes of required nanosize. The objective of the study was to investigate the hydrodynamic size and zeta potential of complexes by dynamic light and electrophoretic light scattering with a view to formulating DNA/DAB complexes of consistent particle size and shape.

Stock solutions of DAB16 (1 & 10 mgmL-[1]) and DNA (Calf thymus) [1 & 10 mgmL-[1]] concentrations were prepared in 5% dextrose solution and deionised water respectively. DNA-Dendrimer Complexes (DNA/DAB16) were formulated based on a nitrogen to phosphate (N:P) ratio. Samples were characterized using a Zeta sizer 3000 HAS for hydrodynamic size; surface zeta potentials and Transmission electron microscope (TEM) for imaging.

Evidence showed that a N:P ratio in excess of 8 and/ or a DNA concentration in excess of 250µg/ml lead to non-colloidal complexes. It was also demonstrated that higher levels of dendrimer to dendriplex lead to a diminished dendriplex surface charge. The results also confirmed that as the binding time of Dendriplexes increases, the particle size of the complexes decreases while the zeta potential increases.

It can be concluded based on our results that a period of between 5 and 8 hour could be appropriate for dendriplex binding that will give a relatively consistent particle size and shape for efficient DNA delivery.

Keywords: DNA-DAB Complex, Polypropylenimine dendrimers, Gene delivery, Equilibration time.

INTRODUCTION

Gene therapy has been defined as the introduction of nucleic acid or genetic materials into defective cells purposely to restore normal function and produce a therapeutic effect. There are a number of obstacles limiting successful gene therapy but the most difficult to surpass has been the inability to transfer the appropriate gene into a target cells/tissues/organs[1] without affecting healthy tissues such that therapeutic concentration is delivered to target organ. The success of gene therapy largely depends on the development of suitable vectors or vehicles that can deliver gene(s) or drug to specific target tissue with minimal toxicity. Currently, there are two major methods of enhancing gene delivery, the physical methods which involve electroporation, gene gun, sonoporation and magnectofection while the chemical method is the use of synthetic or non-viral vectors such as Oligonucleotides, lipoplexes,polyplexes, phospholipids[2,3] etc. Though the use of viral vectors have been very efffective but the issue of toxicity, immunogenicity, inflammatory responses coupled with low concentration of virus being produced have limited their use[1,4,5,6]. The non-viral vector or synthetic carriers despite their ease of use have also suffered from transfection insufficiency.[7] The inherent advantage of flexibility and safety of non-carrier system has increased the level of research on the carriers.

The task of developing a non-viral vector system that is needed for efficient delivery of genes then becomes an issue of great concern. Quite a number of non-viral carriers for gene transfer have been synthesized, these include cationic liposomes, polylysines,[8] polypeptides, polymeric vesicles, recombinant histones, lipopolyplexes[7], Dendrimers[10,11,12] and other reagents that can convalently bind to biological molecules such as nucleic acid, proteins, peptides etc.[13] Several efforts at formulating deoxyribonucleic acid (DNA)-polycation complexes that show a relatively consistent particle size, shape and functions with overall ability for efficient in-vivo gene transfection have not really yielded perfect outcomes. It is regrettable, despite these efforts, non-uniform, irregular particle aggregates, toroid structures coupled with gel-like formation of complexes with some of these synthetic delivery systems were still beyond mechanistic explanation.

It has been proven that the double stranded helical structure of DNA is not completely rigid or stiff but can bend backward and forward thereby adopting different structures. This conformational change varies the spartial position of the binding sites of DNA to synthetic vectors such as polymers.[14] The volume or concentration of dendrimers necessary to neutralize or create polycation charge excess (CE) varies with the adopted DNA conformation which ultimately affect the particle size and shape of the final complex. Therefore shapes and sizes of gene-polymer complexes become very critical in assessing the ability of complexes to permeate membranes and other endothelial enzymes for effective transfection.

Dendrimers and dendritic polymers have played an impressive role in the delivery of nucleic acids. The finger-like structures of these molecules have also made conjugation and electrostatic binding with nucleic acid a plausible task. High molecular weight polymers such as Polypropylenimine dendrimers (DAB) function to change the topological structure of DNA molecules to form compact particles by forming an electrostatic interaction with DNA. There is also a growing consideration that apart from the terminal nitrogen, the internal tertiary nitrogen in the dendrimers may be involved in electrostatic binding with DNA phosphate groups.[15]

It is however hypothesized that to produce stable colloids of consistent particle size and shape, the binding/conjugation /equilibration rate are skillfully controlled and monitored in order to avoid particle agglomeration.

One of the objectives of this work is to investigate the hydrodynamic size and zeta potential of complexes by dynamic light and electrophoretic light scattering with a view to formulating DNA/DAB complexes of consistent particle size and shape. Secondly to provide a possible explanation for the particle variables which control in-vivo gene transfection.

MATERIALS AND METHODS

Preparation of 50ml 5 % Dextrose

Dextrose powder (2.5 g) was weighed into 50 mL volumetric flask and made up to volume with deionised water. The solution was then sonicated and filtered with 0.2 µm syringe micro-filter.

Preparation of DAB16 stock solutions (10 & 1 mgmL^{-1})

Polypropylenimine decaamine dendrimer [DAB16] (100 mg) was weighed into 10 ml volumetric flask and made up to volume with dextrose (5% w/v) and sonicated for about 5 minutes to give a concentration, 10 µgµL^{-1}. One ml of the above solution was diluted to 10 ml with 5 % dextrose solution to arrive at a concentration of 1 µgµL^{-1} of DAB.

Preparation of DNA (10 & 1 mgmL^{-1})

The sodium salt of Calf Thymus DNA (100mg) was procured from Sigma-Aldrich sample catalog No. 89380-100mg. The sample was transferred into a 10 ml volumetric flask and made up to volume with deionised water to arrive at concentration of 10 mgmL^{-1}. One ml of the stock solution was also made up to 10 ml with water to produce a concentration of 1 mgmL^{-1}. All the samples prepared for the analyses were made in 2 ml with 5 % dextrose solution including the Transmission Electron Microscope (TEM) (FEI CM120 BioTwin (Eindhoven, Netherlands) samples.

Preparation of DNA/DAB formulation based on Nitrogen to Phosphate ratios (N:P).

Stock solutions of DAB16 (1 & 10 mgmL^{-1}) and DNA (Calf thymus) [1 and 10 mgmL^{-1}] concentrations were prepared as shown above. Complexes (DNA/DAB16) were formulated based on N:P ratios as shown in table 2. These complexes were formed by addition of DAB solutions to DNA solutions and the complexes left undisturbed for about 3 minutes before mixing or stirring. This procedure is very important and crucial in order to avoid precipitation of DNA, most especially at DNA concentrations of 250 µgmL^{-1} and above. The complexes were allowed to equilibrate for 60 minutes for a single point sizing while samples used for the time-dependent assay were measured immediately after stirring at various time intervals for 24hrs at 37^{0}C. The table and calculations below show the formulation based on nitrogen to phosphate (N:P) ratio i.e. An N:P ratio of 1 requires 53g of DAB16 to complex with 340g DNA or alternatively an N:P ratio of 1 may be obtained if 0.16g of DAB16 are complexed with 1g of DNA.

Table 1: Materials source

Material	Source
DNA sodium salt Calf Thymus-89380-100mg	Sigma-Aldrich, UK
D-(+) Glucose monohydrate-D9559-100mg	Sigma-Aldrich, UK
Polypropylenimine decaamine dendrimer (DAB 16)	Sigma-Aldrich, UK
Tubes Sample S/C short 7ml	Scientific Lab. Supplies, UK

Table 2: Examples of Formulations Tested.

N:P Ratio	Vol. of DNA taken in µL (stock mgmL^{-1})	Vol. of DAB taken in µL (stock mgmL^{-1})	Final DNA/ DAB Complex µg/mL^{-1}
2	50 (1)	16 (1)	25/ 8
2	200 (10)	64 (10)	1000/320
3	50 (1)	24 (1)	25/12
3	200 (10)	96 (10)	1000/480
8	100 (1)	128 (1)	50/64
8	200 (1)	256 (1)	100/128
8	300 (1)	384 (1)	150/192
8	500 (1)	640 (1)	250/320
30	500 (1)	240 (10)	250/1200
60	500 (1)	480 (10)	250/2400

Determination of Hydrodynamic diameter measurement

The formulations were prepared in 5% dextrose solution (as dispersion medium) in a total volume of 2 ml. This was placed using a Zeta sizer 3000 HAS (Malvern Instrument Ltd., UK) three times. All samples passed instrumental tests such as baseline, polydispersity (less than 1) etc.

For the time-dependent (kinetic studies) particle size assay, the sample particle size was determined only once as assay time per instrument run was 15 minutes. The replicates were also in a polystyrene cuvet and measured at 25^{0}C. The triplicate samples were prepared at different days and particle size of each was measured measured once for the 24 h samples.

Determination of Zeta potentials of formulation

Manufacturer's standard solution (Malvern Zeta potential Transfer standard, DTS 1050) was filled into the in-built cuvet after cleaning with soapy solution, followed by de-ionized water and finally rinsed in 70% ethanol. The measurement was 47 mV conformed to manufacturer's specification of zeta potential of 50 ± 5 mV.

Sample (2 ml) was filled slowly into the Zeta-sizer using a 2 ml syringe to avoid formation of bubbles which could interfere with measurements. The equipment auto- regulates the temperature to 25^{0}C followed by the measurement. Replicate samples were prepared and measured on different days.

Transmission Electron Microscope measurement

A drop of formulation was placed onto a copper grid (300 mesh, Taab Laboratories, UK) that had a Formvar/carbon support film. Excess sample was removed with filter paper (N0.9 Hardened) then a drop of heavy metal stain (1% aqueous Uranyl acetate) added for contrast. The prepared grid was then examined under the Transmission Electron Microscope, which was an FEI CM120 BioTwin TEM (Eindhoven, Netherlands). Images were captured with AMT (Stowmarket, UK) Digital Camera.

RESULTS AND DISCUSSION

Figure 1a shows evidence that a N:P ratio in excess of 8 and / or a DNA concentration in excess of 250µg/ml leads to non-colloidal complexes. The area encircled in figure 1a seems to follow a pattern and the particle size within the region was found to be below 500nm. The other parts of the curves where the N:P were higher than 8 with increase in DNA gave bigger particles of micro-sized as confirmed by the Zeter sizer.

Figure 1b was selected for monitoring because the particle size formed was in the nanoscale and in this region increased particle size correlates with increase in DNA concentrations which tends to form a pattern. The field selected are the formulations between N:P 2 and 8 with the DNA concentration below 250µgmL^{-1}. All formulations with a DNA concentration in excess of 250 µgmL^{-1} at an N:P ratio above 10 formed particle sizes in the microscale.

Figure 2 demonstrates that higher levels of dendrimer to dendriplex lead to a diminished dendriplex surface charge. Similarly at DNA concentration lower than or equal to 100µg/ml, the zeta potentials tend to be high and subsequent decreases as dendrimer concentration increases.

All the selected formulations (Fig.1a) of DNA concentrations 25 – 400 µgmL^{-1} showed good physical stability (clear solution) as no precipitation was observed compared to DNA concentrations 500 and 1000 µgmL^{-1} which precipitated the DNA instantly as shown in figure 8.

The positive surface charge is essential because it also facilitates cell absorption and mediates efficient endosomal uptake into cells.[9] Figure 3a,b,c and d showed that as DNA concentration increases the particle size increases with attendant decrease in zeta potentials of the complexes.

The particle size and shape of the DAB/DNA complexes examined using transmission electron microscope (TEM) as shown in Figure 6 were found to be below 200nm nanoscale while sample of higher N:P 60 gave larger particle size in the range of microscale as presented in figure 7. This corroborates the results emanated from Zeter sizer measurement of particle size of the same formulations. The bigger particle size formed at higher N:P's could be as a result of aggregation of complexes.

It becomes obvious from the figure 4 that as zeta potential is increased, the particle size of the complexes decreases. This translates into formation of more compact particles that would be able to permeate cell membranes better than the bigger particle size formed from DNA concentrations higher than 400ug/ml or N:P higher than 10.

Figure 5 shows the effect of varying concentration of DNA on the particle size and zeta potential with the equilibration time of 24 hours. DNA concentration less than 250µgmL^{-1} brings about formation of Dendriplexes of particle size less than 400 nanograms. This result shows that as the formulation ratio increases to 60 N:P, the toroid strucures formed (Figure 7) from the complex were noticed to align in chain. The consequence of the high particle sizes and shapes might make the dendriplex not to be able to move across membranes and cells so easily. Perhaps, this might also explain why complexes failed to reach target organs and consequently lead to poor gene transfection.

Various incubation or binding time has been postulated by several authors ranging from 5 to 30 minutes.[16, 17,18, 19] The rate of complex synthesis was monitored for 24 hours. It becomes clearer from the result as shown in figure 4 that dendriplex formation requires some time for efficient binding. Our study shows that the DNA-dendrimer binding occurs earlier than 8 hour. We therefore suggested that a time-period of 5 h is needed for efficient dendriplex synthesis.

The order of mixing DNA with dendrimer and the waiting time lag of 30 minutes before mixing and agitation are also very important and crucial for effective dendriplex synthesis and for prevention of complex precipitation/aggregation. We cannot really say that the 3 minutes time lag is peculiar to this DNA as no other commercial DNA samples were compared. The size of the DNA could not be measured because of lack of facilities to do so and the manufacturer could not give the same information either.

It was observed from the TEM imaging of the complexes that the DNA wraps round the polymer since the region of dark staining or intercalation is formed outside the complex structures.

In conclusion, it is evident from this study that particle size of dendriplexes decreases with time and zeta potential also varies with time. Stability was achieved between N:P 2 to 8 and within DNA concentration of not more than 250 µgmL^{-1}. A binding time of 5 hour is hereby suggested for DNA/DAB16 complex formation. Therefore it is posited that stable colloids of consistent particle size and shape are formed if the binding time, mixing process and zeta potentials of dendriplexes are skilfully controlled and monitored in order to avoid complex agglomeration. It is hoped that if the established formulation ratio (2 to 8 N:P) is utilized for in-vivo trial, we might likely achieve a better in-vivo gene transfection.

RESULTS

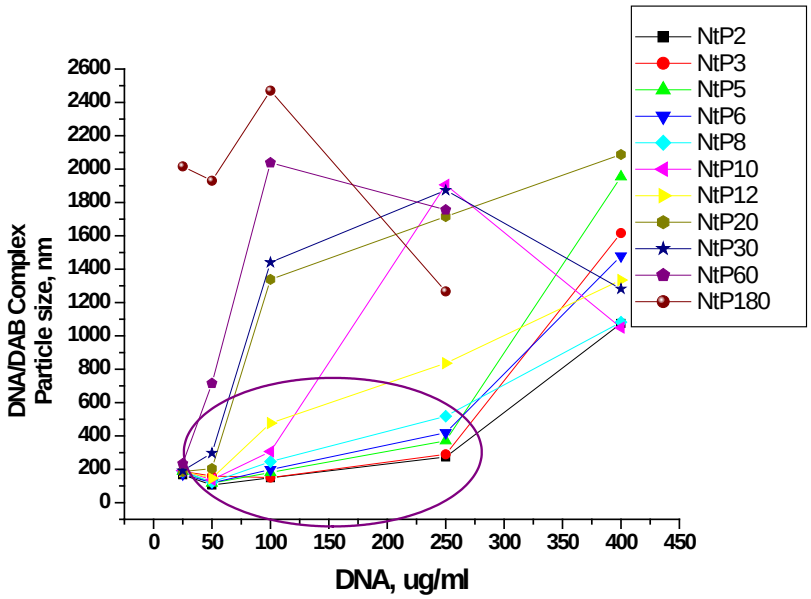


Fig. 1a: Effect of DNA concentration on dendriplex particle size after an incubation time of 1 h.

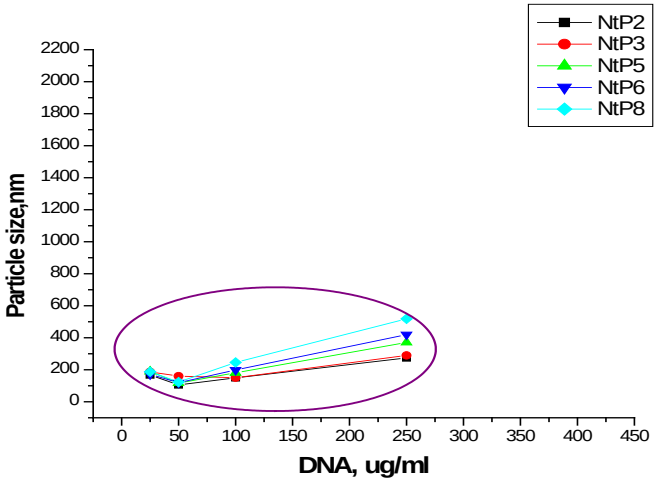


Fig. 1b: **Dendriplex colloid dormain**

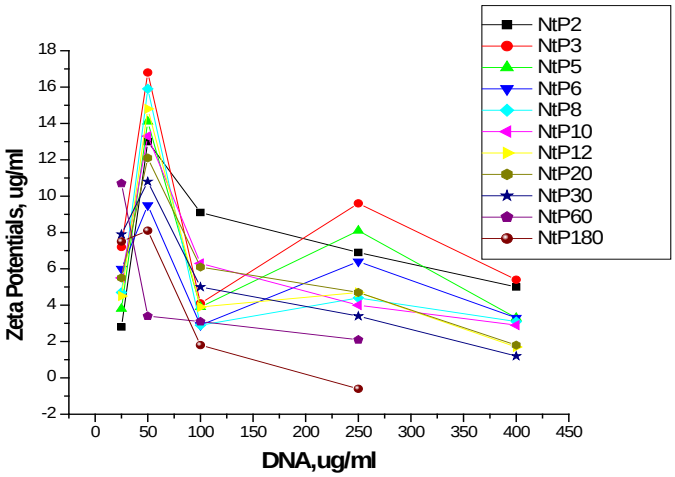


Fig. 2a: Effect of DNA concentrations on dendriplex zeta potentials after a 1 h incubation time

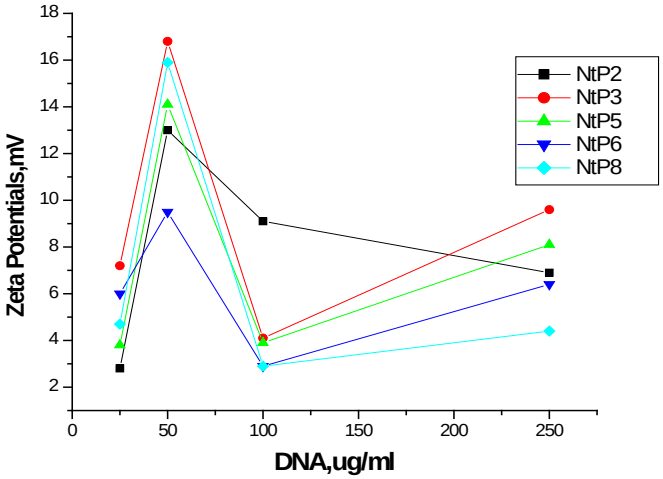
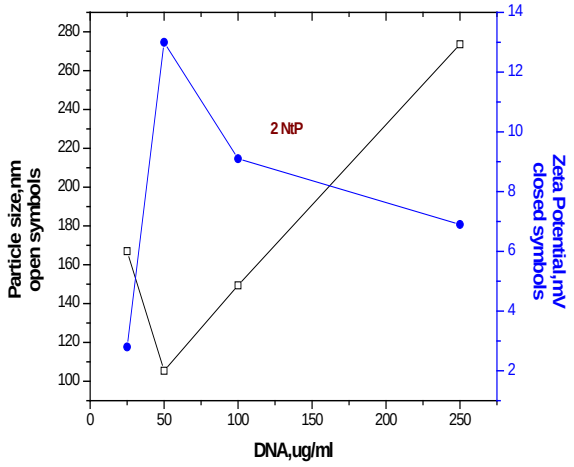
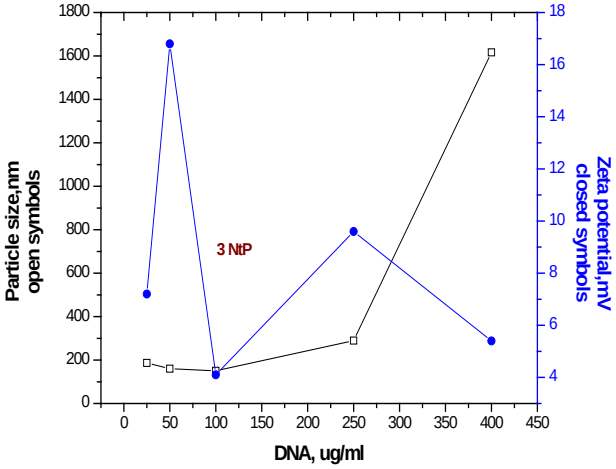


Fig. b: Dendriplex zeta potential at low N:P ratios

N:P ratio = 2 N:P ratio = 3

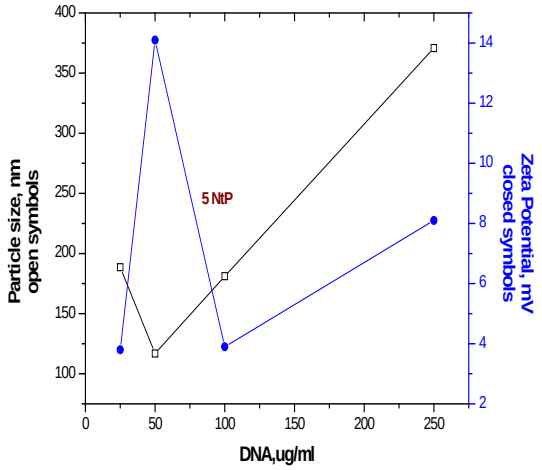


a

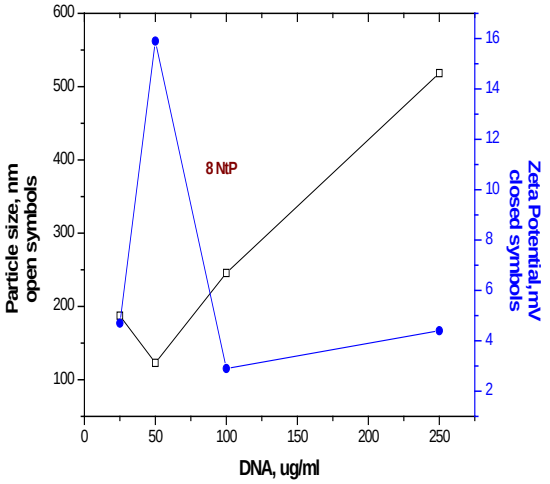


b

N:P ratio = 6 N:P ratio = 8

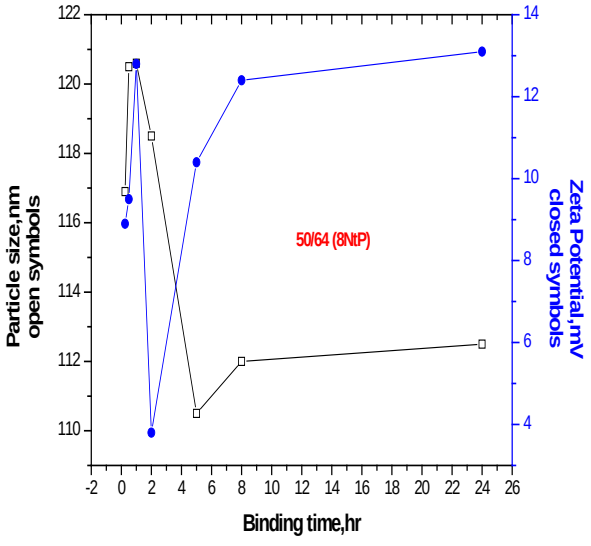


c

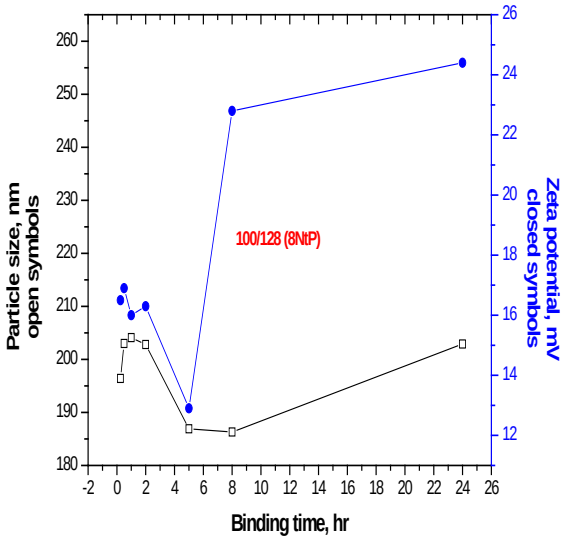


d

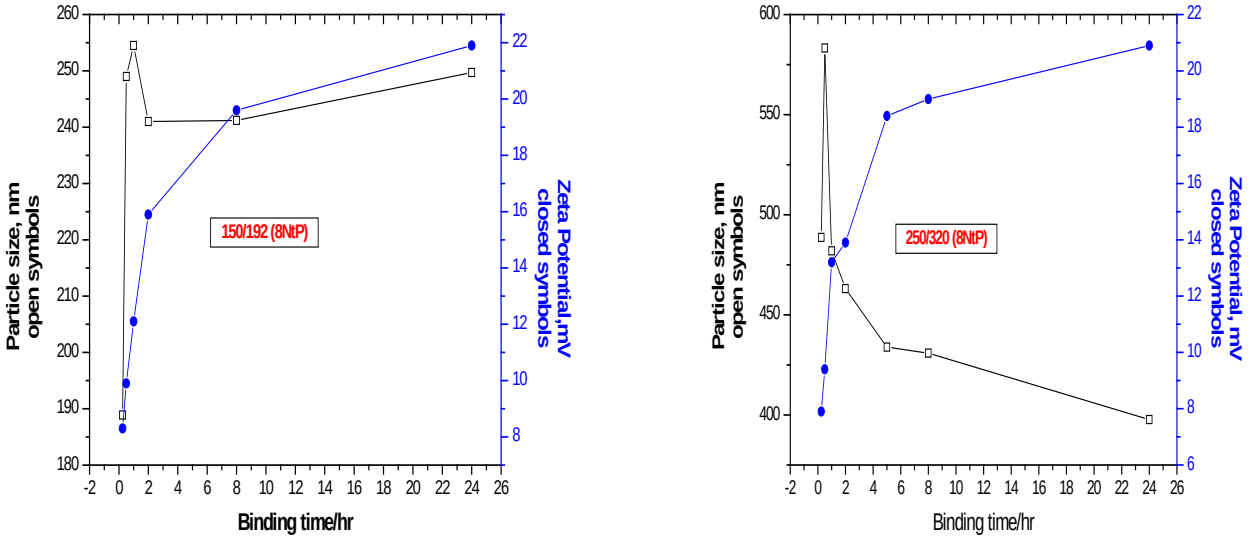
Fig. 3a-d: The effect of N:P ratio on particle size and zeta potential



A



B



C **D**

Fig. 4: Effect of Binding time on the particle size and zeta potential of DNA/DAB complexes A 50/64; B 100/128; C 150/192 and D 250/320 using samples prepared at N:P ratio of 8.

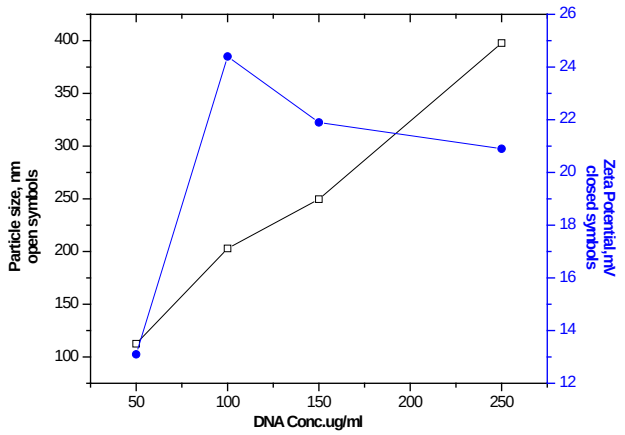
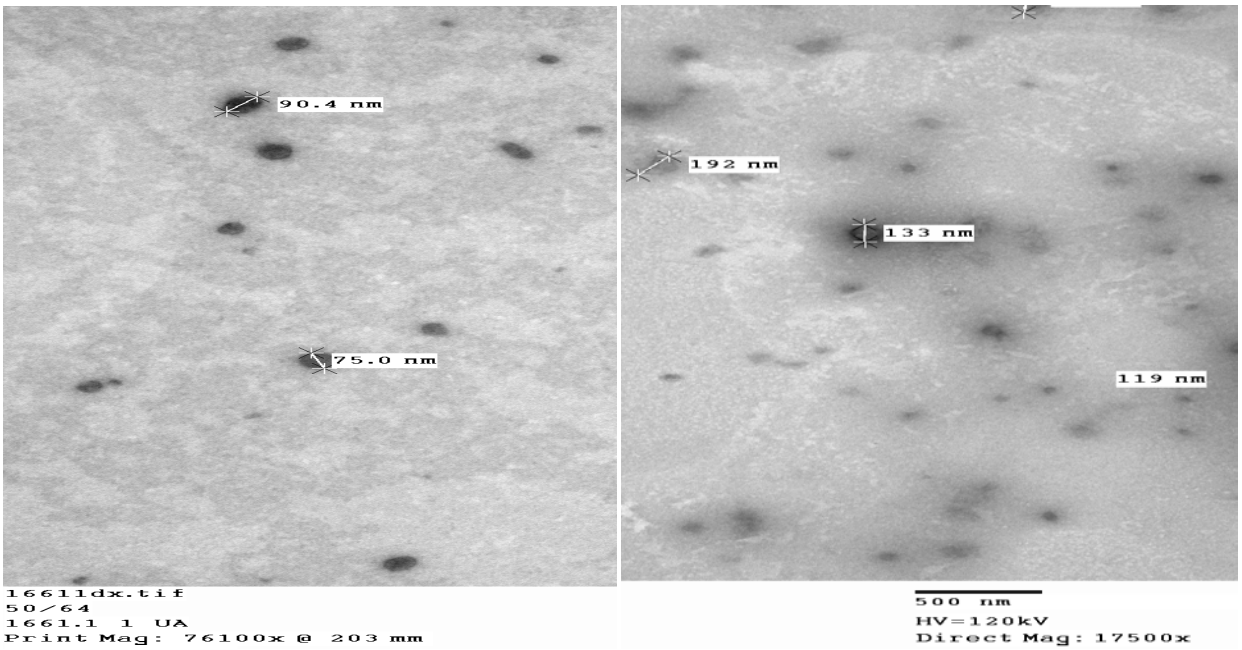
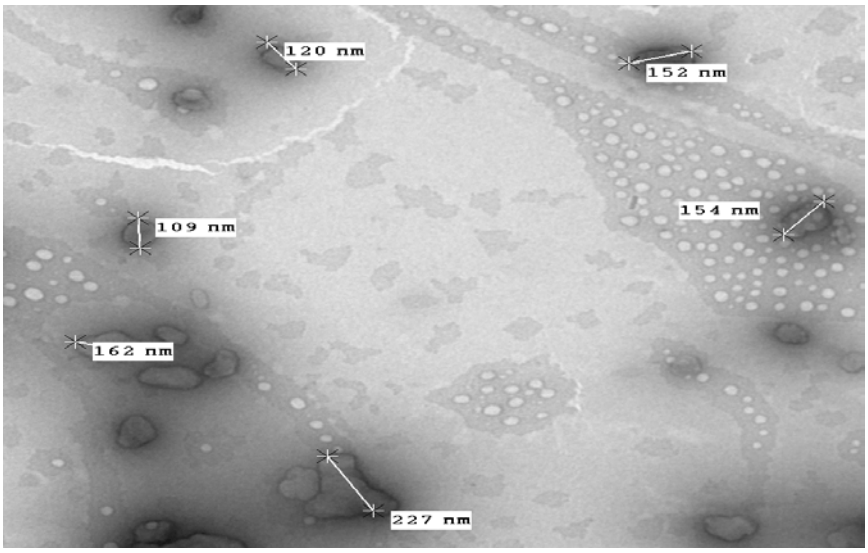


Fig. 5: Effect of increase in DNA concentrations on particle size and zeta potential of dendriplex formed at 24 h time point.



A **B**



C

Fig. 6: Negative stained Transmission Electron Micrograph of DNA/DAB16 formulations A 50/64; B 100/128; C 250/320 at N:P ratio 8. Samples were imaged 5h after preparation and presented as clear liquid.

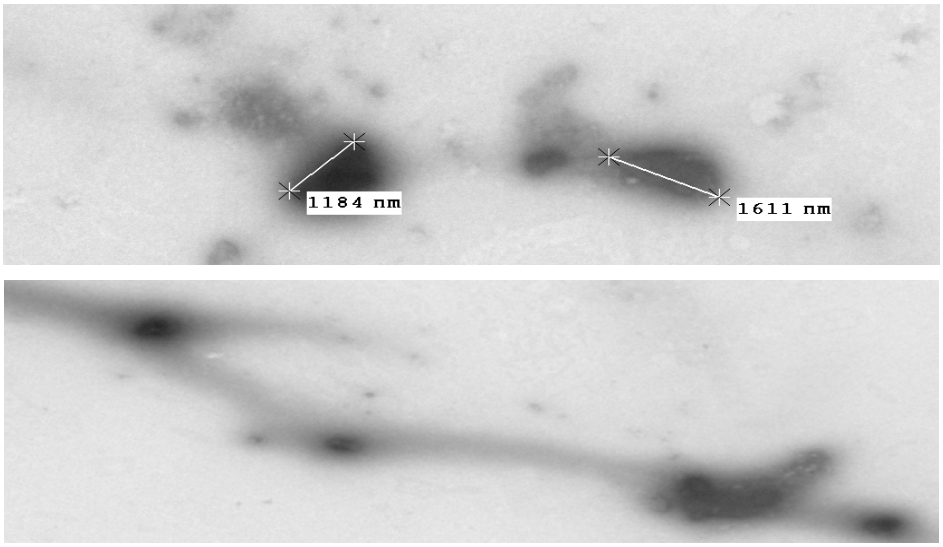


Fig. 7: Negative stained Transmission Electron Micrograph of a DNA/DAB16 formulation (DNA concentration = 250µg/ml, DAB16 concentration = 2400µg/ml, N:P ratio = 60). Sample was imaged 5h after preparation and presented as a slightly cloudy solution.

A B C D

Fig. 8: Photographs of dendriplex dispersions DNA/DAB 16 concentration: A 250/320; B 250/7200; C 1000/320; D 1000/320 at N:P ratio 2, 180, 2 and 2 respectively. Formulations were imaged 24 h after preparation. DAB 16 was added to DNA solution in sample A, B & D while DNA was added to DAB 16 in sample C. A precipitate is clearly visualized in the dispersion C & D.

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