AFM and TEM Study of Biocomplexes between Cationic Polymers and DNA

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INTRODUCTION
The ability to characterize gene delivery systems is of immense importance both scientifically and pharmaceutically. Various vectors have been investigated to introduce DNA into cell’s nucleus, of which non-viral vector polymer-based systems form one of the most important branches [2, 9]. Biocomplexes of cationic polymers associate with DNA through electrostatic interaction and are the most widely studied for both in-vitro and in-vivo transfection [3]. The cationic nature of the complexes facilitates the cellular uptake of condensed DNA via electrostatic adsorption onto anionic cell membrane surfaces [5, 8]. Moreover, the binding of cationic compounds to DNA reduces the repulsion between the double-helical segments and produces DNA-collapse in vitro [4].

Figure 1 is a schematic showing the principle behind DNA condensation with cationic polymers. It is important to condense plasmid DNA to as small a size as possible to facilitate gene transfer, and indeed the size of the condensed DNA complex may be one of the most important factors for successful in vivo gene delivery. A polymeric gene delivery complex of polymer-DNA can be classed as a large polyelectrolyte, which interacts electrostatically and lead to neutralize the charges. At low amounts of cationic polymer the complexes have a net negative charge due to the high number of negative phosphate bases on the DNA. Increasing the amount of polymer counteracts the net negative charge of the complex and the cationic polymer will distribute along the DNA backbone until a critical composition of polyelectrolyecomplex is reached. At this point, disproportionation occurs and leads to the formation of many populations of complexes.

In this article we describe our investigation of the complexes formed between the polymer dimethylaminoethylmethacrylate (DMAEMA) and pBR322 plasmid DNA. AFM and TEM imaging of the morphology of the resultant condensates was conducted under physiological pH and the biocomplexes were classed according to their structural appearance on the substrate surface.

MATERIALS AND METHODS
Specimen Preparation
pBR322 plasmid DNA (Sigma-Aldrich, Poole, Dorset, UK) was used. pBR322 is a 4365 base pair (bp) DNA oligonucleotide that was chosen because it is a well characterized plasmid in the literature, often used as the starting material in the construction of new cloning vectors and is readily available in a pure form.

The polymer was synthesized and obtained from the laboratories of Prof. S. Armes, School of Chemistry, Physics and Environment Science, University of Sussex, Brighton.

DMAEMA polymer stock solutions of 1 mg ml⁻¹ were prepared. The polymer was supplied as freeze-dried solid, and prior to use was dissolved in water to give solution of a chosen concentration. DMAEMA polymer was prepared to a concentration that allowed polymer and DNA solutions to be combined in equal volume. The solutions of polymer and DNA were combined to produce biocomplexes with a predetermined ratio of ionisable polymer nitrogen ions to DNA phosphate ions. All polymer-DNA complexes were routinely prepared by the addition of a single aliquot of polymer solution to buffered DNA solution. Biocomplexes have been investigated over a range of ratios, 0:5:1, 1:1 and 5:1, in a physiological pH environment.

Figure 1: A schematic showing the principle behind DNA condensation with cationic polymers. Adapted from Uchechukwu, I. and A. Gachatztein, Genesics Manufacturers Should Exploit Drug Delivery Technologies for Improved Therapeutics, in Business Briefing: Pharmatech. p. 203-208. 2003.
Conducted in tapping mode, at 512 x 512 pixel resolution, 100 µm in length, were selected for Digital Instruments (Bruker Nano, Santa Barbara, CA) to prepare by addition of equal volumes, (20-30 µl), of polymer solution to DNA solution. After 10 minutes incubation, 40 µl of the resulting solution was spotted as a single aliquot onto a 1 cm² freshly cleaved mica disc. Imaging was conducted under 10% PBS.

A Dimension 3000 AFM manufactured by Digital Instruments (Bruker Nano, Santa Barbara, CA) proved particularly useful for the liquid imaging of DNA condensates. A G type scanner used for the D3000 that has a lateral scanner used for the D3000 that has a lateral size of about 90 µm and a Z-scale of about 10 µm. Thin-arm, silicon nitride, oxide-sharpened, triangular cantilevers were selected, operating at resonant frequencies of approximately 8 kHz. Generally, short armed cantilevers, 100 µm in length, were selected for their low force constants, 0.06-0.6 N m⁻¹, because of the delicate nature of the sample.

All imaging was conducted on a PicoScan AFM and carried out in a liquid environment, using single lever cantilevers. These were 220-230 nm in length with an average spring constant of 1.2-5.5 N m⁻¹. All imaging was conducted in tapping mode, at 512 x 512 pixel resolutions, at a scan speed of approximately 2-6 Hz and at ambient conditions. To reduce sample distortion through compression due to the tapping AFM probe, data were acquired at a set-point chosen to minimise tip-sample interaction.

The post-imaging analysis was carried out using NanoScope Illa software, version 4.2.3/3. Background slope was removed using a first or second order polynomial function and some images were subjected to a median filter.

Transmission Electron Microscopy

A 10 µl drop of polymer-DNA complexes containing suspension was placed onto a copper grid coated with polyvinylbutyral (Piolofor) resin, (TAAB Laboratory Equipment, Reading, UK) and left for 1 minute, followed by removal of excess liquid by blotting with filter paper. This process was repeated, and after air-drying, the grid was inverted onto a drop of a saturated solution of uranyl acetate in 50 % alcohol. This was covered and left for 5-15 minutes to stain, before washing with 50 % alcohol and two stages of deionised water, blotting between each wash.

Grids of polymer-DNA complex samples were analysed using a JEOL JEM-1010 TEM (Jeol (UK) Ltd, Welwyn Garden City), operating at a voltage of 80 kV. Micrographs were taken at magnifications ranging between 60,000 and 200,000× with a Kodak Megaplus digital camera 1.6i, utilising the AnalySIS 3.0 software package (ensemble supplied by Soft Imaging System GMBH, Münster, Germany). This software was also used to take measurements of condensate size.

RESULTS AND DISCUSSION

When polymer-DNA biocomplexes formed, different structural morphologies were observed. The results revealed a number of recognizable classes of condensate structures among the AFM and TEM experiments. In order to interpret the data, these structures were divided into seven categories according to their structural appearance on the substrate surface: open circular, toroidal, plectonemic, rod-like or linear, flower-like, globular, and aggregate structures. Examples of the AFM and TEM images of each of these are shown in Figure 2.

Previously open circular structures have been interpreted as molecules, which were forced into a tangle ring by DNA self-repulsion [7]. Toroidally supercoiled structures were considered to be intermediates between open circular and plectonemic structures [12]. It is worth mentioning that there is a similarity to some extent between relaxed molecules throughout the experimental work and non-supercoiled strands of DNA itself, particularly for the low ratio DMAEMA:DNA biocomplexes. It is considered that this is not likely to be a significant problem in the interpretation of our results, due to the absence of free DNA.

Plasmid DNA morphology was found to be in predominantly supercoiled form, which agrees with the general belief that plasmid DNA adopts this superstructure under physiological conditions. Rod-like structures were interpreted as highly supercoiled forms. These we believe arise from plectonemic structures of polyplexes formed by further winding into more tightly coiled structures, which then curl up into dense, linear structures.

The flower-like condensate structures were observed at a few ratios of polymer to DNA. The current work agrees with the model suggested by Montigny that the flower-like structures are thought to result from the binding of cations with the DNA molecules leading to the DNA looping and subsequent loop condensation. The resultant complex with a single cation core and multiple free
cationic molecules were present in an excess of DNA in terms of charge.

**CONCLUSIONS**

The polymer-DNA biocomplexes appeared in different structures, which have been classified into seven categories to facilitate characterization and interpretation of the resulting data according to their structural appearance. These classifications are believed to represent various intermediate condensate structures, for instance open circular, toroids, plectonemic, rod-like, flower-like structure, and also represent end-like condensate structures such as globular structures. Moreover, the condensate structures had no direct relation between the technique used or sample preparation.

**REFERENCES**