

Article

Camptothecin-Bearing PEGylated Polypropylenimine Dendriplexes for Prostate Cancer Gene Therapy: Impact of Microfluidic Processing on Physicochemical Properties and Transfection

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Abstract

Background/Objectives: Prostate cancer is the most commonly diagnosed cancer in men and a leading cause of cancer-related mortality, highlighting the need for delivery systems capable of efficiently transporting both chemotherapeutic drugs and therapeutic genes to tumor cells. Generation-3 diaminobutyric polypropylenimine (DAB) dendrimers display low toxicity, high drug loading capacity and efficient gene delivery, and can be engineered as camptothecin-bearing PEGylated carriers complexed with plasmid DNA. The aim of this study was to compare microfluidic processing with conventional hand mixing for the preparation of camptothecin-bearing PEGylated DAB dendriplexes and to evaluate the impact of formulation methods and microfluidic parameters on their physicochemical properties, cellular uptake and gene expression in prostate cancer cells. **Methods:** Camptothecin-bearing PEGylated DAB dendrimers were synthesized and complexed with plasmid DNA to form dendriplexes. Formulations were prepared either by microfluidics, using different total flow rates and aqueous: organic flow rate ratios, or by conventional hand mixing. The resulting dendriplexes were characterized for DNA condensation, particle size, polydispersity index and zeta potential. Morphology was assessed by transmission electron microscopy. Cellular uptake of fluorescein-labelled DNA and β -galactosidase reporter gene expression were evaluated in PC3-Luc and DU145 prostate cancer cells. **Results:** Both microfluidic and hand-mixed methods produced stable, nanosized, positively charged dendriplexes with efficient and sustained DNA condensation (more than 99% over 24 h). Microfluidic processing, particularly at an aqueous: organic flow rate ratio of 3:1, yielded dendriplexes with hydrodynamic diameters and zeta potentials comparable to or slightly improved over hand-mixed formulations. These microfluidic conditions significantly enhanced cellular uptake in both PC3-Luc and DU145 cells. In PC3-Luc

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cells, this translated into β -galactosidase expression levels comparable to hand-mixed dendriplexes and higher than naked DNA, whereas in DU145 cells, transfection efficiencies remained modest for all formulations despite increased uptake. **Conclusions:** Microfluidic processing enables the reproducible and scalable preparation of camptothecin-bearing PEGylated DAB dendriplexes with tunable physicochemical properties. Under selected conditions, in vitro cellular uptake and gene expression were comparable to conventional hand mixing, supporting microfluidics as a robust alternative platform for the manufacture of dendrimer-based systems for combined chemo-gene delivery in prostate cancer.

Keywords: dendrimer; dendriplex; camptothecin; microfluidics; prostate cancer; cellular uptake; gene expression

1. Introduction

Prostate cancer is one of the most frequently diagnosed malignancies in men and a leading cause of cancer-related mortality worldwide, with more than 1.4 million new cases and 375,000 deaths estimated in 2020 alone [1,2]. The disease is highly heterogeneous, ranging from indolent tumors that may never become clinically significant to aggressive forms that progress rapidly and metastasize despite similar clinicopathological features at diagnosis [3]. Conventional management strategies such as radical prostatectomy, radiotherapy, androgen-deprivation therapy and newer systemic agents have significantly improved patient outcomes but are often associated with substantial toxicity and the eventual development of treatment resistance. This has driven the search for novel, more selective therapeutic approaches that can eradicate cancer cells while sparing healthy tissues.

Gene therapy has emerged as a promising strategy for the treatment of cancer, offering the possibility to deliver therapeutic nucleic acids that can restore, silence or modulate specific molecular pathways involved in tumorigenesis [4]. Over the past three decades, gene therapy has progressed from proof-of-concept studies to clinical reality, with more than 3900 clinical trials conducted worldwide and an increasing number of approved products for a variety of indications [5]. In oncology, both viral and non-viral vectors have been explored extensively. Although viral vectors possess high transduction efficiency, safety concerns, immunogenicity, limited packaging capacity and manufacturing complexity continue to restrict their widespread use. In contrast, non-viral vectors such as cationic polymers, lipids and dendrimers offer more favorable safety profiles, are easier to manufacture and can be engineered to provide tunable physicochemical and biological properties [6–8].

Dendrimers are highly branched, monodisperse macromolecules with a well-defined, tree-like architecture, internal cavities and a large number of surface functional groups that can be tailored for drug and gene delivery. Their unique structure allows efficient complexation of nucleic acids and loading of hydrophobic drugs, while surface modification enables targeting and modulation of toxicity [9,10]. Among the different families of dendrimers, poly(propylenimine) (DAB) dendrimers have attracted particular interest as non-viral gene delivery systems due to their high buffering capacity, strong interaction with nucleic acids and ability to promote endosomal escape. Targeted poly(propylenimine) dendrimers functionalized with ligands such as transferrin or peptides have shown efficient gene delivery in vitro and in vivo, including to the brain and to tumors, highlighting their potential as versatile platforms for nucleic acid delivery [11–13].

Camptothecin is a potent topoisomerase I inhibitor with broad antitumor activity, but its clinical use is hampered by poor solubility, chemical instability of the active lactone ring and dose-limiting toxicity. To overcome these limitations, several dendrimer–camptothecin conjugates have been developed to improve aqueous solubility, enhance tumor accumulation and enable controlled release of the drug [14–16]. In particular, camptothecin-based dendritic systems have been engineered to self-assemble into dendrimersomes capable of co-delivering both chemotherapeutic agents and nucleic acids, thereby enabling combination therapy within a single nanocarrier [16,17]. In the present work, camptothecin is therefore incorporated as an integral component of an established chemo–gene delivery scaffold (DAB–CPT), providing both a cytotoxic payload and a hydrophobic domain that supports nanostructure self-assembly and colloidal stability. Importantly, this study focuses on how microfluidic processing impacts dendriplex formation and gene delivery readouts. Anticancer activity of camptothecin is therefore not assessed here and has been reported previously for related camptothecin-bearing DAB systems [16]. Such systems are especially attractive for prostate cancer, where simultaneous modulation of oncogenic pathways and delivery of cytotoxic agents may help to overcome resistance mechanisms and improve therapeutic efficacy.

Despite these advances, the clinical translation of dendrimer-based gene delivery systems is still limited by challenges associated with their formulation. Conventional bulk mixing methods used to prepare dendriplexes and dendrimersomes often yield particles with broad size distributions and batch-to-batch variability, as the self-assembly process is highly sensitive to parameters such as mixing time, order of addition, concentration, ionic strength and temperature [18,19]. This variability can in turn affect colloidal stability, cellular uptake, biodistribution and transfection efficiency, making it difficult to achieve the level of quality control required for clinical applications.

Microfluidic technologies have emerged as powerful tools to address these limitations by enabling precise control of fluid flow, rapid and reproducible mixing and scalable fabrication of nanocarriers [19–22]. Microfluidic platforms have been successfully applied to the manufacture of a wide range of nano-drug delivery systems, including lipid nanoparticles, polymeric nanoparticles and nucleic acid polyplexes, providing improved control over particle size, polydispersity and encapsulation efficiency compared with conventional bulk methods [18,23]. In the context of gene delivery, microfluidic preparation of polymer–nucleic acid nanocomplexes has been shown to produce smaller, more homogeneous and more stable particles, resulting in enhanced transfection efficiency and reduced variability [18,19,24].

While NanoAssemblr™ microfluidics has been widely adopted for the controlled manufacture of lipid and polymer nanocarriers, fewer studies have systematically mapped how microfluidic operating parameters translate into physicochemical and functional performance for dendrimer–DNA complexes, particularly for multifunctional chemo–gene delivery scaffolds. Here, we address this gap by performing a parameter-controlled comparison of microfluidic processing versus conventional hand mixing for camptothecin-bearing PEGylated DAB dendriplexes, interrogating how total flow rate and aqueous flow-rate ratio govern nanoparticle attributes (DNA condensation kinetics, size/PDI, zeta potential and morphology) and *in vitro* delivery outcomes (quantitative uptake and reporter gene expression) in prostate cancer cell models. This positioning emphasizes the novelty of the present work as a robust and scalable formulation/manufacturing study for a dual-function dendrimer platform.

Building on previous work describing camptothecin-based DAB dendrimers capable of forming dendrimersomes with combined gene and drug delivery functions [16], the aim of this study was to investigate whether microfluidic processing can reproducibly formulate DAB–CPT-based dendriplexes for prostate cancer gene delivery and how key

processing parameters (total flow rate and aqueous: organic flow-rate ratio) influence physicochemical properties and in vitro performance. Microfluidic and bulk preparation methods were compared in terms of physicochemical characteristics, colloidal stability and in vitro gene delivery performance in prostate cancer cell lines, to identify formulation conditions that maximize transfection performance while reducing batch-to-batch variability.

2. Materials and Methods

2.1. Cell Lines and Reagents

Generation-3 diaminobutyric polypropylenimine (DAB) dendrimer was obtained from SyMO-Chem (Eindhoven, The Netherlands). Orthopyridyl disulfide glycol succinimidyl carboxymethyl ester (OPSS-PEG-SCM with PEG, 3500 Da) was purchased from JenKem Technology (Plano, TX, USA). Camptothecin (CPT), 3-tritylsulfanylpropionic acid, 4-dimethylaminopyridine (DMAP), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 2-nitrophenyl- β -D-galactopyranoside (ONPG), anhydrous dichloromethane, triethylsilane, trifluoroacetic acid, diethyl ether, the deuterated solvents DMSO- d_6 , $CDCl_3$ and D_2O , and all other analytical-grade solvents/reagents not specified below were sourced from Sigma-Aldrich (Poole, UK). Passive lysis buffer was purchased from Promega (Southampton, UK). Tris-acetate-EDTA (TAE) (10 $\times$) and Tris-EDTA (TE) buffers were supplied by Fisher Scientific (Loughborough, UK).

The β -galactosidase reporter plasmid (pCMVsport β -galactosidase) was purchased from Invitrogen (Paisley, UK) and purified using an endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany).

Bioware[®] PC-3M-luc-C6 (PC3-Luc) androgen-insensitive human prostate adenocarcinoma cells expressing firefly luciferase were purchased from Caliper Life Sciences (Hopkinton, MA, USA). Androgen-insensitive DU145 and androgen-sensitive LNCaP prostate cancer cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK).

Minimum essential medium (MEM), Quanti-iT[®] PicoGreen[®] dsDNA reagent, fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin-streptomycin, TrypLE[®] Express, and Alexa Fluor[®] 647 were obtained from Life Technologies (Paisley, UK). Vectashield[®] mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories (Peterborough, UK). Label IT[®] Fluorescein Nucleic Acid Labeling Kits were purchased from Cambridge Biosciences (Cambridge, UK).

2.2. Synthesis of Camptothecin-Bearing PEGylated Dendrimer

The synthesis of camptothecin-bearing PEGylated dendrimer was done according to a method previously described by Laskar et al. [16], with some modifications (Figure 1). Briefly, camptothecin (CPT, 40.1 mg, 0.1148 mmol), 3-tritylsulfanylpropionic acid (40.11 mg, 0.1148 mmol), DMAP (24.22 mg, 0.1263 mmol) and EDC (15.5 mg, 0.1263 mmol) were mixed in anhydrous dichloromethane (4 mL) and allowed to react overnight at 25 °C. The next day, triethylsilane (0.5 mL) and trifluoroacetic acid (0.8 mL) were added sequentially, and the mixture was stirred for a further 2 h at 25 °C before being left in the fume hood for an additional 2 h to facilitate solvent removal. The residue was re-dissolved in dichloromethane (0.5 mL) and precipitated by dropwise addition into cold diethyl ether (15 mL). The precipitate was collected, dried under vacuum and the product recovered as a yellow powder (90% yield). A ¹H-NMR spectrum of thiolated camptothecin (1 mg in 500 μ L DMSO- d_6) was acquired on a Bruker Avance[®] III-HD500 spectrometer (Billerica, MA, USA).

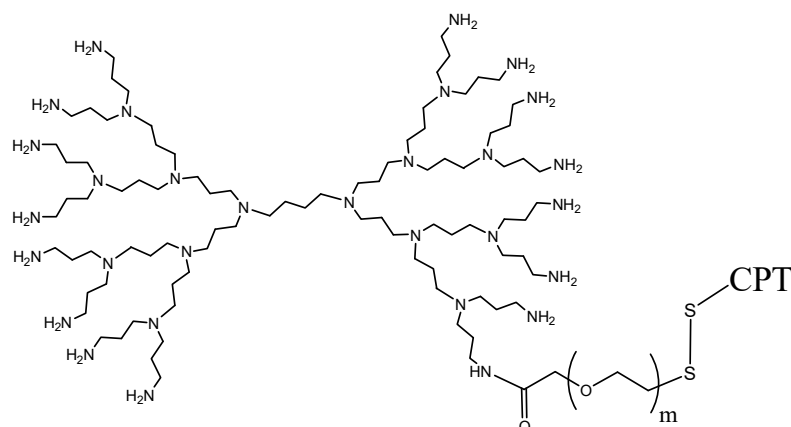


Figure 1. Chemical structure of camptothecin-bearing PEGylated dendrimer ($m = 45$).

Generation 3-diaminobutyric poly(propyleneimine) dendrimer (DAB) (1 equivalent, 60 mg) was dissolved in 1.5 mL of dimethyl sulfoxide (DMSO) and stirred at 25 °C for 10 min. In parallel, OPSS-PEG-SCM (1 equivalent, 81.59 mg) was dissolved in 1.5 mL of DMSO, and then added dropwise to the DAB solution. The reaction mixture was protected from light and stirred for 6 h at 37 °C. After 6 h, a freshly prepared solution of thiolated camptothecin (2 equivalents; 31.03 mg in 1.5 mL DMSO) was added to the PEGylated-DAB and the mixture was stirred for 16 h at 37 °C in the dark.

The product was purified by dialysis (with a molecular weight cut-off of 3500 Da) to remove unreacted species, first against 500 mL DMSO for two days, then against 500 mL methanol for 2 days, and finally against 2 L of distilled water for 2 days at 25 °C, with the dialysis medium replaced twice daily. The dialysate was freeze-dried using a Christ Epsilon 2-4 LSC freeze dryer (Osterode am Harz, Germany) to yield a yellow powder, stored at −20 °C until use. The final product was characterized by ^1H -NMR (3.12 mg in 0.5 mL CDCl_3) on a Bruker Avance® III-HD500 NMR spectrometer (Billerica, MA, USA), as described in Laskar et al. [17].

2.3. Preparation of Dendriplexes

Camptothecin-bearing dendrimers were complexed with plasmid DNA to form dendriplexes using either microfluidic processing or conventional hand mixing.

Microfluidic dendriplexes were produced with a NanoAssemblr™ Benchtop microfluidic system (Precision Nanosystems Inc., Vancouver, BC, Canada). Stock solutions of DAB–CPT dendrimer and plasmid DNA were prepared in phosphate-buffered saline (PBS) and adjusted to a dendrimer: DNA weight ratio of 20:1 and a final DNA concentration of 10 $\mu\text{g}/\text{mL}$. The dendrimer (organic phase) and DNA (aqueous phase) solutions were loaded into separate 1–3 mL syringes and connected to the right and left inlets of the cartridge, respectively. Dendriplexes were generated at total flow rates (TFRs) of 4, 12, or 20 mL/min and aqueous-to-organic phase flow rate ratios (FRRs) of 1:1, 3:1, or 1:3. The resulting dendriplex solutions were collected from the outlet channel of the NanoAssemblr™ system for subsequent characterization.

For manual preparation, 0.5 mL of plasmid DNA solution at the desired concentration was combined with 0.5 mL of DAB–CPT dendrimer stock solution and mixed by pipetting. The dendrimer concentration was adjusted as required to obtain a final dendrimer: DNA weight ratio of 20:1 for all hand-mixed formulations.

2.4. DNA Condensation

Dendriplexes prepared by microfluidics or by hand mixing were assessed for DNA complexation using agarose gel electrophoresis. Complexes were formulated at a

dendrimer: DNA weight ratio of 20:1 in 5% (*w/v*) glucose solution, with a fixed final DNA concentration of 10 µg/mL. Aliquots were loaded on a 0.7% (*w/v*) agarose gel prepared in 1X Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (0.4 µg/mL). The running buffer was also 1x TAE containing ethidium bromide (0.4 µg/mL). 1 kb Plus DNA ladder (New England Biolabs, Hitchin, UK) was used as a DNA size marker. Electrophoresis was carried out at 50 V for 1 h and gels were subsequently visualized under UV illumination to assess DNA migration.

DNA condensation was quantified using a PicoGreen® assay according to the manufacturer's instructions. Complexes were prepared at a dendrimer: DNA weight ratio of 20:1 at total flow rates of 4, 12, and 20 mL/min in 5% (*w/v*) glucose solution. On the day of the experiment, PicoGreen® reagent was diluted 200-fold in 5% (*w/v*) glucose solution. Diluted PicoGreen® solution (1 mL) was added to glucose solutions containing the dendrimer/DNA complexes or DNA alone (control), vortexed immediately, and fluorescence was recorded (λ_{exc} : 480 nm, λ_{em} : 520 nm, excitation and emission slits: 5 nm) using a Varian Cary Eclipse® fluorescence spectrophotometer (Palo Alto, CA, USA). Measurements were taken at defined time points, and results were represented as a percentage of DNA condensation.

2.5. Determination of Size and Zeta Potential of Dendriplexes

The hydrodynamic diameter, polydispersity index (PDI) and zeta potential were measured for dendriplexes prepared as described above. Measurements were performed in quadruplicate by photon correlation spectroscopy and laser Doppler electrophoresis using a Malvern Zetasizer Nano-ZS® (Malvern Instruments Ltd., Malvern, UK). All measurements were carried out at 37 °C immediately after complexation.

2.6. Morphology of Dendriplexes

Dendriplex morphology was examined by transmission electron microscopy (TEM). DAB–CPT dendriplexes complexed with plasmid DNA were prepared by microfluidics (TFR 4 or 12 mL/min) or by hand mixing in 5% (*w/v*) glucose solution. Samples were diluted in purified water (1/10) and a 3 µL aliquot of each sample was deposited onto carbon-coated copper grids (400 mesh size). The grids were left to dry overnight in a desiccator at room temperature prior to imaging. Imaging was performed using a JEOL JEM-1200EX® transmission electron microscope (Jeol, Tokyo, Japan) operating at an accelerating voltage of 80 kV.

2.7. Cellular Uptake

The cellular uptake of fluorescein-labelled plasmid DNA complexed with the dendrimer and formulated either by hand mixing or by microfluidics was assessed qualitatively by confocal microscopy and quantitatively by flow cytometry. PC3-Luc and DU145 cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere in minimum essential medium (MEM) supplemented with 10% (*v/v*) fetal bovine serum, 1% (*v/v*) L-glutamine and 0.5% (*v/v*) penicillin–streptomycin. Plasmid DNA was labelled with fluorescein using a Label IT® Nucleic Acid Labelling Kit according to the manufacturer's protocol.

For confocal imaging, cells were seeded onto glass coverslips in 6-well plates at a density of 200,000 cells/well and incubated for 24 h. Dendriplexes were prepared in PBS by microfluidics or hand mixing to give a final DNA dose of 2.5 µg/well, diluted in MEM, and incubated with the cells for 24 h. Cells were then washed three times with 3 mL PBS, fixed with 3 mL of 3.7% (*w/v*) formaldehyde for 15 min at room temperature, washed again three times with 3 mL PBS and permeabilized with 3 mL of 0.1% (*v/v*) Triton X solution for 15 min at 37 °C. The cells were then washed three times with 3 mL PBS, blocked with 3 mL of 1% (*w/v*) bovine serum albumin (BSA) in PBS for 45 min at room temperature,

and incubated with Alexa Fluor® 647 (diluted in PBS) for 1 h at room temperature. Coverslips were mounted on glass slides using Vectashield® mounting medium containing DAPI and kept in the dark for 2 h at room temperature before imaging on a Leica TCS SP5® confocal microscope (Leica Microsystems, Wetzlar, Germany). DAPI (used to stain the nucleus) was excited with a 405 nm laser (emission bandwidth: 415–491 nm), fluorescein-labelled DNA with a 514 nm laser (emission bandwidth: 550–620 nm), and Alexa Fluor® 647 (used to stain the cell membrane) with a 633 nm laser (emission bandwidth: 650–685 nm).

For quantitative analysis of cellular uptake, cells were seeded in 6-well plates at a density of 200,000 cells/well, incubated for 24 h, and treated with dendriplexes under the same conditions as for confocal microscopy. After 24 h, treatments were removed and cells were washed three times with 3 mL PBS. Cells were detached by adding 250 µL trypsin per well and incubating for 5 min at 37 °C, followed by the addition of 500 µL flow cytometry buffer (PBS containing 0.5% BSA and 2 mM EDTA). Fluorescence associated with fluorescein-labelled DNA was measured using an Attune NxT® flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA), acquiring 10,000 gated events per sample. Data were analyzed using a broad forward- and side-scatter (FSC/SSC) gate to define the main cell population, followed by doublet exclusion and quantification of the fluorescein signal. A 488 nm (blue) laser was used for excitation.

2.8. Gene Expression

The gene expression efficiency of plasmid DNA complexed with camptothecin-bearing DAB dendrimer and formulated either by microfluidics or hand mixing was assessed in PC3-Luc and DU145 prostate cancer cells using a β -galactosidase reporter plasmid. Dendriplexes were prepared using the optimized microfluidic conditions (total flow rates of 4, 12 and 20 mL/min and aqueous-to-organic phase flow rate ratios of 1:1, 3:1 and 1:3) and the corresponding hand-mixed formulations, maintaining the same dendrimer: DNA ratio and physicochemical characteristics throughout the study.

Cells were seeded in 96-well plates at a density of 2000 cells/well in triplicate and cultured for 72 h at 37 °C in a humidified 5% CO₂ atmosphere prior to treatment. They were then exposed to the dendriplex formulations at a fixed DNA dose of 1 µg/well and incubated for a further 72 h under the same conditions. β -galactosidase activity was determined after cell lysis with 50 µL of 1× Passive Lysis Buffer (PLB) for 20 min at room temperature. An ortho-nitrophenyl- β -D-galactosidase (ONPG) solution (1.33 mg/mL) was prepared in an assay buffer (200 mM sodium phosphate buffer, pH 7.3, containing 2 mM magnesium chloride and 100 mM β -mercaptoethanol) and added to lysates (50 µL/well). Plates were incubated for 2 h at 37 °C, and absorbance was read at 405 nm using a Multiskan Ascent® plate reader (MTX Lab Systems, Bradenton, FL, USA).

2.9. Statistical Analysis

Results were expressed as the mean $\pm$ standard error of the mean (S.E.M.). Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-test. For two-group comparisons, an unpaired t-test was used (version 22.0, Minitab® LLC, State College, PA, USA). Differences were considered statistically significant for *p* values lower than 0.05.

3. Results

3.1. Synthesis of Camptothecin-Bearing PEGylated Dendrimer

The ¹H-NMR results confirmed the synthesis of camptothecin-bearing PEGylated dendrimer as follows: ¹H-NMR (500 MHz, CDCl₃): δ 2.3–2.5 ppm, DAB (NCH₂-CH₂-CH₂-

N); δ 3.5–3.8 ppm, PEG (NH-CO-CH₂-OCH₂CH₂); δ 7.3–7.4 ppm, CPT (CH-CH-N-CO) (Figure 2).

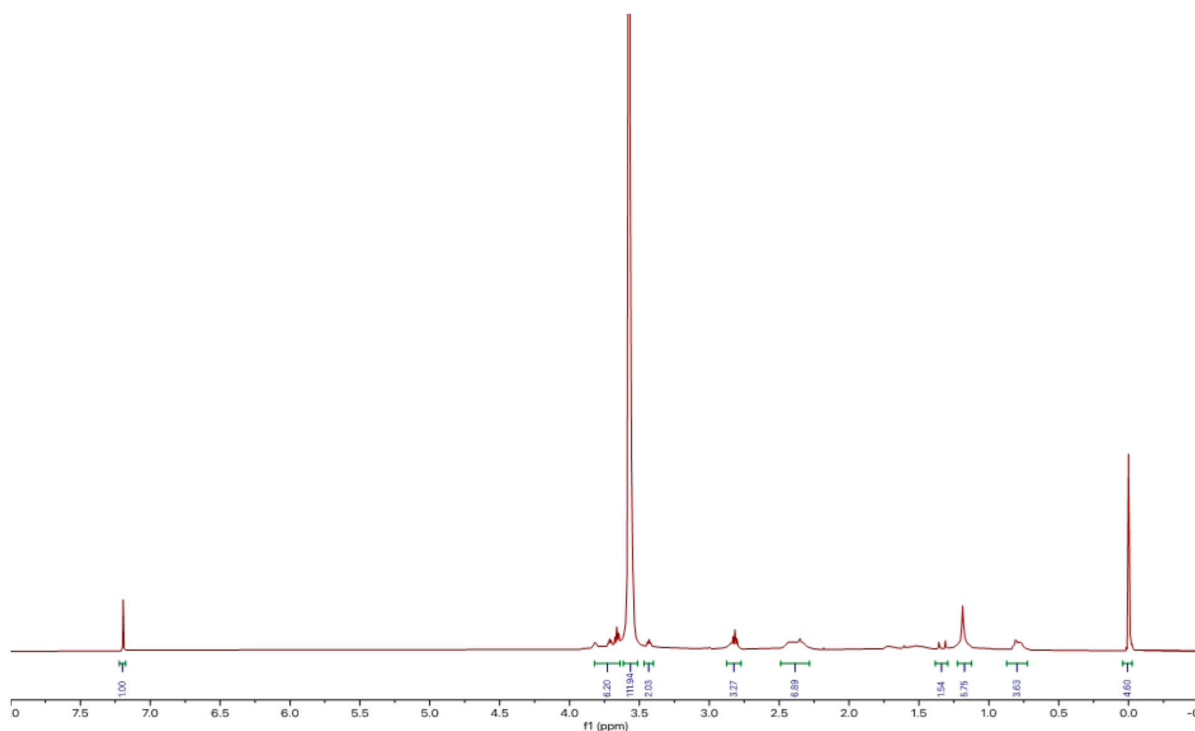


Figure 2. ¹H-NMR spectrum of camptothecin-bearing PEGylated dendrimer (in CDCl₃, 500 MHz).

3.2. DNA Condensation

The ability of camptothecin-bearing DAB dendrimers to complex plasmid DNA was first evaluated by agarose gel electrophoresis (Figure 3). Dendriplexes were prepared either by hand mixing or by microfluidics at different total flow rates and flow-rate ratios. Across all microfluidic conditions tested (TFR 4, 12 and 20 mL/min; FRR 1:1, 3:1 and 1:3), as well as for the hand-mixed formulations, no migration of free plasmid DNA was observed, indicating complete complexation of DNA within the dendriplexes. By contrast, naked DNA (control) showed a distinct band migrating through the gel, confirming the expected retardation effect for dendrimer–DNA complexes. These data demonstrate that DAB–CPT dendrimers efficiently condense DNA independently of the mixing method or microfluidic operating parameters.

DNA condensation was further quantified using the PicoGreen® assay (Figure 4). Because DNA condensation was consistently very high across all conditions, Figure 4 is displayed with an expanded *y*-axis (95–100%) to visualize small differences that would be compressed on a 0–100% scale. Across all total flow rates and FRR values, as well as for hand-mixed dendriplexes, DNA condensation remained very close to 100% throughout the 24 h monitoring period, with only minimal differences between formulations. These findings confirm the strong and sustained DNA-binding capacity of the camptothecin-bearing dendrimer and indicate that microfluidic processing does not compromise, and may subtly enhance, DNA condensation relative to conventional hand mixing.

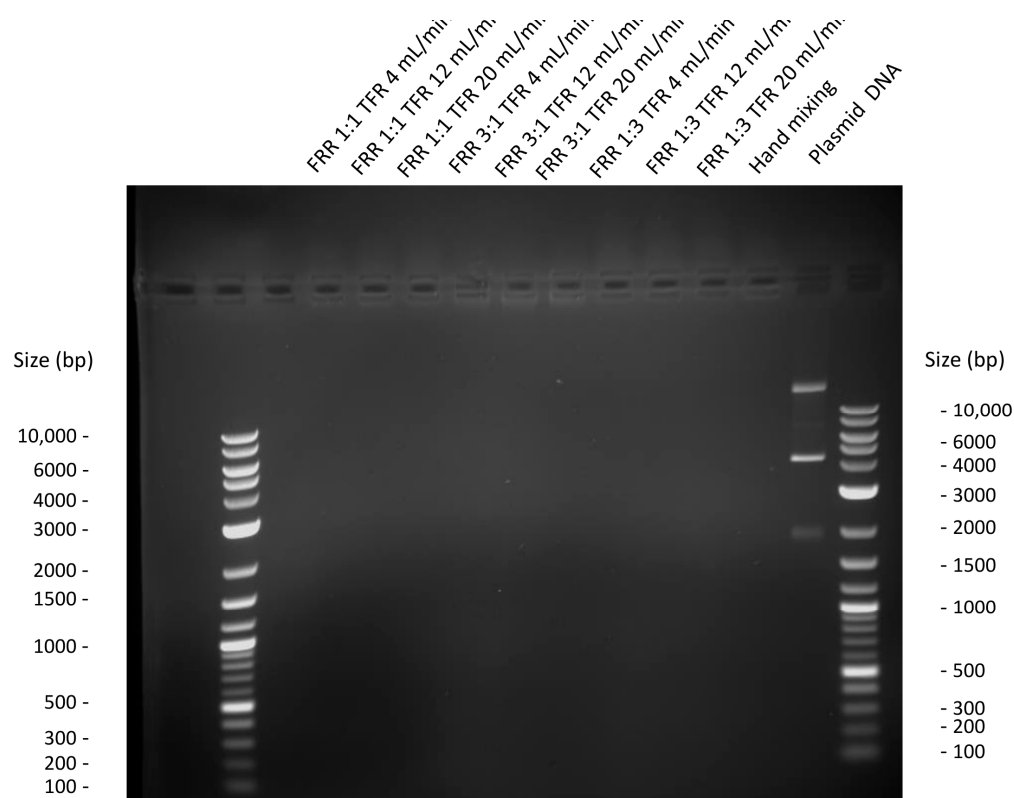


Figure 3. Gel retardation assay of camptothecin-bearing DAB dendrimer (DAB-CPT) dendriplexes prepared by hand mixing and by microfluidics at different total flow rates (4, 12, and 20 mL/min) and aqueous: organic flow rate ratios (1:1, 3:1, and 1:3). Naked plasmid DNA was used as a control.

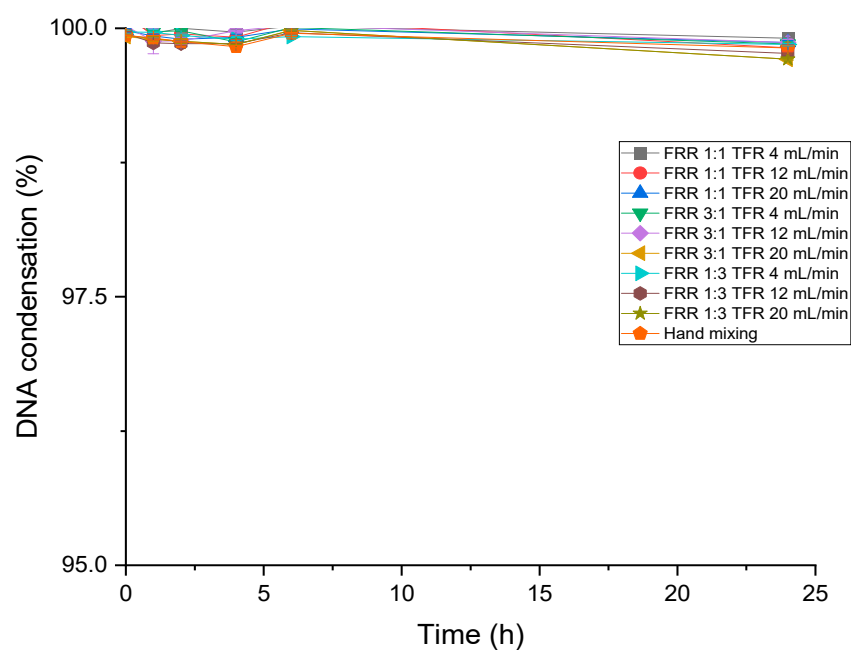


Figure 4. Quantitative assessment of plasmid DNA condensation by camptothecin-bearing DAB dendriplexes prepared by microfluidics at different total flow rates (4, 12 and 20 mL/min) and aqueous: organic flow rate ratios (1:1, 3:1, and 1:3), compared with hand-mixed complexes, as determined by PicoGreen® assay ($n = 3$).

3.3. Determination of Size and Zeta Potential of Dendriplexes

Microfluidic processing had a marked influence on the physicochemical properties of the camptothecin-bearing DAB dendriplexes (Figure 5). Hand-mixed complexes displayed the smallest mean hydrodynamic diameter (80 ± 1 nm), closely followed by dendriplexes generated using the NanoAssemblr™ at an aqueous: organic FRR of 3:1 and TFR of 4 and 12 mL/min (80 ± 2 nm and 95 ± 3 nm, respectively). Most other microfluidic conditions also produced nanosized complexes, whereas formulations prepared at FRR 1:3 and TFR 4, 12, and 20 mL/min gave slightly larger particles (128 ± 2 nm, 119 ± 1 nm, and 131 ± 2 nm, respectively). In all cases (including the hand-mixed dendriplexes), the polydispersity index remained below 0.2, indicating narrow size distributions and good colloidal homogeneity. All dendriplexes carried a positive surface charge: microfluidic formulations exhibited zeta potential values between 11 ± 2 mV and 23 ± 1 mV, while hand-mixed complexes were slightly more positively charged (22 ± 2 mV).

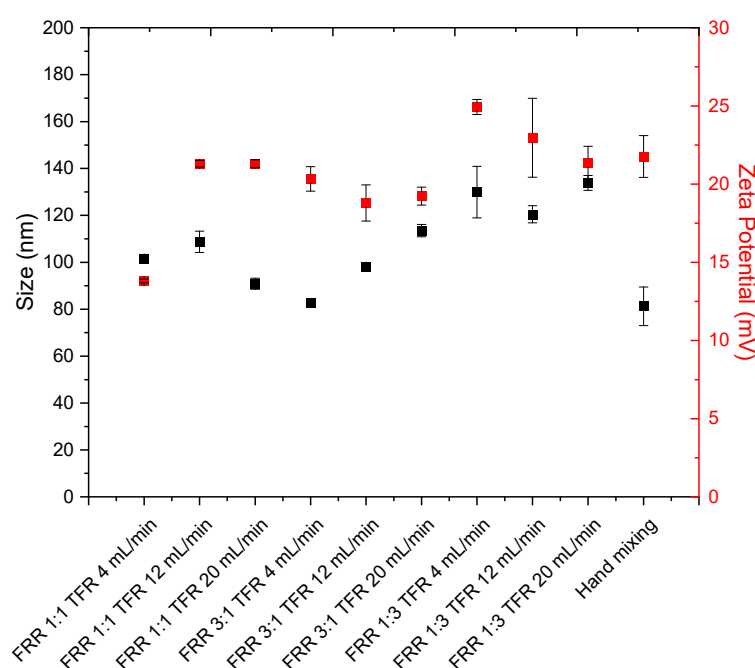


Figure 5. Hydrodynamic diameter and zeta potential of camptothecin-bearing DAB dendriplexes prepared by microfluidics at different total flow rates and aqueous: organic flow rate ratios, compared with hand-mixed complexes, measured immediately after formulation ($n = 3$).

3.4. Morphology of Dendriplexes

Transmission electron microscopy confirmed that camptothecin-bearing PEGylated DAB dendriplexes formed by both hand mixing and microfluidics were predominantly spherical nanoparticles with diameters below 200 nm (Figure 6). Across most conditions, the complexes appeared smooth and uniform, consistent with the narrow size distributions measured by DLS. In contrast, dendriplexes generated using the NanoAssemblr™ at a FRR of 1:1 and a TFR of 12 mL/min displayed higher heterogeneity in size and noticeable deviations from a regular spherical morphology, suggesting that this particular microfluidic condition may promote less controlled self-assembly compared with the other formulations and the hand-mixed dendriplexes.

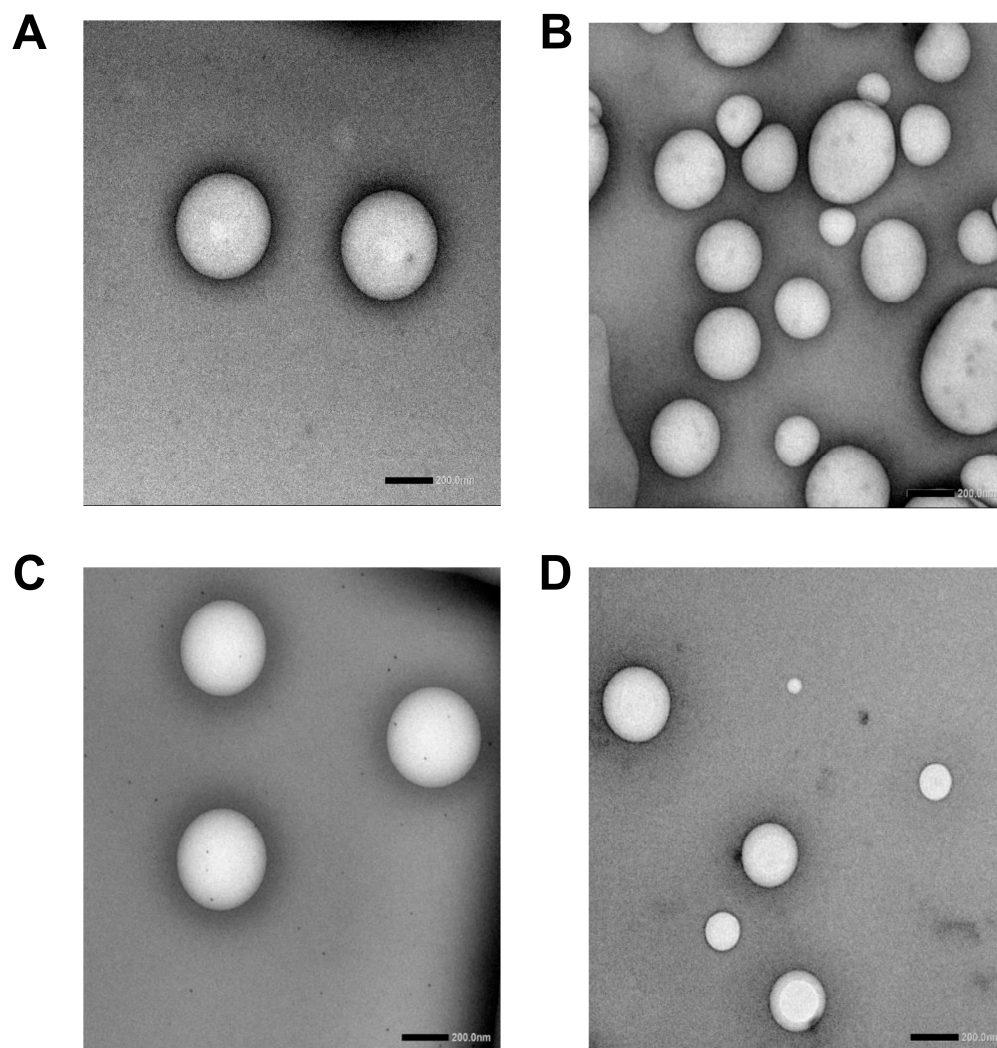


Figure 6. Transmission electron microscopy images of camptothecin-bearing PEGylated DAB dendriplexes prepared by microfluidics at a flow rate ratio of 1:1 and total flow rates of 4 mL/min (A) and 12 mL/min (B), compared with dendriplexes prepared by hand mixing (C) and DAB–DNA complexes prepared by hand mixing (D). Scale bar = 200 nm.

3.5. Cellular Uptake

Confocal microscopy confirmed efficient cellular uptake of fluorescein-labelled plasmid DNA delivered by DAB–CPT dendriplexes in both PC3–Luc (Figure 7) and DU145 (Figure 8) cells. In both cell lines, green fluorescence corresponding to internalized DNA was markedly higher in cells treated with dendriplexes prepared by microfluidics at an FRR of 3:1 and TFR of 4, 12, and 20 mL/min than in cells treated with complexes generated under other microfluidic conditions or in untreated controls. Hand-mixed dendriplexes also produced substantial intracellular fluorescence, although generally lower than that observed for the 3:1 FRR microfluidic formulations. By contrast, dendriplexes prepared at FRR 1:1 showed only moderate fluorescence, while those produced at FRR 1:3 yielded very weak or sparse green signal, indicating limited DNA uptake. In all cases, untreated cells exhibited only background fluorescence. These observations suggest that microfluidic preparation at FRR 3:1 across the tested TFR range enhances the cellular uptake or stability of DAB–CPT dendriplexes compared with other formulation conditions.

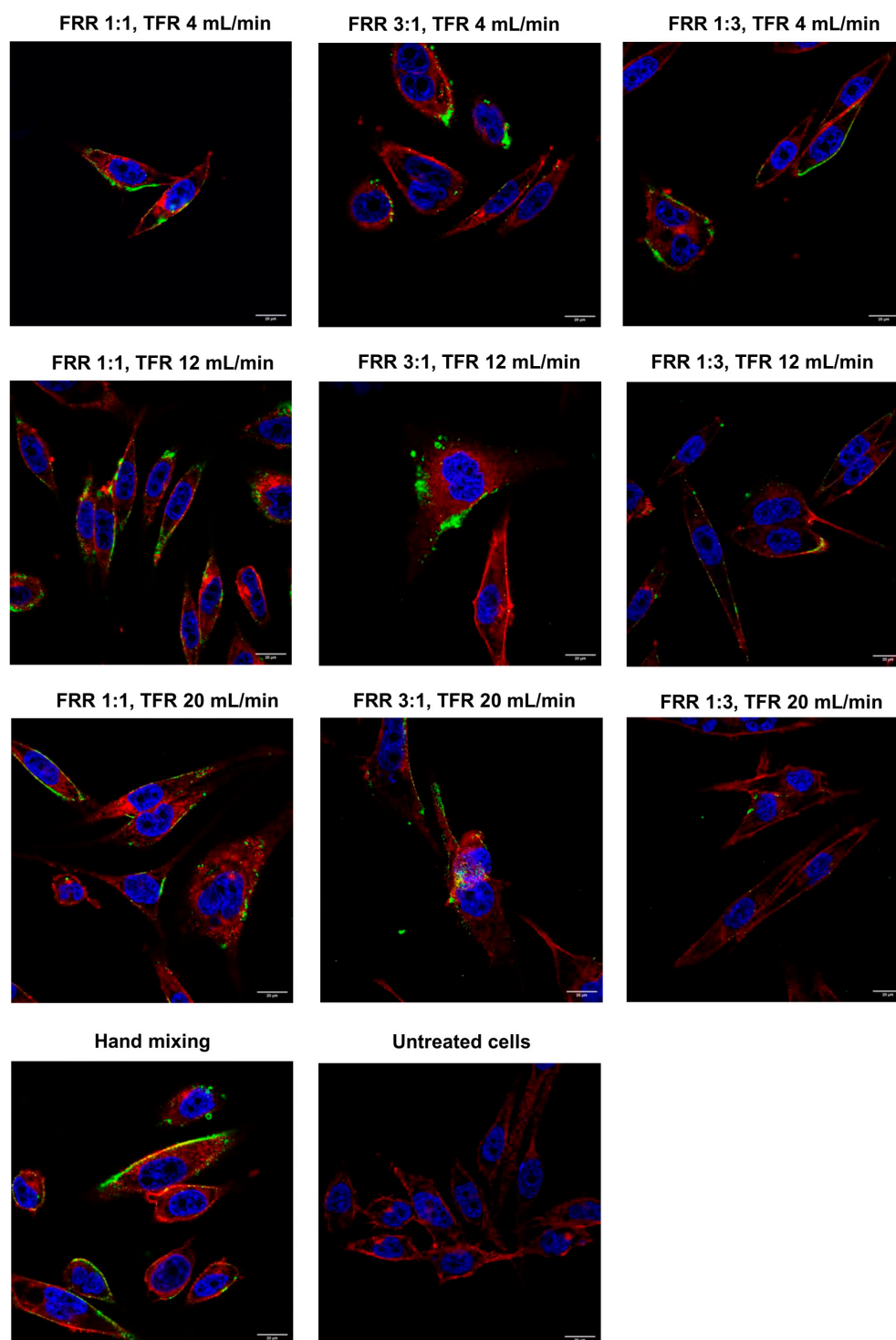


Figure 7. Confocal microscopy images of PC3-Luc cells showing uptake of fluorescein-labelled plasmid DNA (2.5 µg/well) complexed with camptothecin-bearing DAB dendrimer (DAB-CPT) and formulated by hand mixing or microfluidics. Nuclei are stained with DAPI (blue; excitation 405 nm, emission 415–491 nm), fluorescein-labelled DNA is shown in green (excitation 514 nm, emission 550–620 nm), and cell membranes are labelled in red (excitation 633 nm, emission 650–685 nm) (Scale bar: 20 µm).

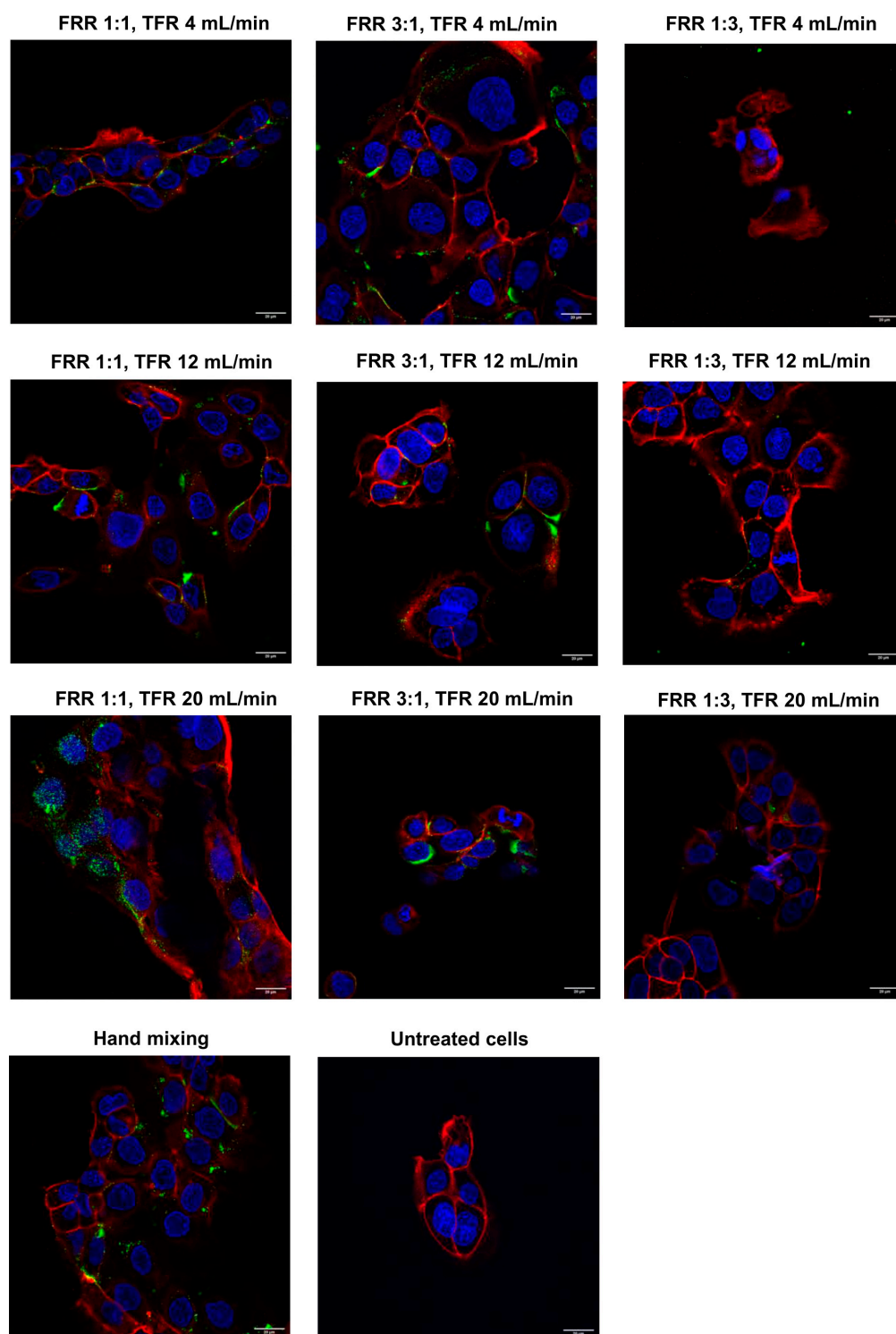


Figure 8. Confocal microscopy images of DU145 cells showing uptake of fluorescein-labelled plasmid DNA (2.5 µg/well) complexed with camptothecin-bearing DAB dendrimer (DAB-CPT) and formulated by hand mixing or microfluidics. Nuclei are stained with DAPI (blue; excitation 405 nm, emission 415–491 nm), fluorescein-labelled DNA is shown in green (excitation 514 nm, emission 550–620 nm), and cell membranes are labelled in red (excitation 633 nm, emission 650–685 nm) (Scale bar: 20 µm).

Cellular uptake of fluorescein-labelled plasmid DNA delivered by DAB-CPT dendriplexes was quantified by flow cytometry in PC3-Luc and DU145 cells (Figure 9). In PC3-Luc cells, dendriplexes prepared by microfluidics at a FRR of 3:1 and TFR of 4, 12,

and 20 mL/min showed the highest uptake, with fluorescence intensities of at least 90 ± 3 a.u., comparable to or slightly higher than complexes formed by hand mixing (85 ± 2 a.u.) (Figure 9A). Formulations produced at FRR 1:1 (TFR 4–20 mL/min) also displayed high uptake (around 78 ± 2 a.u.), whereas dendriplexes generated at FRR 1:3 (TFR 4–20 mL/min) exhibited a marked reduction in cellular uptake (35 ± 4 a.u.) compared with the 3:1 and 1:1 conditions.

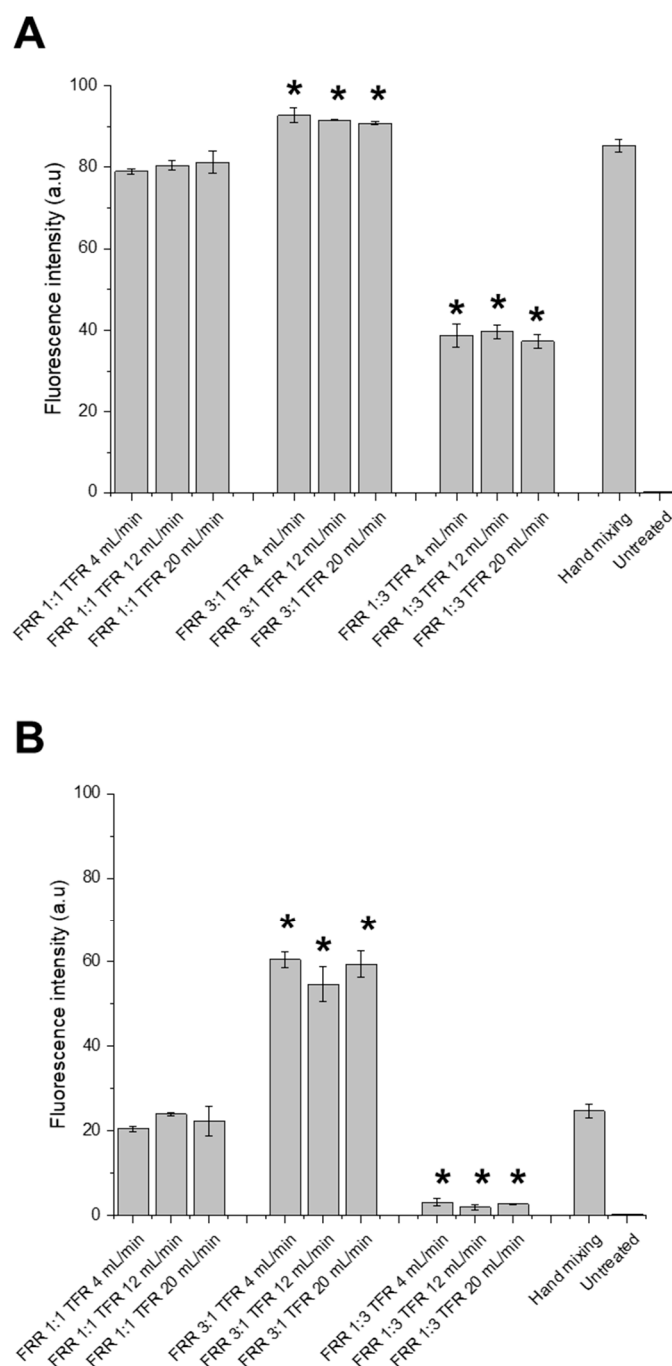


Figure 9. Quantitative analysis of cellular uptake of fluorescein-labelled plasmid DNA delivered by DAB-CPT dendriplexes prepared by hand mixing or microfluidics in (A) PC3-Luc and (B) DU145 cells. Uptake is expressed as fluorescence emission intensity (mean $\pm$ SEM, $n = 3$). * $p \leq 0.05$ versus hand-mixed dendriplexes.

A similar trend was observed in DU145 cells, although overall uptake levels were lower than in PC3-Luc cells. Dendriplexes prepared by microfluidics at FRR 3:1 and TFR

4, 12 and 20 mL/min achieved the highest uptake (60 ± 4 a.u.), substantially exceeding that obtained with hand-mixed complexes (24 ± 3 a.u.) (Figure 9B). In contrast, formulations produced at FRR 1:1 (TFR 4–20 mL/min) showed intermediate uptake (around 20 ± 4 a.u.), while those prepared at FRR 1:3 (TFR 4–20 mL/min) resulted in minimal fluorescence (2 ± 4 a.u.), close to untreated controls. Overall, these data indicate that microfluidic preparation at FRR 3:1 consistently maximizes cellular uptake of DAB–CPT dendriplexes in both prostate cancer cell lines, with a particularly pronounced benefit in DU145 cells.

3.6. Gene Expression

The transfection efficiency of camptothecin-bearing DAB dendriplexes was evaluated in PC3-Luc and DU145 cells using a β -galactosidase reporter plasmid (Figure 10).

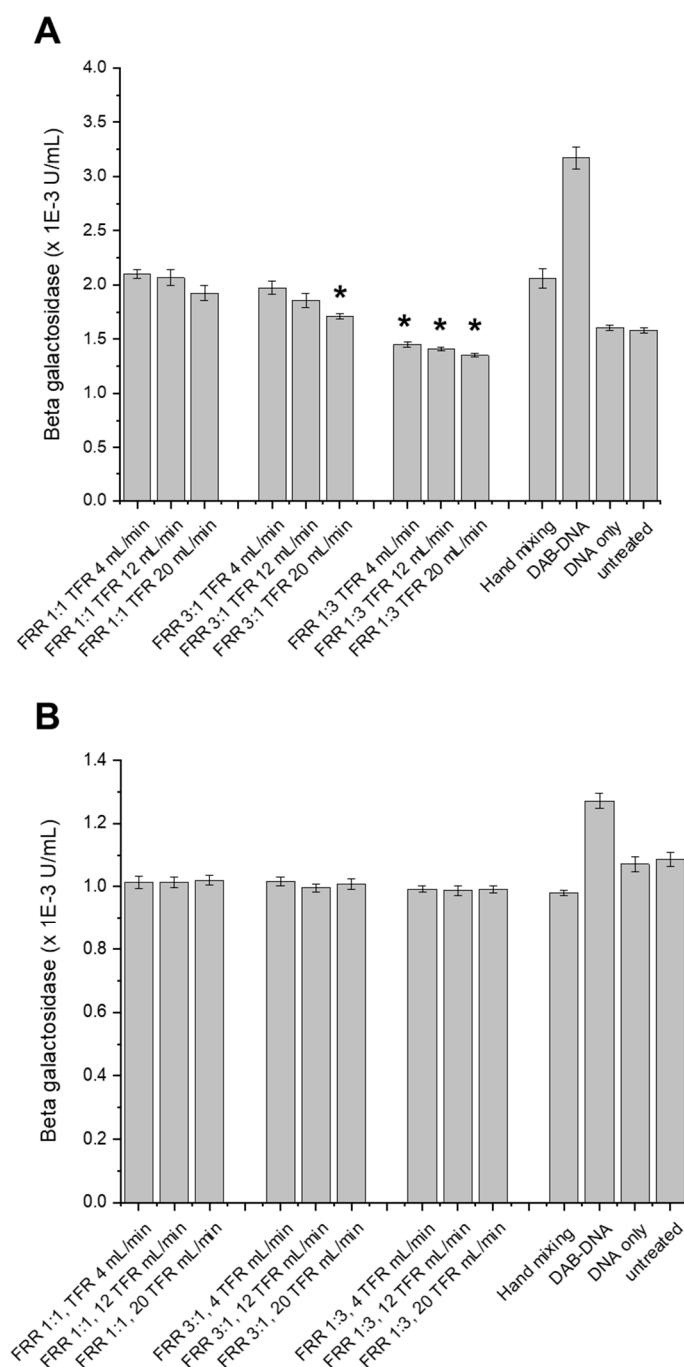


Figure 10. β -Galactosidase expression in (A) PC3-Luc and (B) DU145 cells following transfection with plasmid DNA encoding β -galactosidase delivered by camptothecin-bearing G3 DAB

dendriplexes prepared by microfluidics at different flow rate ratios and total flow rates or by hand mixing, compared with DAB–DNA (5:1 *w/w*), naked DNA and untreated controls ($n = 3$). * $p \leq 0.05$ versus naked DNA.

In PC3-Luc cells, dendriplexes prepared by microfluidics at FRR 1:1 or 3:1 and all TFR values (4, 12 and 20 mL/min) produced β -galactosidase activities of approximately 2×10^{-3} U/mL, which were comparable to those obtained with hand-mixed complexes (about 2.1×10^{-3} U/mL) and higher than naked DNA (1.6×10^{-3} U/mL) (Figure 10A). In contrast, dendriplexes generated at FRR 1:3 gave lower expression levels (around 1.4 – 1.5×10^{-3} U/mL), indicating that this condition is less favorable for functional gene delivery. As expected, the gold-standard DAB–DNA complex (5:1 *w/w*) achieved the highest reporter expression in PC3-Luc cells (3×10^{-3} U/mL), outperforming all camptothecin-bearing formulations.

In DU145 cells, overall β -galactosidase expression was lower than in PC3-Luc cells, consistent with the lower transfection efficiencies typically reported for this cell line (Figure 10B). Dendriplexes prepared by microfluidics at FRR 1:1 and 3:1 across all TFR values (4, 12, and 20 mL/min) produced β -galactosidase activities of around 1.0×10^{-3} U/mL, which were comparable across these conditions and slightly below the level obtained with naked DNA (1.1×10^{-3} U/mL). In contrast, dendriplexes generated at FRR 1:3 and those prepared by hand mixing yielded lower expression, around 0.9×10^{-3} U/mL. As in PC3-Luc cells, the DAB–DNA complex at a dendrimer: DNA ratio of 5:1 remained the most efficient formulation in DU145 cells, giving the highest β -galactosidase activity within this cell line. However, even under the best conditions, reporter expression in DU145 cells did not reach the levels observed in PC3-Luc cells, highlighting a clear cell line dependence of transfection performance and suggesting that, in DU145 cells, the enhanced cellular uptake achieved with some microfluidic formulations does not fully translate into increased gene expression.

4. Discussion

This work evaluated microfluidic processing versus manual hand mixing for the formation of camptothecin-bearing PEGylated G3 DAB dendriplexes and their in vitro performance in two prostate cancer cell lines. Overall, the data show that microfluidics can generate DAB–CPT dendriplexes with efficient DNA condensation, favorable nanoscale size and surface charge, and spherical morphology, while maintaining gene expression comparable to hand-mixed complexes in PC3-Luc cells. In DU145 cells, gene expression remained modest for all formulations, underscoring the importance of cell-line-dependent factors in non-viral gene delivery.

Cationic dendrimers such as polypropylenimine (PPI) and polyamidoamine (PAMAM) have long been recognized as versatile non-viral vectors because their highly branched architecture and dense surface amines enable efficient condensation of nucleic acids into compact nanoparticles and facilitate endosomal escape [25]. In particular, camptothecin-bearing PEGylated G3 DAB dendrimers were previously shown to self-assemble into dendrimersomes capable of delivering plasmid DNA while enabling redox-responsive release of camptothecin in cancer cells [16]. Building on this platform, the present study examined whether replacing manual mixing by microfluidics would improve the reproducibility and potentially the biological performance of DAB–CPT dendriplexes, while retaining the dual gene–drug delivery potential of the system.

The gel retardation and PicoGreen® assays confirmed that DAB–CPT dendrimers efficiently condensed plasmid DNA under all microfluidic conditions tested (TFR 4–20 mL/min; FRR 1:1, 3:1, 1:3), with more than 99% condensation maintained for at least 24 h, comparable to hand-mixed complexes. Efficient condensation is essential to protect DNA

against nuclease degradation and to promote cellular uptake, but overly strong binding can hinder intracellular DNA release and limit gene expression. The sustained high level of condensation observed here suggests that microfluidic processing does not compromise DNA complexation, and that any differences in transfection between formulations are more likely related to size, morphology and intracellular trafficking than to initial DNA binding.

Microfluidic formulation enabled fine control over dendriplex size and surface charge. Across microfluidic conditions, nanoparticles were typically 80–140 nm with low polydispersity, and zeta potentials of approximately 11 to 23 mV. Differences in zeta potential across TFR/FRR conditions are expected because zeta potential is sensitive to the assembly pathway and resulting surface composition [26]. In microfluidics, FRR and TFR modulate mixing intensity, residence time, and may influence the relative exposure of PEG/CPT-modified dendrimer versus DNA at the particle surface, which can alter the apparent surface potential measured for the resulting complexes [24,27,28]. These values fall within the size range generally considered suitable for systemic administration and tumor accumulation via the enhanced permeability and retention (EPR) effect, while remaining below 30 mV, a threshold often associated with increased aggregation and toxicity [29].

FRR had a marked impact on size and charge: FRR 3:1 typically yielded smaller, more uniform particles with moderately positive zeta potentials, whereas FRR 1:3 tended to produce larger complexes with slightly altered surface charge. These trends are consistent with microfluidic mixing principles, where rapid, diffusion-limited mixing at appropriate flow-rate ratios promotes homogeneous nucleation and limits particle growth, whereas sub-optimal conditions can lead to broader size distributions and aggregation [19,30]. The FRR values (1:1, 3:1 and 1:3) were selected to cover balanced, aqueous-dominant and organic-dominant mixing conditions, allowing the influence of solvent composition on dendriplex self-assembly to be systematically investigated. This FRR range was chosen based on our previous work using microfluidic preparation of dendriplexes [24] and PEGylated zein micelles [28], where similar ratios produced reproducible and stable nanoparticles. Comparable FRRs have also been successfully applied for other nanoparticle systems, including liposomes and lipid nanoparticles [27,31], confirming that this range provides efficient mixing and uniform particle formation across diverse delivery platforms.

To place these physicochemical properties in a broader gene-delivery context, it is useful to compare dendriplexes with other widely used non-viral nanocarriers such as liposomes/lipid nanoparticles and polymeric nanoparticles. Across these platforms, successful nucleic-acid delivery systems are typically engineered in the tens-to-hundreds of nanometres range, with a balanced surface charge: sufficiently cationic to promote nucleic acid complexation and cellular interaction, but not so high as to drive excessive serum protein adsorption and cytotoxicity [32]. For lipid systems, cationic lipoplexes commonly fall below 150 nm and often display positively charged zeta potentials, reflecting the need for membrane interaction and uptake [33]. In contrast, clinically translated ionizable lipid nanoparticles are frequently designed for a hydrodynamic size lower than 100 nm and low surface charge at physiological pH to support systemic circulation and tolerability [34]. Similarly, for liposomal gene delivery, moderate zeta potential values (less than 30 mV) are often favored to reduce toxicity while maintaining performance [26]. In this context, the DAB–CPT dendriplexes reported here (80–140 nm; 11 to 23 mV) fall within the physicochemical window commonly targeted for non-viral gene delivery and are broadly comparable to many liposomal and polymeric systems. Liposomes/lipid nanoparticles and polymeric vectors remain the principal non-viral approaches explored in prostate cancer gene delivery, supporting the relevance of benchmarking dendriplex performance against these classes [8,35].

The influence of total flow rate on dendriplex size was less pronounced in this system than in some lipid or polymeric nanoparticles, where increasing TFR typically decreases particle size by reducing mixing time and enhancing supersaturation [36,37]. This may reflect the different assembly mechanisms and rigidity of the DAB-CPT scaffold compared with more fluid lipid systems. Nonetheless, the ability to generate dendriplexes with similar sizes at different TFRs is advantageous for scale-up, as it allows production rates to be increased without compromising key physicochemical properties, in line with recent reports on microfluidic manufacturing of dendriplexes and other nanocarriers [19,24].

TEM imaging corroborated the DLS data by showing predominantly spherical dendriplexes for both microfluidic and hand-mixed formulations. Microfluidic complexes prepared at FRR 3:1 and 1:1 displayed well-defined, compact nanoparticles with limited aggregation, whereas certain conditions (for example, FRR 1:1 at intermediate TFR) produced slightly more heterogeneous morphologies. The hand-mixed DAB-CPT dendriplexes also appeared spherical, while hand-mixed DAB-DNA controls showed a somewhat different morphology, which may reflect the absence of the bulky CPT and PEG moieties. Taken together, these data indicate that microfluidic processing can reliably generate nanoscale dendriplexes with morphology comparable to manual methods, but with the added benefit of tunability through adjustment of FRR and TFR.

The cellular uptake studies in PC3-Luc and DU145 cells revealed a strong influence of microfluidic parameters on internalization of fluorescein-labelled DNA. Dendriplexes prepared at FRR 3:1 across TFR 4–20 mL/min consistently exhibited the highest uptake in both cell lines, whereas complexes formulated at FRR 1:3 showed substantially reduced uptake. A likely explanation is that FRR 3:1 provides rapid and homogeneous solvent exchange during microfluidic mixing, promoting more uniform self-assembly [27,38]. Consistent with this, FRR 3:1 formulations tended to exhibit smaller hydrodynamic diameters with low PDI and moderately positive zeta potentials, a combination that is favorable for membrane interaction and endocytic uptake while maintaining dispersion stability under the *in vitro* conditions used [39,40]. By contrast, FRR 1:3 produced larger complexes and lower uptake, which may reflect less favorable assembly kinetics and a higher propensity for aggregation [27]. This trend has been reported across different carrier classes in microfluidics, where FRRs favoring the aqueous phase (3:1 or higher) often produce nanoparticles with narrower size distributions compared with lower aqueous fractions [31]. These findings are consistent with our previous microfluidic studies on polypropylenimine dendriplexes and PEGylated nanocarriers for prostate cancer, where analogous FRR regimes yielded reproducible, biologically active formulations [24,28]. Although a broad array of nanocarrier types has been explored for prostate cancer gene delivery (including lipid nanoparticles, polymeric vectors, and dendritic systems), a common conclusion is that small, low-polydispersity carriers with controlled surface properties show enhanced cellular interactions [8,35]. The FRR 3:1 conditions identified here conform to these desirable physicochemical attributes and therefore offer a formulation regime that is both mechanistically justified and consistent with trends in related delivery platforms. Formulations produced at FRR 1:1 generally showed intermediate to high uptake, particularly in PC3-Luc cells. These trends likely reflect the combined effects of particle size, surface charge and colloidal stability: smaller, moderately cationic dendriplexes are expected to interact more efficiently with the negatively charged cell membrane and to be internalized via endocytosis, while larger or more aggregated complexes may sediment or be taken up less efficiently. Similar flow-ratio-dependent effects on nanoparticle uptake and *in vivo* performance have been reported for microfluidically prepared lipid nanoparticles and nanolipomers, where an aqueous: organic FRR of 3:1 at a TFR of 4 mL/min yielded small, uniform particles with high gene-silencing efficacy or sustained cellular uptake [38,41]. Despite these pronounced differences in uptake, β -galactosidase expression in PC3-Luc cells was

broadly similar for dendriplexes prepared by microfluidics at FRR 1:1 and 3:1 and those formed by hand mixing, whereas FRR 1:3 complexes showed significantly lower expression. This suggests that once a threshold level of cellular internalization is reached, gene expression is governed not only by uptake but also by intracellular processing, including endosomal escape and DNA release from the dendrimer. The strong and sustained condensation observed for all microfluidic formulations may limit DNA dissociation within the cytosol, thereby constraining transcription, as previously proposed for other microfluidically prepared dendriplexes [24]. In this context, the similar transfection levels observed for hand-mixed and microfluidic dendriplexes in PC3-Luc cells are encouraging, as they indicate that microfluidics can at least match the performance of conventional methods while offering improved process control and scalability.

In DU145 cells, gene expression levels were generally lower than in PC3-Luc cells for all formulations, with microfluidic and hand-mixed dendriplexes yielding comparable, modest β -galactosidase activity and naked DNA showing similarly low expression. Only microfluidic formulations prepared at FRR 3:1 tended to produce slightly higher expression, in line with their superior uptake, but the overall transfection remained limited. These differences between PC3-Luc and DU145 cells likely reflect cell-line-specific variations in size, morphology, membrane composition, receptor expression and endocytic pathways, all of which can influence nanoparticle internalization, trafficking and nuclear delivery of plasmid DNA. This interpretation is supported by recent work in prostate cancer cells showing that uptake mechanisms (including clathrin- and caveolae-mediated endocytosis) and formulation variables can drive divergent intracellular accumulation across PC-3-Luc, DU145 and LNCaP models [28]. In addition to these general differences, several biological factors could contribute to the lower transgene expression in DU145. Differences in cell-surface proteoglycans and glycocalyx density can alter polycation binding and internalization efficiency [42–44], while variations in membrane lipid composition and membrane tension can influence which endocytic routes predominate [45]. Importantly, endocytic trafficking fate strongly impacts expression: uptake via pathways that favor lysosomal maturation can increase degradation and reduce productive delivery, whereas caveolar routes or macropinocytosis can in some contexts reduce lysosomal exposure [45,46]. Furthermore, polycation-mediated transfection is often limited by intracellular steps downstream of uptake, including endosomal escape, cytosolic unpacking of DNA from tightly condensed complexes, nuclear import, and the transcriptional competence of the delivered plasmid [47–50]. Thus, the modest expression observed in DU145 despite measurable uptake is consistent with more restrictive intracellular processing in this cell line. Similar cell-line-dependent behavior has been reported for other targeted and non-targeted nanocarriers in prostate cancer models, highlighting the need to evaluate gene delivery systems across multiple cellular backgrounds.

Comparing the present findings with earlier work on DAB-based vectors is informative. Unmodified DAB dendriplexes have previously shown high gene expression and antitumor efficacy in various cancer models, particularly when combined with targeting ligands such as transferrin or epidermal growth factor [11–13]. The PEGylated DAB-CPT system used here introduces both steric shielding and a covalently attached cytotoxic payload, which are expected to improve colloidal stability and enable combined chemo-gene therapy, but may also reduce the effective surface charge density and alter the dynamics of DNA complexation and release [16]. The observation that DAB-CPT dendriplexes produced either manually or by microfluidics achieve gene expression approaching that of DAB-DNA controls in PC3-Luc cells suggests that these trade-offs are acceptable in this context, especially given the additional chemotherapeutic functionality of the carrier. CPT inclusion within the PEGylated DAB dendrimer provided both structural and therapeutic advantages. Conjugated CPT contributes to nanoparticle self-assembly and stability and

offers the prospect of combined chemo-gene therapy, as previously demonstrated for DAB-CPT systems [16,24]. Future work will assess CPT release and cytotoxicity under biologically relevant conditions to confirm preservation of its anti-tumor activity following microfluidic formulation.

From a technological perspective, the present work reinforces the value of microfluidic platforms such as NanoAssemblr™ for the controlled, scalable preparation of gene delivery systems. Microfluidics offers precise control over mixing, FRR and TFR, enabling the production of dendriplexes with reproducible physicochemical properties and potentially simplifying translation from bench to batch manufacturing [19,51,52]. Hand mixing remains the most widely used laboratory method for dendriplex preparation and therefore serves here as the benchmark reference method rather than a control group. In this context, the primary advantage of microfluidics is improved process standardization, tunability and scalability, even when *in vitro* gene expression is comparable to hand mixing. Moreover, the optimal conditions identified here (FRR 3:1, TFR 4 mL/min) parallel those used for highly potent siRNA-loaded LNPs *in vivo*, suggesting that similar design principles may apply across nanocarrier classes [38]. Importantly, DAB-CPT dendriplexes showed comparable performance whether formulated by microfluidics or hand mixing, supporting the feasibility of transitioning established manual dendriplex formulations to microfluidic manufacture without loss of activity.

While microfluidics offers tight control of mixing parameters, potential limitations can arise during translation to larger production scales. Scaling-up typically relies on parallelization of microchannels or adaptation to higher-throughput geometries, which can influence shear and diffusion times. Nevertheless, several studies have demonstrated that microfluidic systems maintain excellent reproducibility and batch-to-batch consistency compared with bulk mixing when operated under well-defined flow and solvent conditions [52–55]. Overall, the microfluidic method used here is scalable and offers greater control over dendriplex formation than conventional manual preparation.

Taken together, these findings demonstrate that microfluidic processing can generate camptothecin-bearing PEGylated DAB dendriplexes with physicochemical properties, cellular uptake and gene expression comparable to those obtained by hand mixing in prostate cancer cells. Microfluidics therefore represents a promising strategy for the standardized, scalable manufacture of dendrimer-based gene and drug delivery systems, and, with further optimization and *in vivo* validation, may facilitate the translation of DAB-CPT dendriplexes as a platform for combined chemo-gene therapy in prostate cancer.

From a field perspective, this study contributes mechanistic and practical guidance on how NanoAssemblr™ parameters translate to dendrimer-based polyplex/dendriplex systems. Across multiple nanocarrier classes, microfluidic formulation is frequently used to narrow size distributions and improve reproducibility. However, for dendrimer-DNA complexes, it is not always clear how changes in microfluidic parameters translate into physicochemical differences and, ultimately, into cellular uptake and transfection performance. By coupling FRR/TFR variation with condensation, size, zeta potential, TEM, cellular uptake and gene expression, we define a reproducible microfluidic operating window (notably FRR 3:1) that consistently maximizes uptake in both prostate cancer cell lines while maintaining transfection comparable to hand mixing. This provides a transferable framework for standardized manufacturing and rational selection of microfluidic conditions for dendrimer-based gene delivery scaffolds.

At the same time, the present *in vitro* assays cannot fully predict *in vivo* performance. *In vivo* efficacy will depend on systemic stability, clearance and biodistribution; PEGylation may improve colloidal/plasma stability and prolong circulation but can also reduce cellular interactions. In particular, dendriplex integrity and nucleic-acid protection can be compromised by dilution, ionic strength, competitive polyanions (glycosaminoglycans),

and serum proteins/nucleases, which may alter cellular uptake and transfection. Our gel retardation and PicoGreen® assays confirm strong DNA complexation under the conditions tested, but do not quantify stability in biologically relevant media. Moreover, because camptothecin was conjugated to the PEGylated dendrimer prior to complexation, the initial fraction of free camptothecin at formulation is expected to be low. However, the concentration of pharmacologically active drug in vivo will depend on linker/drug stability and release in biological media, as well as maintenance of the active lactone form. Future work should therefore quantify dendriplex stability and DNA protection in serum and relate this to transfection under serum-containing conditions, alongside drug release and lactone stability measurement. In vivo, pharmacokinetics, biodistribution, and anti-tumor efficacy studies will be required to confirm whether the microfluidic-tuned properties translate into improved tumor delivery.

5. Conclusions

This study successfully prepared and characterized camptothecin-bearing PEGylated DAB dendrimers complexed with plasmid DNA using either microfluidics or conventional hand mixing, and systematically compared their physicochemical and biological properties in prostate cancer cells. Both methods generated stable dendriplexes with efficient and sustained DNA condensation, nanoscale hydrodynamic diameters, low polydispersity indices and positive zeta potentials. Microfluidic processing, particularly at an aqueous: organic flow rate ratio of 3:1, yielded complexes with sizes and surface charges comparable to or slightly improved over hand-mixed formulations, while maintaining the same high level of DNA binding. Morphological analysis confirmed that dendriplexes prepared by both approaches were predominantly spherical nanoparticles with limited aggregation, consistent with their favorable size distributions. In vitro, microfluidically prepared dendriplexes at 3:1 flow rate ratio displayed enhanced cellular uptake in both PC3-Luc and DU145 cells, translating into gene expression in PC3-Luc cells that matched hand-mixed DAB–CPT complexes and approached that of DAB–DNA controls. In DU145 cells, gene expression remained modest for all formulations, underlining the importance of cell-line-dependent factors in non-viral gene delivery.

Overall, these results demonstrate that microfluidics can reliably produce camptothecin-bearing PEGylated DAB dendriplexes with physicochemical characteristics, cellular uptake and transfection efficiency comparable to those obtained by hand mixing, while offering superior control over formulation parameters and facilitating scale-up. Microfluidic processing therefore represents a robust and versatile platform for the reproducible manufacture of dendrimer-based gene delivery systems and provides a solid basis for future optimization of targeted, combined gene–drug therapies for prostate cancer. Further translational assessment will require serum stability studies (including DNA protection against nucleases/polyanion displacement) and drug release/lactone stability testing, and in vivo pharmacokinetics and biodistribution studies.

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Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	One-way analysis of variance
BSA	Bovine serum albumin
CDCl ₃	Deuterated chloroform
CPT	Camptothecin
DAB	Generation-3 diaminobutyric polypropylenimine dendrimer
DAB-CPT	Camptothecin-conjugated generation-3 diaminobutyric polypropylenimine dendrimer
DAPI	4',6-diamidino-2-phenylindole
DMAP	4-dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
D ₂ O	Deuterium oxide
DLS	Dynamic light scattering
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FRR	Flow rate ratio
MEM	Minimum Essential Eagle's Medium
NMR	Nuclear magnetic resonance
ONPG	Ortho-nitrophenyl-β-D-galactopyranosidase
OPSS	Orthopyridyl disulfide
OPSS-PEG-SCM	Succinimidyl carboxymethyl ester
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PLB	Passive lysis buffer
S.E.M.	Standard error of the mean
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TFR	Total flow rate

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