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Comparison of the endocytic properties of linear and branched PEIs, and cationic PAMAM dendrimers in B16f10 melanoma cells

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Abstract

Many different polymers and architectures are now being developed as polymer therapeutics and non-viral vectors for cytosolic delivery, and cationic dendrimers, and linear and branched poly(ethylenimine)s (PEIs) have been widely used. For rational design and safe transfer into the clinic, it is important to better understand the cellular pharmacokinetics of the carrier, even if this will likely change when it is conjugated to, or complexed with, a targeting residue or therapeutic payload. The aim of these studies was to compare binding, endocytic capture and intracellular trafficking of linear and branched PEIs (Mw 25,000 g/mol) and cationic PAMAM dendrimers (generations (gen) 2– 4) using B16F10 murine melanoma cells. FITC-dextran was used as a control for comparison. All polymers were first conjugated to Oregon Green (OG) and carefully characterised in respect of pH- and concentration-dependence of fluorescence. Throughout, non-toxic concentrations of polymer were used. Flow cytometry showed that all the cationic polymers were internalised by "adsorptive" endocytosis, with maximum uptake seen for PAMAM gen 4>>branched PEI>linear PEI>PAMAM gen 3>PAMAM gen 2. The PAMAM gen 4 uptake rate was 130 fold greater than seen for FITC-dextran. Branched PEI had the highest extracellular binding (accounting for >50% of total cell-associated fluorescence) whereas for the linear PEI, binding was only 13% of the cell-associated fluorescence. Unlike FITC-dextran, all cationic polymers lacked significant exocytosis over the time period studied. Whereas PAMAM gen 4 and the branched PEI were predominately internalised by cholesterol-dependent pathways, internalisation of linear PEI appeared to be independent of clathrin and cholesterol. A perception of the rate and mechanisms of cellular uptake of these vectors will be important in the context of their proposed use as drug delivery systems.
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1. Introduction

The most successful polymer therapeutics have arisen from careful optimisation of chemical composition bearing in mind the proposed use, the route of administration and cellular pharmacokinetics (reviewed in [1–3]). A growing number of linear polymer- and dendrimer-drug conjugates (particularly anticancer agents) are being developed either as lysosomotropic delivery systems or as polymer-based non-viral vectors for endosomotropic delivery of peptides, proteins, and genes. Endocytic internalisation and appropriate intracellular trafficking is a prerequisite for effective performance. Relatively few studies have systematically evaluated the effect of fundamental polymer characteristics on endocytosis and intracellular trafficking. Using poly(vinylpyrrolidone) (PVP) we (RD) defined the differential effect of polymer molecular weight on uptake by a rat epithelial model (rat visceral yolk sac) and macrophages [4]. In subsequent studies libraries of *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers were used to study the effect of charge [5] and hydrophobicity [6] on endocytic capture. Others have shown that murine bone marrow-derived macrophages fractionate different molecular weight dextrans during pinocytic capture [7]. They found that following internalisation, dextran recycling to the extracellular medium was more efficient for the 4000 g/mol polymer than for a larger dextran (150,000 g/mol) which showed preferential accumulation in lysosomes. Similar observations have been reported in

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Chinese hamster ovary (CHO) cells [8] and L929 cells (mouse fibroblasts), where ~50% of internalised dextran was delivered to the extracellular fluid within 15–30 min [9].

Linear polymers have been most widely investigated clinically as polymer therapeutics (reviewed in [1,3]). However, modified poly(lysine) dendrimers recently entered clinic trial as a topical, vaginal virucide being tested as an anti-HIV agent [10]. Also the dendrimer Gadomer 17 has been tested clinically as a blood pool magnetic resonance imaging (MRI) agent [11]. An increasing number of studies have shown that dendrimers display unusual pharmacokinetics. Wiwattanapatapee et al. [12] found that polyamidoamine (PAMAM) dendrimers traverse the rat intestinal tissue at much higher rates than linear polymers of similar molecular weight and charge and others have witnessed similar dendrimer properties in Caco2 monolayers *in vitro* [13,14]. A recent study demonstrated topology-dependent endocytosis of 1, 2 or 4 armed poly(ethylene glycol) (PEG) polyester dendrons-(gen 1–4) [15]. High exocytosis rates were observed for the more branched dendrons, with higher intracellular retention for more linear dendrons. Few studies have quantified the endocytosis/exocytosis of dendrimers, and this information is urgently needed to ensure optimised design for specific drug delivery and imaging applications.

Therefore, the aim of this study was to measure the binding, endocytic internalisation and exocytosis of cationic PAMAM dendrimers gen 2–4 in B16F10 murine melanoma cells, and to compare their rate and mechanism of uptake to that of FITC-dextran (a widely used marker for endocytosis) and linear and branched polyetheleneimines (PEIs) of similar molecular weight (25,000 g/mol). First, the PAMAMs and PEIs were fluorescently labelled using Oregon green (OG), and the products were carefully characterised in terms of bound and free OG, and the pH- and concentration-dependence of fluorescence output. As the toxicity of cationic dendrimers and PEIs has been well documented [16,17], the cytotoxicity of all polymers was first determined in B16F10 cells (72 h) using the MTT assay to ensure that non-toxic concentrations could be used in all endocytosis studies. Flow cytometry was then used to measure cell surface binding (4 °C), endocytic internalisation (37 °C) and, in pulse chase experiments, exocytosis. Preliminary experiments were also carried out to investigate the route(s) of polymer internalisation into B16F10 using methods previously described [18]. The putative pinocytic inhibitors methyl-β-cyclodextrin (MβCD), chlorpromazine and wortmannin were selected as they are reported to predominately block the cholesterol-dependent [19], the clathrin-mediated [20], and the phosphoinositide 3-kinase-dependent macropinocytosis [21] pathways respectively. Live cell confocal microscopy was also conducted to visualise conjugate binding and internalisation.

2. Materials and methods

All chemical reagents were of analytical grade and used as supplied, and were from Sigma-Aldrich (UK), Fisher Chemicals (UK), Fluka (UK) or BDH (Germany) unless otherwise stated. FITC-dextran (Mw 10,000 g/mol), branched PEI (Mw 25,000 g/mol) as well as all PAMAM dendrimers (ethylenediamine core gen 2–4) were from Sigma-Aldrich (UK), whereas linear PEI (Mw 25,000 g/mol) was from Polysciences (UK). OG 488 succinimidyl ester (OG-SE) was obtained from Molecular Probes (USA). B16F10 cells were obtained from ATCC (USA) and cultured in RPMI medium 1640 supplemented with filtered 10% foetal bovine serum (Gibco Invitrogen Co-operation, UK) in an humidified atmosphere at 37 °C and 5% CO_2 to a confluence of 80–90%. The characteristics of the polymers used are summarised in Table 1.

2.1. Synthesis and purification of polymer-OG conjugates

Reaction conditions were optimised using PAMAM gen 4 as a model compound (see Scheme 1). Briefly, a 1 M equivalence of OG-SE : PAMAM gen 4 (20 mg) was dissolved in methanol (MeOH) and reacted for 2 h under N_2 at room temperature in the dark. The reaction was monitored by thin layer chromatography (Alugram SIL-G/UV_{254} Macherey-Nagel, Germany) using MeOH as a mobile phase. The solvent was evaporated and the crude product dissolved in 2 ml of phosphate buffered saline (PBS; 0.1 mol/l; pH 7.4), and fractionated with a Sephadex G25 column using PBS as the mobile phase (fraction volume 1 ml). Fractions were routinely analysed by thin layer chromatography for the absence of free OG. Pooled fractions were subjected to a final analysis by gel permeation chromatography (GPC) to confirm the purity of the sample. To recover the conjugate from solution and to remove PBS salts, the conjugate was extensively dialysed (Mw cut-off 2000) against distilled H_20 and subsequently freeze-dried and stored at −20 °C until use. To estimate bound and free OG, a small sample (25 μl) of the crude reaction mixture was retained and analysed by GPC using a PD10 column.

Conjugation of OG-SE to all other polymers was also carried out in MeOH as described above. When necessary, the pH of the reaction mixture was adjusted with triethylamine to pH ~8.5. For conjugate purification (bulk) and profiling (to estimate loading), 25 cm and 6 cm GPC columns were used respectively. Sephadex G25 was used as stationary phase for the purification of all other PAMAM dendrimers and linear PEI using PBS (pH 7.4, 0.1 mol/l) and citrate buffer (pH 5.0, 0.1 mol/l) as mobile phase respectively. Branched PEI was purified using Sephadex LH20 and MeOH/H_20 mobile phase (60:40 *v/v*) containing 0.1 mol/l NaCl.

Table 1
Polymer characteristics

Polymer	Topology	Nominal Mw (g/mol)	Estimated [a] Mw (g/mol)	Estimated [a] Mn (g/mol)	Estimated [a] Polydispersity (Mw/Mn)
Dextran	Linear	10,000 [b]	10,311	4422	2.33
PEI linear	Linear	25,000 [b]	21,258	6430	3.31
PEI branched	Branched	25,000 [b]	25,324	9723	2.61
PAMAM G4	Dendritic	14,215 [c]	11,245	7404	1.52
PAMAM G3	Dendritic	6909 [c]	6452	4316	1.50
PAMAM G2	Dendritic	3256 [c]	3497	2397	1.46

[a] Estimated by GPC using polysaccharide standards.
[b] Determined by the manufacturer by laser light scattering.
[c] According to [38].

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