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Cytotoxicity of polycations: Relationship of molecular weight and the hydrolytic theory of the mechanism of toxicity

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ABSTRACT

The mechanism of polycation cytotoxicity and the relationship to polymer molecular weight is poorly understood. To gain an insight into this important phenomenon a range of newly synthesised uniform (near monodisperse) linear polyethylenimines, commercially available poly(L-lysine)s and two commonly used PEI-based transfectants (broad 22 kDa linear and 25 kDa branched) were tested for their cytotoxicity against the A549 human lung carcinoma cell line. Cell membrane damage assays (LDH release) and cell viability assays (MTT) showed a strong relationship to dose and polymer molecular weight, and increasing incubation times revealed that even supposedly "non-toxic" low molecular weight polymers still damage cell membranes. The newly proposed mechanism of cell membrane damage is acid catalysed hydrolysis of lipidic phosphoester bonds, which was supported by observations of the hydrolysis of DOPC liposomes.

1. Introduction

Polycations are materials that find application as delivery vectors in the field of DNA and RNA based therapies, because they condense polyanionic nucleic acids. This is a field with enormous promise, but one that has failed to achieve its full potential despite great clinical interest; mostly due to the toxicity of the polycationic carriers. (Behr, 2012; Gary et al., 2007; Pack et al., 2005) Further problems arise due to the fact that polymeric transfection methods require a large excess of polycations, which is not associated with the poly(nucleic acid), (Boeckle et al., 2004; Dai et al., 2011; Yue et al., 2011) and this present major limitations on *in-vivo* transfection due to different trafficking of the two populations.

Polyethylenimine (PEI) is a cationic polymer which is commonly available with branched and linear structures, and has been synthesised in hairy, comb and cyclic structures. The branched structure is synthesised via the aqueous cationic polymerisation of aziridines, whilst the linear form is typically synthesised by the hydrolysis of a poly(2-oxazoline). (Monnery and Hoogenboom, 2015) Poly(L-lysine) is also commonly available via the ring-opening polymerisation of *N*-carbobenzoxy-L-lysine-*N*-carboxy anhydride. (Fasman et al., 1961) Since these polycations are some of the most frequently used in cell transfection, this work concentrates on assaying these materials.

The mechanism of cytotoxicity of polycationic materials, such as PEI, is a poorly understood matter. (Parhamifar et al., 2010) It is known that polycationic materials do not produce an apoptotic response, but rather cell death is due to necrosis, (Fischer et al., 2003) and that a variety of organelles are damaged. (Grandinetti et al., 2011; Grandinetti et al., 2012; Moghimi et al., 2005) The mechanism of the necrotic damage is not yet understood. Here we aim to investigate the effect of the properties of the polymer on cell membrane damage.

There is a significant body of evidence that polycations open pores in cellular membranes. Banaszak-Holl and coworkers have shown that a wide variety of cationic macromolecules open pores in a supported phospholipid bilayers. (Hong et al., 2004; Hong et al., 2006; Leroueil et al., 2008; Mecke et al., 2005) Poration of the cells is a common way of transfecting cells, with various physical

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methods (electroporation etc.) and chemicals such as surfactants allowing polynucleic acids to simply diffuse into the cells. (Hapala, 1997) However, commonly used polycations such as PEI and PLL should not act as surfactants.

It has been shown that the presence of uncomplexed polycation is responsible for the cell damage, but without their presence there is limited transfection. (Boeckle et al., 2004; Fahrmeir et al., 2007; Hanzlikova et al., 2011; Yue et al., 2011)Kichler et al. reported that PEI has no effect on cell membranes. (Kichler et al., 2001) However, the underlying experiments were carried out in citrate buffer, and the PEI was hence sequestered into an uncharged PEI-citrate complex(Eberhardy et al., 2009), so no free polymer was present.

The effect of cationic polymer molecular weight on the cytotoxicity is poorly understood. Fischer et al. claim that higher molecular weight materials are more toxic, (Fischer et al., 2003) but this is based on a rather random selection of cationic polymers as only one molecular weight of a range of different polymers is assayed and compared. Long et al. performed a systematic survey of the toxicity of poly(*N*,*N*-dimethylaminoethyl methacylate)s, but unfortunately the lowest molecular weight assayed (43 kDa) was so toxic that no relationship can be discerned, although toxicity was clearly due to membrane lysis. (Layman et al., 2009) Two groups (Coll et al. and Wurm et al.) have independently fractionated a commercial broadly-polydisperse linear PEI (l-PEI) and assayed the individual fractions for transfection efficacy. They both reported that fractions below 4 kDa show little transfection activity and little toxicity, and fractions above 20 kDa show little activity but high toxicity, with a maximum transfection efficiency at ca. 15 kDa. (Falco et al., 2009; Kadlecova et al., 2012) However, neither group performed an LDH or similar assay to assess the effect of the molecular weight on cell membrane disruption. Boe et al. assayed a limited range of commercial PEI samples of both linear and branched structures on osteosarcoma cells, with mitochondrial activity measured by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) after 24 h exposure. However, the limited range of materials (i.e. no polycations between 2.5 and 25 kDa) and lack of LDH or similar assays make further interpretation difficult (Boe et al., 2008). In none of these cases the degree of cell membrane disruption has been assessed (i.e. by the lactose dehydrogenase assay), and since the toxicity is clearly due to the disruption and poration of cellular membranes this is clearly a major gap in our knowledge, which will be addressed in the current work.

The mechanism by which polycations induce pores on cellular membranes remains obscure, and essentially two reasonable models exist: either the polycations act as a surfactant(Vaidyanathan et al., 2016) or as a proton transfer catalyst. (Seddon et al., 2009) In the latter, the polycations acts as a proton transfer catalyst that could lead to the hydrolysis of the phospholipids and changes in the curved elastic stress of the membrane. This induces the formation of inverted hexagonal phases in the lipid bilayer ("pores") and phase separation of lysophospholipids which form blebs, this being demonstrated for low molecular weight cationic materials. (Baciu et al., 2006; Casey et al., 2014; Casey, 2011; Shearman et al., 2007) The latter requires the cationic polymer to stabilise a pore, and thus remain localised in the pore. This has never been observed, although the surfactant effect may explain poration by amine functionalized silica or gold nanoparticles, which has been observed by Banaszak-Holl and coworkers.

The hypothesis of this work is that the toxicity of polycations increases with molecular weight (assuming the same structure and architecture), and is due to a greater degree of membrane disruption. To test this a systematical series of l-PEI covering a broad molar mass range has been synthesised (Monnery et al., 2015) and tested for mitochondrial activity and cell membrane damage in the A549 cell line, in comparison to 25 kDa hyperbranched PEI (b-PEI) and a series of poly(L-lysine)s (PLL). To address the postulated membrane disruption by either hydrolysis or surfactant mechanism, experiments were undertaken to evaluate the hydrolysis of 1,2-dioleoyl-sn-*glycero*-3-phosphocholine (DOPC) in presence of PEI by HPLC.

2. Materials and methods

2.1. Materials

Calcium hydride (93%), 2-ethyl-2-oxazoline (99%), methyl tosylate (98%), dimethyl sulfoxide (99%), thiazolyl blue tetrazolium bromide (98%), various poly(L-lysine)s, 1,2-dioleoyl-sn-*glycero*-3-phosphocholine, various poly-L-lysines and 25 kDa hyperbranched PEI were purchased from Sigma-Aldrich. Water used was purified by reverse osmosis to 18 MΩ (Barnstead Nano-pure). Hydrochloric acid (37%), sodium hydroxide (99%+, pellets), DCM (99.8%+) and diethyl ether (99.8%+) were purchased from VWR (Lutterworth, UK). Phosphate buffered saline, DMEM (Glutamax), OptiMEM and fetal calf serum were purchased from Gibco (Thermo Fisher Scientific Inc., Loughborough, U.K.). LDH release assay were purchased from Promega (Madison, WI, U.S.A.) under the name "CytoTox 96® Non-Radioactive Cytotoxicity Assay".

Acetonitrile was stirred over calcium hydride overnight and then refluxed (2 h) before being distilled under argon into a Schlenk equipped two necked round bottom flask. 2-Ethyl-2-oxazoline was stirred over calcium hydride overnight and then refluxed (2 h) before being distilled onto activated 3 Å molecular sieves (activated by heating >300 °C for >1 h under <1 mbar vacuum, cooling and backfilling with dry nitrogen). Nitrogen gas (BOC) and argon (BOC pureshield) were dried through sodium hydroxide and self-indicating silica gel. Syringe filters (0.22 μm polysulfonate filter with polypropylene housing) were purchased from Fisher (Loughborough, UK).

Standard glassware was used throughout. The polymerisation vessel was an oven dried two neck flask equipped with a tap (for addition of liquid reagents and sampling) and a condenser with an isolation tap connected to the Schlenk line via rubber tubing and was flame dried under vacuum before use. Liquid reagents were handled with vacuum dried gas-tight syringes (Hamilton, Bonaduz, Switzerland) using Schlenk technique. Glassware for hydrolysis to l-PEI etc. was similar, although less rigorous procedures were adopted in light of the aqueous solvent.

2.2. Instrumentation and polymer characterisation

Polymers were analysed by Size Exclusion Chromatography (SEC) and Nuclear Magnetic Resonance Spectroscopy (NMR). The SEC was a Polymer Laboratories GPC-50 with 2 x PLGEL MIXED-D (300 × 75 mm) columns and a guard column (50 × 7.5 mm MIXED-D), using DMF (1% (v/v) triethylamine and acetic acid); poly(methyl methacrylate) (PMMA) standards were used as calibrants. A sample of the crude reaction mixture was mixed with an equal volume of deuterated chloroform and analysed via ^{1}H NMR spectroscopy on a Bruker DX-400 machine and the degree of conversion was determined by integrating the polymer backbone (δ = 3.1–3.8 ppm) and the two methylene peaks (4 and 5 position) of the oxazoline ring (δ ~3.8 and 4.1) using the equation:

$$p = \frac{\sum \delta(backbone)}{\sum (\delta(backbone) + \delta(monomer4') + \delta(monomer5'))}$$

A multi-angle light scattering DAWN EOS (Wyatt Technologies Corporation) was placed in series between the SEC column and the refraction index detector and used to determine $Đ_{LS}$. (Shortt, 1994)