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Intracellular fate of bioresponsive poly(amidoamine)s *in vitro* and *in vivo*

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ABSTRACT

Linear poly(amidoamine)s (PAAs) have been designed to exhibit minimal non-specific toxicity, display pH-dependent membrane lysis and deliver genes and toxins *in vitro*. The aim of this study was to measure PAA cellular uptake using ISA1-OG (and as a reference ISA23-OG) in B16F10 cells *in vitro* and, by subcellular fractionation, quantitate intracellular trafficking of ^{125}I-labelled ISA1-*tyr* in liver cells after intravenous (i.v.) administration to rats. The effect of time after administration (0.5–3 h) and ISA1 dose (0.04–100 mg/kg) on trafficking, and vesicle permeabilisation (*N*-acetyl-b-D-glucosaminidase (*NAG*) release from an isolated vesicular fraction) were also studied. ISA1-OG displayed ~60-fold greater B16F10 cell uptake than ISA23-OG. Passage of ISA1 along the liver cell endocytic pathway caused a transient decrease in vesicle buoyant density (also visible by TEM). Increasing ISA1 dose from 10 mg/kg to 100 mg/kg increased both radioactivity and *NAG* levels in the cytosolic fraction (5–10 fold) at 1 h. Moreover, internalised ISA1 provoked *NAG* release from an isolated vesicular fraction in a dose-dependent manner. These results provide direct evidence, for the first time, of PAA permeabilisation of endocytic vesicular membranes *in vivo*, and they have important implications for potential efficacy/toxicity of such polymeric vectors.

1. Introduction

The majority of gene therapy clinical trials currently use viral vectors, and effective cytosolic delivery of all therapeutically important macromolecular drugs (e.g. small interfering ribonucleic acids (siRNAs), DNA, proteins, peptides) is still the major challenge restricting their clinical development. Even viral vectors have major limitations [1]. They can be immunogenic [2,3], may produce insertional mutagenesis (causing cancers such as leukaemia) [1], and their pharmacokinetics can be non-target specific. Although an attractive alternative, and despite more than 20 years of research and development, lack of clinically measurable success using polymeric non-viral vectors has diminished credibility of this approach. Such synthetic vectors often show poor transfection efficiency coupled with transient gene expression [3,4]. They can display an unfavourable pharmacokinetic profile with high levels of liver/lung capture [5,6] and many have unacceptably toxicity [4,7,8]. Moreover most polyplexes are ultimately impractical to manufacture on an industrial scale and/or use clinically. Many of these problems are due to either the cationic charge of the polymers used [9] and/or lack of homogeneity of the products prepared. Acknowledged as a key biological rate-limiting step for the development of effective biocompatible, polymer-based cytosolic delivery systems is still poorly efficient endosomal escape which precludes adequate and reproducible intracellular delivery (cytosolic or nuclear) of a therapeutic dose [10–12]. Rather than simply screening for *in vitro* therapeutic activity using libraries of novel vectors, there is a clear need for rational design based upon a fundamental understanding of material physico-chemical properties.

During the development of linear poly(amidoamine)s (PAAs) as pH-responsive endosomolytic polymers (Fig. 1) we identified specific structures and salt form (ISA23·HCl and ISA1·HCl) that showed considerable promise [13–16]. These PAAs (ISA1 IC_{50} for B16 was 3.1 ± 0.7 mg/mL, ISA23 IC_{50} for B16 was >5 mg/mL [13]) have relatively low toxicity compared to other polycationic vectors (>100 fold less toxic than poly(ethyleneimine) (PEI (M_w 70,000 g/mol)) and poly-L-lysine (PLL M_w 72,000 g/mol)), and the ability (ISA23·HCl) to escape reticuloendothelial system (RES) clearance after intravenous (i.v.) administration allowing tumour targeting by the enhanced permeability and retention (EPR) effect [13]. Although cytosolic delivery of genes by ISA23·HCl [15], and the delivery of non-permeant protein toxins such as gelonin and ricin A chain by ISA1·HCl [16] has been demonstrated *in vitro*, titration studies which systematically varied PAA:toxin ratio showed that delivery efficiency was likely to be impractical for repeated clinical dosing.

Therefore, to guide the development of more effective PAA chemistries better able to promote rapid (within <10 min), transient,

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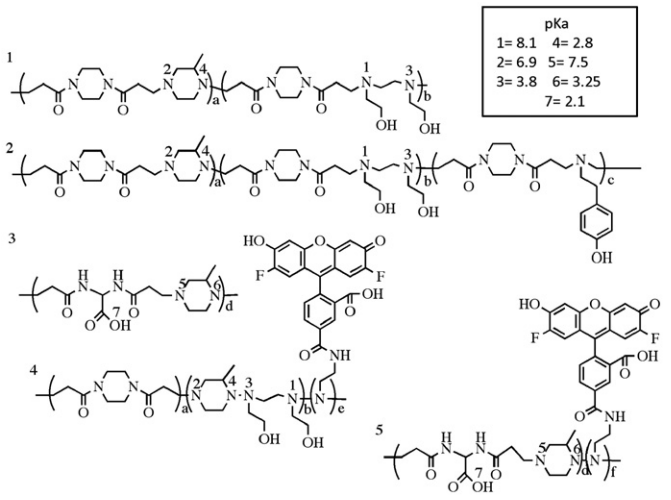

Fig. 1. Poly(amidoamine)s structures. ISA1 (1); ISA1-*tyr* (2); ISA23 (3); ISA1-OG (4); ISA23-OG (5).

and more efficient endosomal escape at pH 6.5 (Fig. 2a), we began to systematically define the physico-chemical properties of ISA23 and ISA1 (Fig. 1; Table 1). PAA shape, size, and pH-responsive changes in conformation were determined using small angle neutron scattering (SANS) [17,18], and the rate of diffusion in solution was defined using pulsed-gradient spin-echo NMR [17]. Most recently PAA interaction with model micelles [19], and model membranes whose composition was chosen to mimic the plasma, endosomal and lysosomal membrane was measured using surface tension and electron plasmon resonance techniques in combination with SANS [19].

The aims of these studies were to define the endocytic uptake and intracellular trafficking of ISA1. First B16F10 cells (previously used to study PAA-mediated toxin delivery [16]), and ISA1-Oregon Green (OG) and ISA23-OG together with flow cytometry were used to quantitate uptake *in vitro*, and fluorescence microscopy was also used to visualise PAA and gelonin-Texas Red (TR) vesicular localisation over time. Subsequently liver subcellular fractionation was used to track the intracellular fate of ISA1 after intravenous (i.v.) to rats. Whereas the amphoteric ^{125}I-labelled ISA23-*tyr* evades liver capture (<10% dose in the liver at 1 h) after i.v. injection, the more cationic ^{125}I-labelled ISA1-*tyr* displays substantial liver localisation (>80% dose, 1 h) [13] thus providing a useful probe for these studies. Both time- and dose-dependent trafficking of ISA1 was quantified and the fractions obtained were also visualised by transmission electron microscopy (TEM). Cytosolic radioactivity levels were used to quantitate ^{125}I-labelled ISA1-*tyr* endosomal escape and cytosolic *N*-acetyl-b-D-glucosaminidase (*NAG*) release provided an indicator of PAA-induced vesicle permeabilisation. Following i.v. administration of increasing doses of ISA1, the ISA1-containing endosomal/lysosome vesicles were isolated from rat liver and the *in vitro* release of *NAG* was measured over time. For comparison, the isolated vesicle fraction was also incubated with ISA1 added to the culture medium (i.e. the outside). These experiments also allowed comparison with vesicle permeabilisation mediated by PEI and (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N,trimethylammonium methylsulfate) DOTAP [20,21].

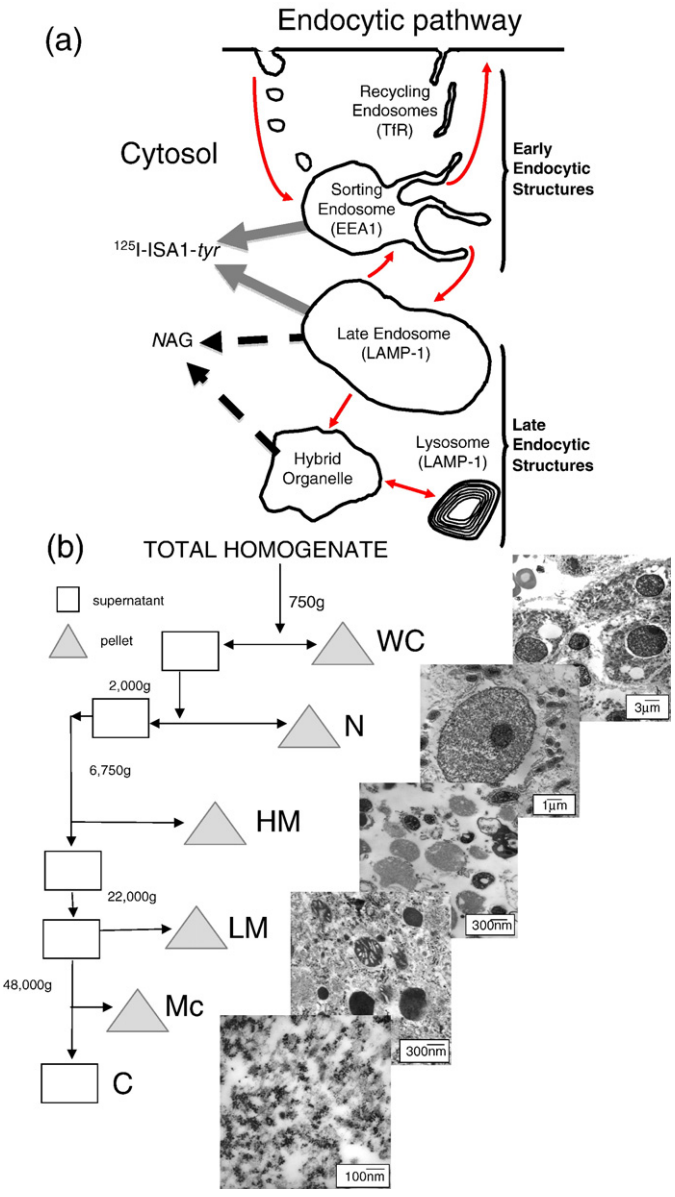

Fig. 2. Markers used and scheme showing methodology used for subcellular fractionation studies. Panel (a) simplified cartoon of the mammalian endocytic system and markers used in this study (modified from [33]). Panel (b) protocol used to perform differential centrifugation with inset TEMs of fractions obtained.

2. Materials and methods

2.1. Materials

Polymers: ISA1, ISA1-*tyr*, ISA23 [13] and ISA1-NH_2 and ISA23-NH_2 (containing a terminal amine group) [22] were synthesised as previously described. The ISA1-*tyr* derivative was radio-iodinated and the PAA-NH_2 derivatives bound to OG as described below. The chemical characteristics of all PAAs used in these studies are given (Fig. 1 and Table 1). PEI (M_n 70,000 g/Mol) was from Sigma-Aldrich (Pool, UK).

General materials: These materials were from Sigma-Aldrich (Pool, UK) unless stated otherwise. PBS tablets were from Oxoid (Hampshire, UK), liquid nitrogen, carbon dioxide and oxygen were from BOC (Surrey, UK). PD10 columns and sodium [^{125}I]iodide were from GE Healthcare (Chalfont St. Giles, UK). Dialysis membranes (14,000 g/mol cut-off), were from Millipore (Dundee, UK).