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In-vitro evaluation of the effect of polymer structure on uptake of novel polymer-insulin polyelectrolyte complexes by human epithelial cells

Ibie C., Knott R., Thompson C.J. *

Institute for Heath and Wellbeing Research, Robert Gordon University, Aberdeen, AB10 7GJ, UK

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ABSTRACT

The biocompatibility and cellular uptake of polymer, insulin polyelectrolyte complexes (PECs) prepared using polyallylamine-based polymers was evaluated *in-vitro* using Caco-2 cell monolayers as a predictive model for human small intestinal epithelial cells.

Poly(allyl amine) (PAA) and Quaternised PAA (QPAA) were thiolated using either carbodiimide mediated conjugation to *N*-acetylcysteine (NAC) or reaction with 2-iminothiolane hydrochloride yielding their NAC and 4-thiobutylamidine (TBA) conjugates, respectively.

The effect of polymer quaternisation and/or thiolation on the IC_{50} of PAA was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay carried out on Caco-2 cells (with and without a 24 h recovery period after samples were removed). Uptake of PECs by Caco-2 cells was monitored by microscopy using fluorescein isothiocyanate (FITC) labelled insulin and rhodamine-labelled polymers at polymer:insulin ratios (4:5) after 0.5, 1, 2 and 4 h incubation in growth media (±calcium) and following pre-incubation with insulin.

MTT results indicated that quaternisation of PAA was associated with an improvement in IC_{50} values; cells treated with QPAA (0.001–4 mg mL^{-1}) showed no signs of toxicity following a 24 h cell recovery period, while thiolation of QPAA resulted in a decrease in the IC_{50}.

Cellular uptake studies showed that within 2–4 h, QPAA and QPAA-TBA insulin PECs were taken up intracellularly, with PECs being localised within the perinuclear area of cells. Further investigation showed that uptake of PECs was unaffected when calcium-free media was used, while presaturating insulin receptors affected the uptake of QPAA, insulin PECs, but not QPAA-TBA PECs.

The biocompatibility of PAA and uptake of insulin was improved by both thiol and quaternary substitution.

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1. Introduction

Oral delivery of insulin, used for the management of type 1 diabetes, could be referred to as one of the major long term goals of pharmaceutical research. Oral administration of insulin is, however, currently not feasible and exogenous insulin formulations are often given subcutaneously (Belchetz and Hammond, 2004). This is a constraint for the management of diabetes as the chronic nature of the condition requires regular injections. It is known that the regular injection regimen required for the management of diabetes predisposes diabetics to physiological stress due to multiple daily injections and has associated risk of infections and/or local reactions at injection sites as well as problems encountered during the insulin administration process such as precipitation of insulin in the injection pump (Wong, 2010). Parenteral administration of insulin also creates a significant difference in the normal physiological distribution of insulin in the body (Chen et al., 2011; Ehud Arbit and Kidron, 2009) which may be associated with the occurrence of peripheral hyperinsulinaemia and insulin resistance resulting in hypoglycaemia, weight gain, neuropathy, retinopathy, atherosclerosis and hypertension in most diabetics (Ehud Arbit and Kidron, 2009).

Oral delivery of insulin has the potential to eliminate these problems and offers an excellent alternative being the easiest and most convenient route of drug administration (Narayani, 2001). Physiological distribution of orally administered insulin also mimics the natural physiological fate of insulin in the body, closely replicating the direct delivery of endogenous insulin to the liver (Satake et al., 2002). Oral insulin delivery is, however, mitigated by the susceptibility of insulin to proteolytic digestion in the gastrointestinal tract (GIT) as insulin is degraded by pepsin in gastric juice and proteases (carboxypeptidase, α-chymotrypsin and trypsin) in the intestinal lumen (Carino and Mathiowitz, 1999;

* Corresponding author.

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Chen et al., 2011; Ehud Arbit and Kidron, 2009; Wong, 2010). Also, insulin a large, hydrophilic macromolecule with log *p*-value < 0 exhibits poor permeation through the GIT epithelium unaided either trancellularly or paracellularly. The presence of a layer of mucus above the intestinal mucosa also constitutes a further permeation barrier (Bendayan et al., 1994; Carino and Mathiowitz, 1999; Morishita and Peppas, 2006).

Polyelectrolyte complexes (PECs) formed spontaneously by electrostatic interaction between oppositely charged polyelectrolytes have been found to have potential applications for the formulation of oral protein delivery systems. At physiologic pH, cationic polymers like chitosan and polyallylamine which feature protonable amine groups can undergo electrostatic complexation with negatively charged insulin forming PECs. These PECs are present in aqueous or buffer solutions as positively charged, spherical nanoparticles, with hydrodynamic sizes between 100–400 nm (Mao et al., 2005). Incorporating insulin intended for oral administration into particles of this size has been shown to enhance transcytotic uptake of insulin-loaded particles through Peyer's patches (Lee 1988; Rao and Ritschel, 1995; Shah et al., 2002). The process of polyelectrolyte complexation also conveniently creates a platform where various functionalities that enhance oral insulin bioavailability can be imparted into the delivery system through rational modification of the carrier polymer structure. Also, the presence of a positive charge on these complexes may provide additional advantages like improved paracellular and transcellular transport of nanocomplexes as a result of electrostatic interaction with anionic components of epithelial cell tight junctions and cell membrane glycoproteins. Transmucosal transport is also facilitated by electrostatic interaction of PECs with anionic sulphate residues and sialic acid present in the intestinal mucosa (Lee 1988; Rao and Ritschel, 1995; Shah et al., 2002).

Polymer quaternisation which stabilises and maximises cationic charge may, therefore, enhance processes like tight junction opening, insulin complexation and mucoadhesion that benefit from charge-based interactions. Quaternisation also improves the biocompatibility of polycationic polymers like PAA and polylysine which have been observed to be cytotoxic as their free protonable amine groups have the ability to interact with anionic portions of glycoproteins on the cell membrane causing apoptosis (Slita et al., 2007). Quaternisation decreases the number of these protonable primary amine groups per molecule minimising toxicity (Brownlie et al., 2004). Other polymer modification processes like thiolation are directed at facilitating polymer-mucin interactions by introducing thiol-disulphide bonding between polymer and mucus thereby improving mucoadhesion of the dosage form. Thiolation has also been shown to reduce efflux of absorbed materials as well as limit some enzymatic degradation of proteins due to the chelating effect thiol groups can have (Lee 1988; Rao and Ritschel, 1995; Shah et al., 2002).

A series of polyallylamine-based amphiphillic polymers (AP) consisting of PAA modified with hydrophobic pendant groups (palmitoyl, cetyl and cholesteryl groups) have been used previously in the formulation of PECs for oral insulin delivery (Thompson et al., 2008, 2010, 2009). These AP were further modified by quaternisation. Complexation with negatively charged insulin was carried out in pH 7.4 Tris buffer resulting mostly in spherical nano-sized complexes. PECs prepared using palmitoyl grafted PAA (Pa)/QPAA (QPa) exhibited the best insulin loading efficiency and protection from peptic and tryptic degradation (Thompson et al., 2010, 2009). Palmitoyl grafted PAA showed 2–3 fold increase in IC_{50} value compared to PAA. The nature of the polymer used in PEC formulation was observed to play a vital role in determining the cellular uptake of the resultant complexes. Major factors that were found to affect the ability of the polymer to facilitate polymer-insulin PEC uptake include structural composition of the polymer, charge density, polymer conformation as well as hydrophilic/lipophilic balance (Fischer et al., 2003; Florence et al., 2000; Malik et al., 2000).

This study aims to further develop this work by modifying PAA to include one of two distinct thiol moieties (*N*-acetyl cysteine or 4-thiobutylamidine) as well as quaternary ammonium moieties. The IC_{50} of these polymers against Caco-2 cells was ascertained using an MTT assay. These novel polymers were then complexed with insulin over a range of polymer:insulin ratios to ascertain their optimal mixing ratio by determination of complexation efficiency (using HPLC), size and zeta potential (using photon correlation spectroscopy) and % transmittance. Optimal ratio PECs were then used to treat a model gut epithelial cell line (Caco-2) to determine the effect of polymer architecture on PEC and polymer uptake (via fluorescence microscopy and fluorimetry, respectively).

2. Materials and methods

2.1. Materials

Poly(allylamine hydrochloride) (average Mw = 15 kDa), tris (hydroxymethyl) aminomethane (Tris base) (≥99%), *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDAC), sodium hydroxide, *N*-hydroxysuccinimide (NHS), *N*-acetylcysteine, 2-iminothiolane hydrochloride, sodium borohydride, iodine solution (0.5 M), starch solution (2%), rhodamine B isothiocyanate (RBITC), fluorescein isothiocyanate (FITC)-insulin, Eagle's minimum essential medium (EMEM), calcium-free EMEM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Triton X-100, dimethylsulfoxide (DMSO) HPLC grade, glycine, Dulbecco's phosphate buffered saline (PBS), sodium dodecyl sulphate (SDS) and trypan blue were from Sigma–Aldrich, UK.

Caco-2 cells were obtained from ECACC, Wiltshire UK and were used passage number 45–70. L-glutamine (200 mM), non-essential amino acids, trypsin-EDTA (0.05%) and DAPI were purchased from Invitrogen, Scotland. Foetal calf serum-activated (FCS) was obtained from Biosera, UK. All other reagents used were of analytical grade.

2.2. Polymer synthesis and characterisation

2.2.1. Extraction of PAA base from hydrochloride and Quaternisation of PAA

The methods used for the purification of the free PAA base from PAA hydrochloride and subsequent quaternisation of PAA to yield QPAA have been previously described in earlier reports published by our group (Narayani and Panduruanga Rao, 1995). The degree of quaternisation of the product was estimated by elemental analysis; samples (1 mg) were analysed using a PerkinElmer series 2 elemental analyser (PerkinElmer, UK) and results were obtained in triplicate.

2.2.2. Thiolation of PAA and QPAA

Thiolation of PAA and QPAA by conjugation to *N*-acetylcysteine via an amide bond was carried out using a similar method to Yin et al. (2009). *N*-acetylcysteine (250 mg; 1.53 mmol) was dissolved in 100 mL of deionised water into which EDAC and NHS were added consecutively up to a final concentration of 200 mM each to activate the carboxylic acid groups of *N*-acetylcysteine. The mixture was adjusted to pH 4–5 using 2 M HCl and left stirring at room temperature for 1 h, after which PAA/QPAA (250 mg) was added into the reaction mixture and the pH of the mixture readjusted to between pH 4–5. The reaction was carried out under nitrogen at room temperature for 5 h without exposure to light. A

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