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The use of fluorescence microscopy to define polymer localisation to the late endocytic compartments in cells that are targets for drug delivery

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Abstract

Macromolecular therapeutics and nano-sized drug delivery systems often require localisation to specific intracellular compartments. In particular, efficient endosomal escape, retrograde trafficking, or late endocytic/lysosomal activation are often prerequisites for pharmacological activity. The aim of this study was to define a fluorescence microscopy technique able to confirm the localisation of water-soluble polymeric carriers to late endocytic intracellular compartments. Three polymeric carriers of different molecular weight and character were studied: dextrin (Mw~50,000 g/mol), a *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer (Mw~35,000 g/mol) and polyethylene glycol (PEG) (Mw 5000 g/mol). They were labelled with Oregon Green (OG) (0.3–3 wt.%; <3% free OG in respect of total). A panel of relevant target cells were used: THP-1, ARPE-19, and MCF-7 cells, and primary bovine chondrocytes (currently being used to evaluate novel polymer therapeutics) as well as NRK and Vero cells as reference controls. Specific intracellular compartments were marked using either endocytosed physiological standards, Marine Blue (MB) or Texas-red (TxR)-Wheat germ agglutinin (WGA), TxR-Bovine Serum Albumin (BSA), TxR-dextran, ricin holotoxin, C6–7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labelled ceramide and TxR-shiga toxin B chain, or post-fixation immuno-staining for early endosomal antigen 1 (EEA1), lysosomal-associated membrane proteins (LAMP-1, Lgp-120 or CD63) or the Golgi marker GM130. Co-localisation with polymer–OG conjugates confirmed transfer to discreet, late endocytic (including lysosomal) compartments in all cells types. The technique

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Appendix A. Supplementary data

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described here is a particularly powerful tool as it circumvents fixation artefacts ensuring the retention of water-soluble polymers within the vesicles they occupy.

Keywords

Endocytosis; Polymer conjugates; Nanomedicine; Intracellular trafficking

1. Introduction

Polymer therapeutics (as defined in [1,2]) include polymer–drug conjugates, polymer–protein conjugates and those complex, multi-component carriers being developed for cytosolic delivery of macromolecular therapeutics such as genes [3], proteins [4] and small interfering (si)RNAs [5,6]. For the effective design of clinically useful constructs it is essential to understand their intracellular trafficking and ultimate fate [7]. For example, it has recently become apparent that the antitumour activity of polyglutamic acid-paclitaxel (XYOTAX™) in women with non-small cell lung cancer (NSCLC) is reliant on the levels of the activating lysosomal enzyme cathepsin B [8,9]. Moreover, not only are cellular enzyme levels crucial, but also it is likely that adequate trafficking of the conjugate to the correct intracellular compartment is crucial [7]. Historically, subcellular fractionation techniques were developed to delineate specific intracellular compartments, and ever since they have been widely used in cell biology to dissect the endocytic, secretory, and biogenic membrane trafficking pathways (reviewed in [10,11]). Although our early studies on *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer anticancer conjugates used subcellular fractionation to confirm lysosomotropic delivery [12–14], and recently we have described new methods able to monitor more subtle compartmentalisation of radio- and fluorescent-labelled polymeric carriers [15–16], such techniques require a high degree of user skill, and need careful standardisation for each cell type to be studied so consequently they are rarely used in drug delivery research.

Epifluorescence, and fluorescence laser scanning confocal microscopy have been used as a technique to study the intracellular fate of macromolecular drugs and delivery systems. A caveat, is that the literature is also peppered with artefacts and many authors make assumptions as to the identity of vesicular profiles containing the endocytosed material (e.g. lysosomes/endosomes) without any definitive identification. However, once validated using well-characterised antibodies chosen to define specific subcellular compartments, this methodology is quicker, less expensive and less labour intensive than subcellular fractionation. In addition, live cell imaging allows the visualisation of trafficking between multiple compartments within individual living cells over time. There are particular challenges associated with fluorescence microscopy when using synthetic polymeric carriers. Traditional fixation methods (e.g. cross-linking with aldehydes followed by detergent extraction or cold-solvent precipitation/extraction [17]) are inadequate as they permeabilise intracellular membranes allowing non-fixed material, such as water soluble polymers, to diffuse out. Live cell imaging circumvents this problem, but does not permit the immuno-staining of intracellular structures and definitive subcellular localisation. Moreover the use of fluorescence probes that show concentration- and/or pH-dependent quenching and

that are released from the carrier following internalisation lead to misinterpretation of images.

Therefore, the aim of this study was to develop a protocol that would facilitate the immobilisation of water soluble polymeric carriers within specific intracellular compartments and thus allow the definition of the identity of the polymer-containing vesicles. Experiments were undertaken with live and fixed cells to eliminate the possibility of fixation artefacts. Both endocytosed physiological markers (wheat germ agglutinin (WGA) [18]; bovine serum albumin (BSA) [19], ricin holotoxin [20] and shiga toxin B chain (STBC)) [21], and immunological markers were used to define specific intracellular compartments (Fig. 1). An early endocytic compartment, incorporating the early sorting endosome, was identified using early endosomal antigen 1 (EEA1) [22], late endocytic structures (including late endosomes, hybrid late endosomes and lysosomes defined as LE/L) were delineated using antibodies to LAMP-1, Lgp120, and CD63 [23], and the Golgi was identified using either a GM130 specific antibody which labels the *cis*- and *medial*-Golgi [24], or C6-7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labelled ceramide which labels the *trans*-Golgi network [21]. Three chemically distinct, widely studied, polymeric carriers of different molecular weight were used as model probes: dextrin (Mw~ 50,000 g/mol), a *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer (Mw~35,000 g/mol) and polyethylene glycol (PEG) (Mw 5000 g/mol). All polymers were labelled with Oregon Green (OG) as this fluorophore has been shown to display minimal pH- and concentration-dependence of fluorescent output [15]. A panel of cells was chosen (THP-1, ARPE-19 and MCF-7 cells and primary bovine chondrocytes) that are currently being used in other studies to evaluate novel polymer therapeutics. NRK and Vero cells were used as reference controls [19,25,26].

2. Materials and methods

2.1. Materials

2.1.1. General chemicals, fluorescent probes, polymers and reagents—

Oregon Green 488-X succinimidyl ester (OG-SE), Oregon Green 488 cadaverine 5-isomer (OG-CAD), Texas Red-*N*-hydroxysuccinimide ester (TxR-SE), lysotracker Green (DnD26), Texas Red (TxR)- or Marina blue (MB)-labelled WGA, C6-7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labelled ceramide and lysine-fixable TxR-labelled dextran (70,000 g/mol), were all from Invitrogen (Paisley, UK). *Streptomyces griseus* pronase was from Roche (Burgess Hill, UK) and *Clostridium histolyticum* type II collagenase was from Worthington (Lakewood, NJ, US). Dextrin (Mw~50,000 g/mol) was from ML Laboratories (Liverpool, UK). *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-GFLG-(9 mol%)-ONp (Mw~ 37,000 g/mol) was from Polymer Laboratories Ltd (Church Stretton, UK) and mPEG-NH₂ (Mw 5000 g/mol), goat serum, Triton-X-100, glycine, paraformaldehyde, leupeptin hydrochloride, BSA, kanamycin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), phorbol 12-myristate-13-acetate (PMA), the BCA assay kit and anhydrous and cell culture grade dimethyl sulfoxide (DMSO) were all from Sigma-Aldrich (Poole, UK).

2.1.2. Cell culture and microscopy—RPMI 1640 medium, Dulbecco's minimal essential medium, glutamax, penicillin/streptomycin, foetal bovine serum (FBS) (for chondrocytes), Dulbecco's Modified Eagles Medium without sodium pyruvate with 450 mg/mL glucose with pyridoxine, gentamicin, kanomycin and fungizone and Hams F-10 were from Invitrogen (Paisley, UK). Foetal calf serum (FCS) (for all non-chondrocyte cells) was from Bio-West (UK). The 6 well treated cluster plates and sterile 22 mm × 22 mm coverslips were from Fisher Scientific, (Loughborough, UK). Normal rat kidney (NRK) cells (ATCC number CRL-6509), Vero cells (ATCC number CCL-81), ARPE-19 cells (ATCC number CRL-2302), and THP-1 cells (ATCC number TIB-202), were from the American Type Culture Collection (ATCC) (Teddington, UK). MCF-7 cells were from the Tenovus Centre for Cancer Research at Cardiff University. The 40 µm nylon cell strainers were obtained from BD Falcon, BD-Biosciences-Discovery Labware (Bedford, USA).

2.1.3. Antibodies—The monoclonal antibodies anti-EEA1 (human), and anti-GM130 (rat) were from BD Bioscience (Oxford UK); the anti-CD63 (bovine) was from Serotec (Oxford, UK); the polyclonal rabbit anti-Lgp120 (rat) was kindly provided by Professor J. P. Luzio, Cambridge University [19]; the monoclonal anti-LAMP-1 (human) was from DSHB (Iowa City, USA) and the polyclonal rabbit anti-RTBC was from AbCam, (Cambridge, UK). The donkey anti-mouse and anti-rabbit secondary antibodies labelled with FITC (for chondrocytes) or Alexafluor488 were from Invitrogen (Paisley UK), and the donkey anti-mouse and anti-rabbit secondary antibodies labelled with Cy5 were from Rockland Immunochemicals (Reading, UK).

2.2. Methods

2.2.1. Synthesis and characterisation of probes (see Scheme 1)—*BSA-TxR* — was prepared using methods previously described [19]. Briefly, TxR-SE (5 mg) was dissolved in DMSO (1 mL) and added to BSA (100 mg, in 5 mL PBS) and then left in the dark at 25 °C for 4 h. The product was purified using PD-10 columns (GE Healthcare, Chalfont St Giles, UK), using PBS as eluant and 0.5 mL fractions were collected. Fractions 2–7 were pooled to give the BSA-TxR conjugate. After dilution (1:100), protein content was determined using the BCA assay, and the total TxR content determined spectrophotometrically (Abs₅₉₄). An aliquot (200 µL) was used to determine the free TxR content using PD-10 column chromatography as described above. The 80×0.5 mL fractions collected were analysed spectrophotometrically (Abs₅₉₄) and free TxR expressed as a percentage of the total. The volume of the final product was adjusted to 10 mg/mL BSA using PBS and then filtered using a 0.2 µm filter. All fluorescent conjugates were stored frozen.

Dextrin-OG: Succinoylated dextrin (~ 50,000 g/mol with ~23 mol% degree of succinoylation) was prepared using methods previously described [27] (Scheme 1a). Briefly, succinoylated dextrin (109.3 mg) was dissolved under stirring in ddH₂O (1 mL). To this, EDC (11.3 mg, 10 molar equiv.), sulfo-NHS (12.8 mg, 10 molar equiv.) and OG-CAD (2.93 mg, 1 molar equiv.) were added, and then NaOH (0.5 M) was added drop-wise to raise the pH to 8.0. The reaction mixture was left stirring for 5 h at room temperature, and progress monitored using thin layer chromatography (TLC-silica gel plates) with methanol as solvent.

The conjugate obtained was purified and characterised by PD-10 column chromatography as described above. The total OG content was determined spectrophotometrically (Abs_{496}) and the free OG content by measuring fluorescence in the PD-10 column fractions as described above (excitation 485 nm and emission 520 nm).

HPMA copolymer-OG: (Scheme 1b) HPMA copolymer-GFLG-ONp (100 mg) was dissolved in dry DMSO (1 mL) under nitrogen. OG-CAD (500 μ L of a 5 mg/mL stock solution in dry DMSO) was then added and the reaction mixture stirred in the dark overnight. TLC, using silica gel plates with methanol:triethylamine (9.5:0.5 v/v) as the mobile phase, was used to monitor the reaction. Finally, an excess of 1-amino-2 propanol (~10 μ L) was added to quench any remaining ONp groups. The conjugate was then purified by precipitation into a mixture (50 mL) of cold acetone/diethylether (9:1 v/v). The precipitate was filtered and washed several times in the acetone/diethylether mixture, and finally dried. The product was dissolved in distilled water and freeze-dried. The conjugate was repurified before use by PD-10 column chromatography using PBS as eluant and characterised as described above in respect of total and free OG.

mPEG-NH₂-OG: (Scheme 1c) mPEG-NH₂ (5000 g/mol, 10 mg) was dissolved in sodium bicarbonate buffer, pH 9 (0.5 mL). OG-SE was dissolved in methanol (5 mg/mL) and an aliquot (0.25 mol. equiv.) of this solution was then added (Scheme 1c). The reaction was left for 4 h at room temperature in the dark, and progress monitored by TLC (silica gel plate) using methanol as the mobile phase. Purification was again carried out using PD-10 columns equilibrated with PBS. Finally samples were desalted using a PD-10 column equilibrated in ddH₂O and the total and free OG determined as described above.

2.2.2. Cell culture—NRK and Vero cells were grown in DMEM containing glutamax (25 mM), penicillin/streptomycin (5 mM) and 10% (v/v) FBS [19]. Cells were split 1:30 twice per week using Trypsin:EDTA, and for the microscopy studies they were plated onto sterile 22 mm×22 mm coverslips at a density of 5×10^5 cells per well in 6 well treated cluster plates.

THP-1 cells were grown in suspension (5×10^4 – 1×10^6 cells per mL) in RPMI 1640 medium with 25 mM glutamax, and 10% (v/v) FBS [28]. They were passaged approximately every 2–3 days. Before the trafficking experiments, these suspension cells were differentiated into adherent macrophages using PMA [28]. The cell suspension (2 mL) was seeded onto coverslips in 6 well plates at a density of 5×10^5 cells/mL and PMA added to give a final concentration of 20 nM. The cells were then left for 2 days to differentiate before the medium was replaced with fresh medium. Cell adherence (differentiation) was confirmed visually by light microscopy, and the cells were left for a further 24 h before being fixed.

MCF-7 cells were grown in RPMI 1640 medium containing 5% (v/v) FBS [29] and were passaged weekly (at ~80% confluence). For microscopy studies, MCF-7 cells were seeded into 12-well plates containing a sterile glass coverslip in each well (0.5 mL, 1×10^6 cells/mL) and then allowed to adhere for 24 h.

ARPE-19 cells were maintained in Hams F-10 medium supplemented with 10% (v/v) foetal calf serum and antibiotics — streptomycin (100 µg/mL), kanamycin (100 µg/mL) and penicillin (60 µg/mL and fungizone (1.25 µg/mL) [30]. For the microscopy studies, cells were seeded at a density of 1×10^5 per well onto coverslips placed at the bottom of a 6 well plate and allowed to adhere overnight.

Bovine chondrocytes were isolated and cultured as previously described [31]. Briefly, articular cartilage was obtained from the metacarpo/metatarsophalangeal joints of 18 month old bovine legs under aseptic conditions. The chondrocytes were isolated by serial digestion in 0.1% (w/v) pronase in DMEM containing 50 µg/mL gentamicin and 5% (v/v) FCS (7.5 mL/g cartilage wet weight) for 3–4 h at 37 °C with constant agitation. Following removal of the pronase solution the tissue was further incubated at 37 °C overnight with 0.04% (w/v) collagenase in DMEM containing 50 µg/mL gentamicin and 5% (v/v) FCS (7.5 mL/g cartilage wet weight) with constant agitation. The isolated cells were filtered through a 40 µm nylon filter and washed by centrifugation twice in DMEM containing 50 µg/mL gentamicin at 1500 rpm for 10 min. Chondrocytes were plated on sterile glass coverslips at 1×10^6 per coverslip. Cells were incubated overnight to adhere prior to incubation in dextrin–OG.

2.2.3. Incubation of cells with fluorescent probes—Cells were incubated with fluorescent probes using a variety of protocols as described schematically in Fig. 2.

Incubation of NRK cells with MB- or TxR-WGA: (Fig. 2) MB- or TxR-WGA and lysine fixable TxR-labelled dextran were dissolved in sterile PBS and centrifuged at $100\,000 \times g$ for 1 h. The supernatant was sterilised through a 0.2 µm filter before use. Cells were incubated with WGA (50 µg/mL) in the presence of leupeptin (200 µM) for 1 h at 37 °C in serum-free medium, and then washed 3× with PBS. Cells were then processed according to one of the following protocols: — (i) Cells were incubated in complete medium (also containing leupeptin) under standard conditions for 48 h before fixation and microscopy (as described below), (ii) Cells were incubated in complete medium for 43 h, and then with either lysine-fixable TxR-dextran (10 mg/mL) or TxR-BSA (10 mg/mL) for 1 h. After again washing with PBS, the cells were incubated under standard conditions for a further 4 h before fixation. (iii) Alternatively, cells were incubated for 48 h in complete medium and the live cells imaged in the presence of lysotracker reagent (Green DnD26) (10 ng/mL). The live cells were washed 3× in PBS and placed in 2 mL of RP media (1 mM MgACh, 1 mM CaCl₂, 5 mM Glucose, PBS, 5 mM Glutamate 10% FBS) and imaged using an Olympus IX70.

Incubation of cells with TxR-BSA and polymer–OG conjugates (Fig. 2): NRK cells were incubated in complete media (+leupeptin; 200 µM) with TxR-BSA (10 mg/mL) or TxR-BSA (10 mg/mL) and polymer–OG (2 mg/mL in PBS) (total volume 2 mL for both) at 37 °C for 1 h (the pulse phase). Then cells were washed 3× in sterile PBS, then complete media containing leupeptin was added and the cells were incubated for 1, 4, or 16 h (the chase phase) under standard conditions. The coverslips were then washed 3× in PBS and fixed as described below. The protocol was modified for Vero, ARPE-19, THP-1 and MCF-7 cells, and bovine chondrocytes. These cells were allowed to internalise TxR-BSA or TxR-

BSA and polymer-OG during a 4 h initial incubation, followed by three washes in PBS and a 16 h incubation under standard conditions prior to fixation.

2.2.4. Incubation of Vero cells with ricin holotoxin and shiga toxin B chain—

Ricin holotoxin was prepared in PBS (100 ng/mL) and was incubated with Vero cells on ice for 30 min (Fig. 2c). The excess toxin was then washed off with ice-cold PBS (3×) and the cells bathed in complete media. The cells were then warmed to 37 °C, and incubated for a further 30 min using standard conditions prior to fixation and immuno-labelling. TxR-labelled shiga toxin B chain (STBC) was prepared as previously described [21], bound to Vero cells at 0 °C for 5 min, and the excess was then washed off as above. The coverslip was placed on a heated stage and bathed in RP media at 37 °C. The C6-NBD-ceramide (5 µM) was then added. Having selected a region of interest and adjusted the photodetector and laser gain accordingly, images were captured every 2 min over a 30 min period. To minimise photobleaching and oxidative cell damage a low pixel dwell time was also selected. Additionally every effort was made to reduce the exposure of the cells to white light generated by the mercury arc lamp. Sequences of images were exported as an AVI file and the images in Fig. 6b were extracted using Final Cut Pro 6.0 (Apple Computers Inc.) at the time points indicated. The channels were separated using Photoshop 7.0 (Adobe). The Zeiss LSM510 confocal microscope was used to collect this data set after the confocal aperture was set to maximum.

2.2.5. Fixation and microscopy—Fixation: For immuno-staining using anti-LAMP-1, anti-CD63 and anti-Lgp120 antibodies (all mark LE/L), cells were fixed in cold methanol (pre-chilled to −20 °C) and incubated for 5 min at −20 °C, but otherwise, cells were fixed using 2% (w/v) paraformaldehyde (20 min at 25 °C) made in PBS, fresh on the day of use. When cells were immuno-labelled after aldehyde fixation they were subject to detergent extraction. When imaging polymer-OG conjugates the detergent permeabilisation step was omitted to ensure maintenance of the integrity of the limiting vesicle membranes. Detergent extraction was performed using PBS containing 50 mM glycine and 0.2% (v/v) Triton-X-100 for 5 min at 25 °C [19].

Immuno-labelling: The cells to be immuno-labelled were first incubated in 2% (v/v) goat serum (in PBS) for 60 min at 25 °C. Primary antibody hybridisations were then conducted over 60 min at 25 °C in the dark. The following antibody dilutions were used: — anti-EEA1 (1:300), anti-Lgp120 (1:1000), anti-GM130 (1:200), anti-LAMP-1 (1:10), anti-CD63 (no dilution) and anti-ricin B chain (1:200). Secondary hybridisations were performed using either anti-mouse or rabbit specific antibodies labelled with Alexafluor488, Cy5 or FITC. An incubation period of 1 h at 25 °C after dilution (1:200 using 1% (v/v) goat serum in PBS) was also conducted in the dark. All samples were mounted using Vectorshield mounting medium (30 µL) to prevent sample photobleaching during microscopic examination.

Microscopy: Epifluorescence microscopy was performed using an Olympus IX70 microscope fitted with a CCD camera, FITC, TxR, DAPI filter cubes and Nomarski optics. Epifluorescence microscopy was used only for the data acquired for Fig. 3a. Confocal microscopy was performed using a Zeiss LSM510 fitted with heated stage (Fig. 6) or a Leica

SP5 system (Figs. 4 and 5). Data was collected using dedicated software supplied by the manufacturers and exported as tagged image files (TIF); at least three representative images were obtained for each sample. Typical results are shown. Examples of colocalisation between the endocytic probe (i.e. TxR-BSA) and the compartment marker (i.e. lysotracker or antibody) are indicated using arrows. Standard procedures were followed to minimise bleed from one channel to another (giving rise to false positives) and sample photobleaching. All images were collected using monochromatic CCD cameras. Merged images were generated using Photoshop. All size bars represent 5 μ m.

3. Results

3.1. Synthesis and characterisation of the probes

The TxR-BSA conjugate contained ~3.4 wt.% TxR corresponding to ~4 molecules of TxR per molecule of BSA and <1% free TxR. The characteristics of the polymer-OG conjugates are summarised in Table 1. Their OG loading varied between 0.3–3.0 wt.% and free OG levels were always <3% the total.

3.2. Optimisation of the time course for pulse-chase experiments using MB- or TxR-labelled WGA and TxR-BSA as reference compartment markers

First, experiments were conducted in NRK cells (using both live and fixed cells) to ensure absence of fixation artefacts and to define the optimum time-frame for the trafficking of reference markers (chased) into late endocytic compartments. Fig. 3a (panel i) shows co-localisation between the late endocytic marker Lgp-120 (which labels a compartment incorporating late endosomes, and lysosomes) and the MB-WGA. These results show that after 48 h the internalised MB-WGA is no longer in an EEA1 positive early endocytic compartment (data not shown), but that it has been transferred to a late endocytic compartment. This WGA +ve, late endosomal/lysosomal compartment was also acidic as evidenced by TxR-WGA co-localisation with lysotracker green in live NRK cells (Fig. 3a: panel ii). Endocytosed TxR-dextran (lysine fixable) (Fig. 3a: panel iii), TxR-BSA (Fig. 3a: panel iv) and cathepsin D (data not shown) were also detected within this WGA +ve compartment after 48 h, confirming the compartment to be a late endocytic compartment that is able to fuse with incoming, ligand-carrying vesicles.

The distribution of TxR-BSA after various incubation times was also followed in NRK cells (Fig. 3b). After a 1 h incubation followed by a 1 h chase there was already little co-localisation between TxR-BSA and EEA1 (Fig. 3b; panel i). However, at this time the TxR-BSA was not exclusively within a late endosomal/lysosomal compartment (arrow labelled with an asterisk in Fig. 3b; panel ii). When the chase time was extended to 4 h (or 16 h in subsequent experiments, i.e. Figs. 4 and 5), TxR-BSA was no longer detectable in early endocytic structures (Fig. 3b; panel iii) being found only in late endocytic structures (Fig. 3b; panel iv).

3.3. Endocytic fate of polymer-OG conjugates in different cell types

Next, experiments were conducted using an optimised protocol (a 4 h incubation with TxR-BSA followed by a 16 h incubation (chase)), to investigate the cellular localisation of

dextrin–OG in different cell types (Fig. 4). When incubated with Vero cells, no TxR-BSA was detectable within an EEA1 +ve early endocytic compartment at this time (Fig. 4a; panel i), and it was found exclusively within the late endosomal/lysosomal compartment (in this instance denoted by the presence of LAMP-1) (Fig. 4a: panel ii). As the dextrin–OG conjugate was detected exclusively within a population of TxR-BSA-containing structures (Fig. 4a; panel iii), it was concluded that dextrin–OG was also exclusively within these late endocytic compartments. Also in ARPE-19 cells (Fig. 4b), THP-1 cells (Fig. 4c), bovine chondrocytes (Fig. 4d) the dextrin–OG was exclusively localised with TxR-BSA within a late endosomal/lysosomal compartment.

3.4. Comparison of the endocytic fate of dextrin–OG, HPMA copolymer–OG and PEG–OG conjugates in MCF-7 cells

To better understand if this methodology could be applied to physico-chemically distinct polymers the intracellular distribution of dextrin–OG, HPMA copolymer–OG and PEG–OG was compared in MCF-7 cells (Fig. 5). Cells were incubated for 4 h with TxR-BSA followed by a 16 h chase. Again, in MCF-7 cells none of the TxR-BSA was detectable within the population of EEA1 +ve structures (Fig. 5; panel i), and it was detectable exclusively within a late endocytic compartment (Fig. 5: panel ii). Furthermore, dextrin–OG (Fig. 5; panel iii), PEG–OG (Fig. 5; panel iv) and HPMA copolymer–OG (Fig. 5; panel v) were also exclusively localised to these TxR-BSA-containing late endocytic structures.

3.5. Endocytic fate of TxR-ricin holotoxin and TxR-shiga toxin B chain

After a 30 min incubation, co-localisation between the antiricin toxin B chain (RTBC) specific antibodies and antibodies specific for the *cis*-Golgi marker GM130 was documented (Fig. 6a; panel i). Furthermore, at this time point, immunolabelled RTBC was not detected within an EEA1 +ve compartment (Fig. 6a; panel ii). The uptake of TxR-shiga toxin B chain (STBC) was monitored with time in live cells. At time zero and at 0 °C, the TxR-STBC was documented on the surface of Vero cells (Fig. 6b; panel i). When the cells were warmed to 37 °C, the TxR-STBC was progressively internalised, and after 20 min was detectable in the *trans*-Golgi, defined by the marker C6-NBD-ceramide (Fig. 6b; panel ii). A supplemental video documenting TxR-STBC Golgi translocation in Vero cells overtime is available online.

4. Discussion

The endocytic and intracellular trafficking pathways are complex and highly regulated processes that serve to help mediate cellular homeostatic control by initiating and attenuating receptor mediated cell signalling, facilitating organelle biogenesis [23,32]. It has long been realised that nano-sized delivery systems can be designed to hijack these physiological transport pathways to facilitate improved drug delivery. Following endocytic uptake, drug delivery systems may be transferred into early sorting endosomes and recycling endosomes (“early endocytic structures”), or transferred from early sorting endosomes to late endosomes, late endosomal-lysosomal hybrid organelles and finally to lysosomes (“late endocytic compartments”). The latter three compartments are, within the context of this study, not easily distinguished from one another and are now being defined as “late

endocytic structures". Non-recycled, natural macromolecules are often transferred into these organelles where they are degraded (Fig. 1), and importantly they contain enzymes that have often been used to degrade, and thus activate, polymer therapeutics by intracellular drug liberation. The catabolic properties of these late endocytic structures make them very hard to study [23]. They are not only capable of degrading any physiological substrates entering them, but also release fluorophores used as probes for drug delivery systems (as well as drugs) and, when isolated may degrade.

Here, techniques have been developed using both endocytosed physiological markers and indirect immuno-fluorescence microscopy that reliably monitor the fate of water-soluble polymers being used, and/or developed, as polymer therapeutics [1,2]. There are many well-acknowledged technical challenges associated with fluorescence microscopy. These include: the optical resolution of the microscope, the small number of cells in the population that can be viewed (relative to subcellular fractionation), artefacts due to fixation techniques, and indeed, without care the microscope laser can permeabilise intracellular membranes allowing vesicular contents to escape (discussed in [33]). Not least the fluorescent probe itself can be problematic [33]. Phenomena such as photo-bleaching, concentration and pH-dependent fluorescence quenching, and probe-driven effects on cellular uptake rate and subsequent trafficking of the conjugate have been well documented [15]. All too frequently authors make assumptions as to the compartments occupied by drug delivery systems (e.g. lysosomes/endosomes) without any definitive characterisation of the compartment. The studies here were undertaken using both live and fixed cells in an effort to define a methodology that would minimise artefacts due to fixation.

Exogenously added endocytic markers can temporally delineate the endocytic pathway, and allow live cell imaging. WGA was chosen as it binds to most cell types [18] unlike other popular probes (e.g. TxR-transferrin [34] and labelled-epidermal growth factor [35]) whose cellular uptake is dependent on levels of receptor expression. Albumin is widely used to characterise endocytic pathways. Albumin is taken up by fluidphase and non-specific, receptor mediated mechanisms (two scavenger receptors (gp18 and pg33), and one highly specific receptor (gp60) have been well characterised [36]). TxR-BSA shows temporal delineation of endocytic sub-compartments, and gold-labelled BSA has been well documented to occupy late endocytic compartments after 1 h [37]. Typically endocytosed markers label early endocytic structures over the first 5–10 min (early sorting endosomes) [19].

For the polymer therapeutic applications we are currently studying in the cell lines reported here, we were particularly interested to learn whether the polymers under study would access late endocytic compartments. Therefore, after a short incubation time, a chase period of 4 to 48 h was used. Leupeptin was added to minimise lysosomal degradation of TxR-BSA and this probe was detected exclusively in late endocytic structures making it a useful marker for this compartment. It should be noted that, without the addition of a protease inhibitor, fluorescence was widely distributed in the cells (data not shown) illustrating the absolute need to prevent TxR-BSA degradation and fluorophore release.

When developing methods to define the intracellular trafficking of drug delivery systems it should be noted that the location of compartments within a cell varies significantly from one cell type to another. Also markers may define a variety of compartments as a consequence of cellular differentiation. While in the majority of cell types, at steady state, syntaxin 6 is a marker of the *trans*-Golgi network (TGN) and is not coincident with LAMP-1 +ve late endocytic structures [38]. In B16F10 cells, antibodies specific for syntaxin 6 may be found decorating a LAMP-1 +ve compartment at the cell periphery. Whilst some researchers favour overexpression of green fluorescent protein-(eGFP) e.g. eGFP-Rab5 [39] to define intracellular compartments without the need for cell fixation, the over-expression of these proteins can result in altered intracellular trafficking of endocytosed materials. For example, over expression of Rab7-eGFP has been shown to alter the kinetics of lysosomotropic trafficking [40].

For the above reasons, post-fixation immuno-labelling techniques were preferred for definition of specific intracellular compartments. The tethering protein EEA1 (which acts as an effector in the vesicle fusion process [22]) was used as a marker of early endocytic structures, and the lysosome-associated membrane proteins (LAMP)s to identify the late endosomal compartment [23]. Due to inter-species differences, it was necessary to use different antibodies to label the late endosomal/lysosomal compartment (designated LE/L) in the different cell types studied. For example, antibodies to rat Lgp-120 and human LAMP-1/LAMP-2 do not cross react. In the case of chondrocytes, as antibodies to bovine LAMP-1 and LAMP-2 were not readily available, antibodies specific for CD63 (LAMP-3) were used. Using these probes, the intracellular location and morphology of the labelled compartments (immuno-probes and fixed cells) correlated well with the labelling pattern seen using the live cells and endocytic probes. The WGA-labelled structures were also shown to be acidic (i.e. +ve for the lysotracker dye) (Fig. 3a: panel ii), positive for the late endocytic marker Lgp-120 (Fig. 3a: panel i) and fusion competent (Fig. 3a: panel iii and iv) as it was possible to chase a second pulse of TxR-BSA or TxR-dextran into them.

Using this optimised methodology it was confirmed that dextrin-OG occupied a late endosomal/lysosomal compartment in a panel of cell lines; THP-1 and ARPE-19 cells and bovine chondrocytes (Fig. 4). In the case of ARPE-19 cells and bovine chondrocytes this is the first demonstration of the endocytosis and intracellular localisation of a polymeric carrier; an observation with important implications for development of polymer therapeutics for treatment of diseases of the eye and the joint respectively. The fact that all three polymer-OG conjugates showed exclusive co-localisation with the late endosomal/lysosomal compartment (Fig. 5) also underlines the versatility of the fixation process and applicability of the methodology to different, clinically relevant polymeric carriers and different cell types.

A major challenge in drug delivery is the achievement of efficient cytosolic access of macromolecular drugs i.e. endosomal/lysosomal escape. Ricin toxin or shiga toxin act by accessing the cytosol via the Golgi and endoplasmic reticulum, and thus conjugation of probes (or drugs) to these toxins would theoretically enable retrograde trafficking and cytosolic access. Here, a method was established that will enable visualisation of this process in the context of macromolecular (polymers and proteins) therapeutics designed

with this function in mind. Here, it was shown that the Golgi could be defined in both fixed (anti-GM130 specific antibodies) and live (C6-NBD-ceramide) Vero cells (Fig. 6). In addition, in both live and fixed cells, TxR-STBC and ricin (Fig. 6) were detected in the Golgi demonstrating the ability to not only label Golgi, but also escape lysosomal degradation. TxR-STBC Golgi translocation (Fig. 6b) is also documented in the supplemental video data available on line. This video shows the appearance of yellow juxtanuclear puncta demonstrating the co-localisation of endocytosed TxR-STBC with C6NBD (i.e. TxR-STBC Golgi accumulation) over time, in live Vero cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A simplified cartoon depicting the endocytic, biogenic and secretory pathways. The principal compartments are shown together with common markers used to define them along with the times taken for material to reach them following endocytic capture.

Fig. 2.
Schematic describing the protocols used for cell culture experiments.

Fig. 3.

Distribution of MB-WGA, TxR-WGA and TxR-BSA in NRK cells. Effect of fixation, incubation (time) and dose (time) upon the localisation of endocytosed probes relative to endocytic compartment markers. In all microscopy figures, the left hand row represents data shown in the red (merged) channel, the second row in the green (merged) channel. Fig. 3a) panel (i) shows fixed cells and panels (ii)–(iv) represent live cells. All cells have been incubated with fluorescently-labelled WGA as described in the methods section. Fig. 3b) shows NRK cells incubated with TxR-BSA and then washed and incubated for 1 h or 4 h as

indicated. Examples of co-localisation between the TxR-BSA and either the labelled polymers or the immunological defined compartments are denoted using arrows. An example of BSA in an unknown compartment is denoted using an arrow marked with an asterisk.

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Fig. 4.

The figure shows the intracellular distribution of dextrin-OG in Vero, ARPE-19, THP-1, and MCF-7 cells, and primary bovine chondrocytes. All cells were incubated with TxR-BSA and polymer-OG for 4 h and then chased for 16 h before as described in Fig. 2. The early endocytic compartments were immuno-stained with EEA1 and the late endocytic compartments (LE/L) with the appropriate marker.

MCF-7 cells-TxR BSA

Fig. 5.

The figure shows the intracellular distribution of Dextrin-OG, PEG-OG and HPMA-OG in MCF-7 cells co-incubated with TxR-BSA (4 h) prior to a second (16 h) incubation without the TxR-BSA and polymer conjugates.

Fig. 6.

a) panel (i) shows ricin specific antibodies co-localising with an antibody specific for a cis-Golgi marker (GM130) in Vero cells. Panel (ii) is a negative control and shows that no ricin is detectable in an EEA1 +ve compartment at the stated time. b) represents Vero cells incubated with TxR-Shiga toxin B chain (STBC). Panel (i) shows TxR-STBC on the plasma membrane at T_0 . Panel (ii) shows that 20 min after heating to 37 °C, TxR-STBC is detected in a C6-NBD-Ceramide (C6-NBDC) +ve Golgi compartment. Please refer also to the online supplemental video.

Scheme 1.
Synthesis of polymer-OG probes.

Table 1

Characteristics of the polymer-OG probes

Compound	Molecular weight (g/mol)	Total OG content (wt.%)	Free OG (% total)
Dextrin-OG	50,000	0.47	ND ^a
HPMA copolymer-OG	37,000	2.89	2.4
mPEG-OG ^b	5,000	0.38	3.0

^aND –not detectable^bThe specific activity of mPEG-OG was 4.2 %.