

### 2.3.2 Evaluation of Cell Growth Using the MTT Assay

Growth curves were determined to ensure that cells used in experiments were within the exponential growth phase. Cell proliferation was assessed by monitoring the conversion of MTT to formazan. The reduction of MTT is catalysed by mitochondrial dehydrogenase enzymes and is therefore a measure for cell viability (Fig 2.1) (Mosmann, 1983).

Briefly, cells (100  $\mu$ l/well) were seeded at seeding densities of  $1 \times 10^5$ ,  $1 \times 10^4$  or  $1 \times 10^3$  cells/ml into 96 well microtitre plates and allowed to adhere for 24 h. Cell viability was assessed on a daily basis by adding 20  $\mu$ l of filter sterilised MTT (5 mg/ml in PBS) to a single row of six wells. Following a 5 h incubation period with MTT, media was removed with a needle and syringe and the blue formazan crystals trapped in cells dissolved in sterile DMSO (100  $\mu$ l) by incubating at 37 °C for 30 min. The absorbance at 550 nm was measured with a plate reader. Only the inner rows of the microtitre plate were used for these studies to minimise cell growth variations due to different medium evaporation rates at the periphery. The growth curve was constructed by plotting absorbance (blanked with DMSO) against time. The growth curves for DU145 cells are shown in Fig. 2.2.

### 2.3.3 Evaluation of Cytotoxicity Using the MTT Assay

The MTT assay (as described above) was also used to assess the *in vitro* cytotoxicity of polymers and polyplexes used in this study (see Chapter 4) (Sgouras & Duncan, 1990). In brief, COS7 and MCF7 cells (100  $\mu$ l;  $1 \times 10^5$  cells/ml) were seeded into 96 well microtitre plates as before and left to adhere for 24 h. The next day, the medium was removed from the wells and replaced with filter sterilised complete medium containing polymer or polyplex. The plates were then incubated with polymer solutions for either 6 h or 24 h. In the case of the 6 h incubation; medium was removed after 6 h and replaced with culture medium only and further incubated to the total time of 24 h. Chitosan derivatives and PEI were applied onto the cells in DMEM at concentrations ranging from 20 to  $10^4$   $\mu$ g/ml (100  $\mu$ l/well) and cell viability compared to cells treated with DMEM only. In the case of the 24 h incubation media containing polymer was removed at 24 h and replaced with complete media. MTT (20  $\mu$ l of 5 mg/ml in PBS) was added to each well of the plates for both incubation times. Plates were incubated for a further 5 h. Then the medium was removed and DMSO (100  $\mu$ l) added before a further incubation of 30 min at 37 °C.

Finally the absorbance at 550 nm of the plates was read with the Tecan plate reader. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only (i.e. no polymer or polyplex added) were taken as 100 % cell viability (i.e. the control).

#### ***2.3.4 Flow Cytometry: General Procedure for the Analysis of Cells***

To exclude non-viable cells and debris from the analysis by flow cytometry, the viable cell population was determined. Viable cells have different scatter characteristics to non-viable cells due to their morphology; the scatter plot was therefore used to select the viable cell population. Flow cytometry was used in Chapter 5 to assess cell association and relative binding affinity of ligands for uPAR. Following incubation with fluorophores all subsequent steps were conducted at 4 °C. After treatment with fluorophores and rinsing with PBS adherent cells were scraped in PBS and centrifuged (2000 RCF, 2 min, 4 °C), the supernatant removed and the cell pellet re-suspended in 300 µl of PBS (4 °C). U937 cells were centrifuged after treatment with fluorophores and rinsed by re-suspension in PBS/BSA (0.1 %, 4 °C) twice. The method was derived from that described by Ormerod et al. (2000). In the side/forward scatter plot viable cells were selected as a region and this region used to collect events, 10000 events were collected for each sample. Forward and side scatter settings for the different cell lines are shown in Table 2.3, as is the gain for FL-1 (530 nm).

#### ***2.3.5 Evaluation of Protein Content Using the Bicinchoninic Acid (BCA) Assay***

The BCA assay is widely used to quantify protein (Smith et al., 1985). The method reported previously was used and scaled accordingly. The protein content of cell homogenates was determined to standardise the amount loaded in western blots (Chapter 5), and to establish the specific activity of luciferase in transfection experiments (Chapters 4 and 6). Briefly, in both cases BCA was added to a CuSO<sub>4</sub> pentahydrate solution (4 % w/v) at a ratio of 1:50 and 200 µl of this reagent was added to 20 µl of cell lysate (Sections 2.3.7 and 2.3.8.1) and incubated at 37 °C for 25 min. The absorbance of the copper-I-BCA complex was measured at 550 nm using the Tecan plate reader. Calibration standards of bovine serum albumin (BSA) in homogenisation buffer were treated identically to samples and used to quantify the amount of protein in the fractions (Fig. 2.3).