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Note

Cell uptake, cytoplasmic diffusion and nuclear access of a 6.5 nm diameter dendrimer

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Abstract

Macromolecular crowding and the presence of organelles in the cytosol present barriers to particle mobility, such that it is unclear how nano-carriers can deliver their active agents to the nucleus. In this work a sixth generation amino terminated polyamide polylysine dendrimer (Gly-Lys_{63} $(NH_2)_{64}$) (MW 8149, diameter 6.5 nm) which is fluorescent allowed the study of nuclear uptake and mobility in living lung carcinoma (SK/MES-1) and colon adenocarcinoma (Caco-2) cells. The dendrimer is found within 25–45 min of incubation inside the cell nuclei. Living cells were then used to develop a method for the dynamic nuclear uptake study using confocal microscopy. The dynamic uptake of the dendrimer demonstrated here allowed the apparent cytoplasmic diffusion coefficient (D) of the dendrimer to be calculated. Values were found in the range 5.99×10^{-11} cm^2 s^{-1} (SK/MES-1 cells) to 9.82×10^{-11} cm^2 s^{-1} (Caco-2 cells). The difference must reflect variation in the intracellular architecture of the cell types.

Keywords: Diffusion coefficient; Fluorescent dendrimer; Nuclear uptake; Nano-probe; Cytosol

The unique dendritic architecture of the dendrimer allows for precise control of size and shape as well as potential targeting ability through surface conjugated ligand to spaced surface groups. Their small size should confer advantages to dendrimers in terms of their ability to diffuse in complex tissues. Interest in the uptake and the translocation of dendrimers inside the cells is undoubtedly crucial for the design and development of highly effective gene carrier systems. We recently reported a sixth generation amino terminated polyamide poly-lysine dendrimer – which we term Gly-Lys_{63} $(NH_2)_{64}$ with a molecular weight of 8149 – as a nano-fluorescent probe (Ruenraroengsak et al., 2005; Al-Jamal et al., 2006). The results of the uptake and mobility of this dendrimer probed with confocal laser scanning microscopy (CLSM) in Caco-2 and SK/MSE-1 cells are discussed in terms of exclusion, obstruction and binding effects within the cytoplasm.

The details of the synthesis and purification of the sixth generation poly-lysine dendrimer (Gly-$Lys_{63}(NH_2)_{64}$) molecular weight 8149 Da are described elsewhere (Sakthivel et al., 1998; Al-Jamal et al., 2006). Hoechst 33342 (trihydrochloride trihydrate) and wheat germ agglutinin, Alexa Fluor® 594 conjugate were purchased from Molecular Probes, UK. Human Caucasian colon adenocarcinoma cells (Caco-2) and Lung carcinoma cells (SKMES-1) were obtained from European Collection of Cell Cultures. Dulbecco's modified eagle medium (DMEM), Foetal bovine serum (FBS), MEM non essential amino acids (MEM), Trypsin-EDTA, Hanks' balanced salt solution and phosphate buffer saline without calcium and magnesium ions (D-PBS) were purchased from Gibco, UK.

The dendrimer possesses a broad emission spectrum and thus, to overcome the possibility of interference with other fluorescent probes used in the system, the excitation and emission spectra at various concentrations of dendrimer were analysed using confocal microscopy. The emission of the dendrimer is concentration dependent (Al-Jamal et al., 2006). The higher the dendrimer concentration the greater the fluorescent intensity detected. A desired concentration of the dendrimer, 0.05–0.2% (w/v), which

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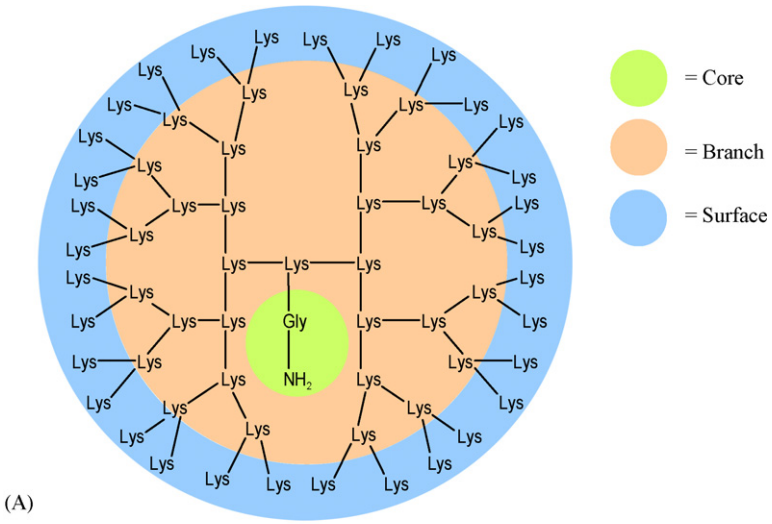
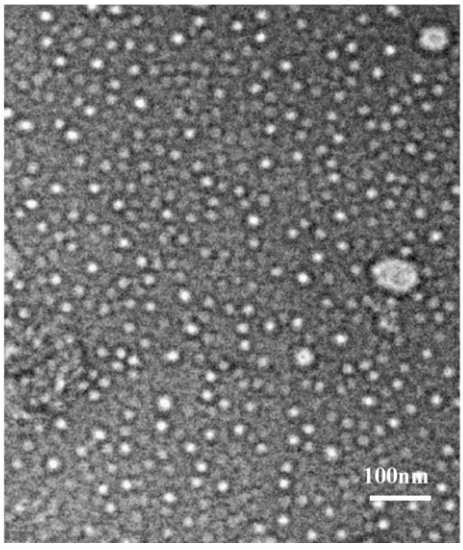


(A)



(B)

Fig. 1. The structure (A) and a transmission electron micrograph (TEM) (B) of the sixth generation poly-lysine dendrimer used in this study: the dendrimers were stained with 1% uranyl acetate and the micrograph was taken by Philips CM 120 (Einhoven, Netherlands) at magnification of 46,000× with an operation voltage of 120 kV.

can be used without interference with other fluorescent probes was selected and was used.

The method for uptake studies of the dendrimer in living cells has been addressed elsewhere (Al-Jamal et al., 2006). In brief, cells were seeded on glass cover slips coated with poly-D-lysine, in six-well plates at a density of 1×10^4 cells/well. After seeding for 24 h, the cell membrane and the cell nuclei were stained with wheat germ agglutinin, Alexa Fluor® 594 conjugate (5 μg/ml) and Hoechst 33342 (2.5–5.0 μg/ml) for 30 min. The cells were rinsed with PBS for 4–5 times. The cover slips were then transferred into a perfusion chamber and the fresh media was added into the chamber. The experiment was performed by adding dendrimers into the media. The uptake of the dendrimers was followed under CLSM (Al-Jamal et al., 2006); *z*-stack images of the cells were made before and after addition of the dendrimer. After the experiment the cells were re-incubated and viability of the cells was assessed. The distance between the plasma membrane and the nucleus of living cells (50 cells, of each type) was measured under the microscope using Leica confocal software (LSC Lite). Then the mean distance between the plasma membrane and nucleus, termed the "cytoplasmic radius", was calculated.

Dynamic uptake of the dendrimer was evaluated using LSC Lite software and custom procedures written in IgorPro software (Wavemetric Inc.). The fluorescent intensity (F) of the dendrimers (detected in green channel, Ch2) inside each *z*-stack images was averaged and standardized by background subtraction. The fluorescent intensity of the dendrimers in the regions of interest (ROIs) at each time point was measured and the apparent diffusion coefficient of the dendrimer was calculated (Ruenraroengsak et al., 2005; Al-Jamal et al., 2006). All experiments were carried out in triplicate.

Fig. 1 shows the chemical structure (A) and transmission electron microscopy (B) of the sixth generation amino terminated polyamide polylysine dendrimer (Gly-Lys_{63} $(NH_2)_{64}$) (MW 8149) which consists of glycine amino acid as a core

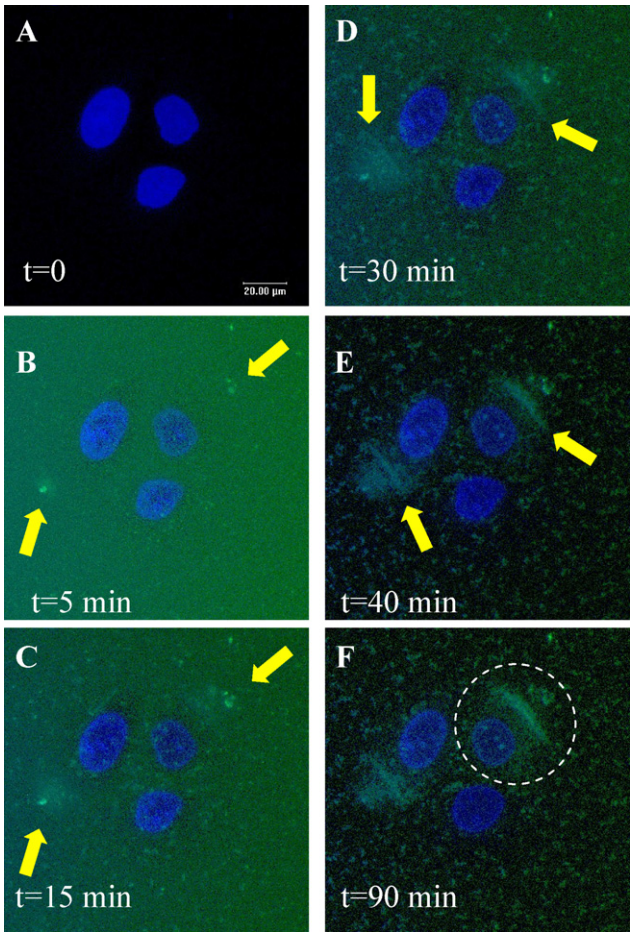


Fig. 2. The overlay of the compressed z-stack images of the dendrimer uptake process (green) in living Caco-2 cells (A–F); the cells were incubated with media containing 0.1% dendrimer and images collected every 5 min before adding the dendrimer (A), $t = 5$ min (B), 15 min (C), 30 min (D), 60 min (E) and 90 min (F) after of incubation period. The nuclei of the cells were stained with Hoechst 33342 represents in blue colour. The scale bar is 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

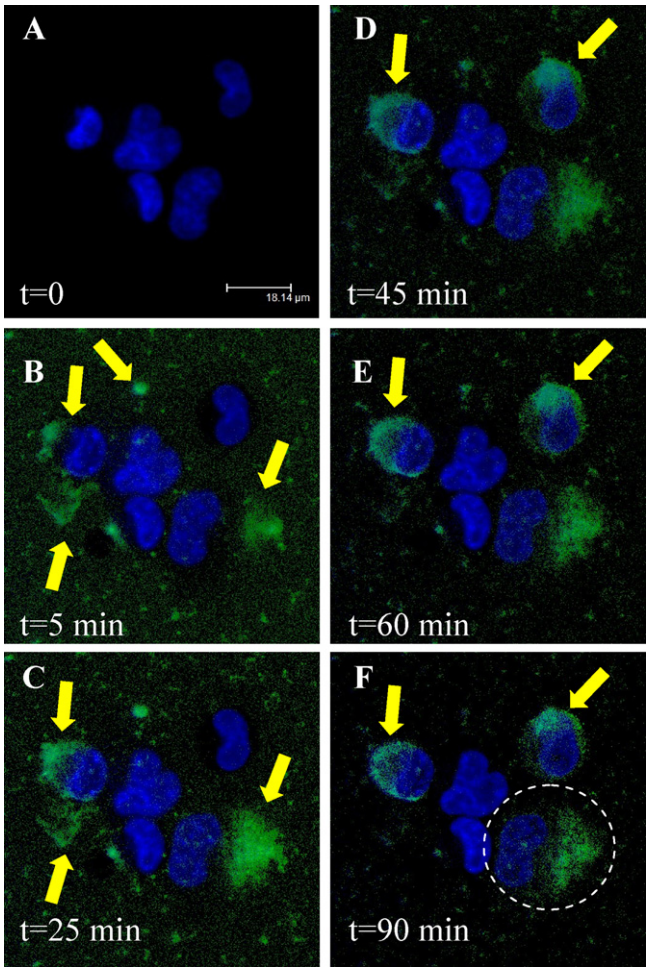


Fig. 3. The overlay images of compressed z-stack images of the dendrimer uptake process (green) in living SKMES-1 (A and B); the cells were incubated with media containing 0.1% dendrimer and images collected every 5 min before adding the dendrimer (A), $t = 5$ min (B), 25 min (C), 45 min (D), 60 min (E) and 90 min (F) after of incubation period. An area in white circle is the cell of interest. The nuclei of the cells were stained with Hoechst 33342 represents in blue colour. The scale bar is 18.14 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

and poly-lysine as a branched chain with 64 amino groups on the surface. The particle size of the dendrimer defined using Quanta/CHARMm (version 96 and 23.2, respectively) software (Accelrys) is 6.48 nm at pH 7.0. Some aggregation of the dendrimer can be seen in Fig. 1B under the conditions of TEM. This may be due to the high concentration use to mimic the conditions used in the uptake experiment. The zeta potential of the dendrimer in double deionised distilled water and in PBS were found to be $+55.6 \pm 3.27$ and $+51.7 \pm 2.83$ mV, respectively (Zetasizer 3500, Malvern Instruments, UK).

A similar pattern of dendrimer uptake was found in both cell types (Figs. 2 and 3). The results demonstrated that the dendrimers were first concentrated in the medium near the cell membrane. Two diffusion processes are occurring. First, the diffusion of the dendrimers within medium from the high concentration to the low concentration presents as a reduction of the green fluorescent signal inside the medium (Figs. 2B–F and 3B–F). Second, the dendrimers diffuse through cell membrane, which may be simultaneous with the movement of the dendrimers toward the cell nuclei (see arrows in Figs. 2 and 3).

The dynamic uptake of the dendrimer into the nucleus was examined by overlaying images of the dendrimers (in green channel (ch2)) over images of the nucleus (in blue channel (ch1). The fluorescent signal in the green channel represents the dendrimers in each ROI with the background fluorescent value subtracted. The relative fluorescent signal of the dendrimer was then plotted (F) against time (t, min) as can be seen in Fig. 4. The results demonstrated that the dendrimer reached the nucleus of Caco-2 cells within 35–45 min of incubation (Fig. 4A) whereas it took 25–30 min in SKMES-1 (Fig. 4B). This might be due to the differences of the intracellular architecture within each cell line and also the size of the cells. Lag times (t_L) required for the dendrimer to develop a uniform concentration gradient within the nucleus were used to calculate a diffusion coefficient. The diffusion layer here is called the cytoplasmic radius which rep-

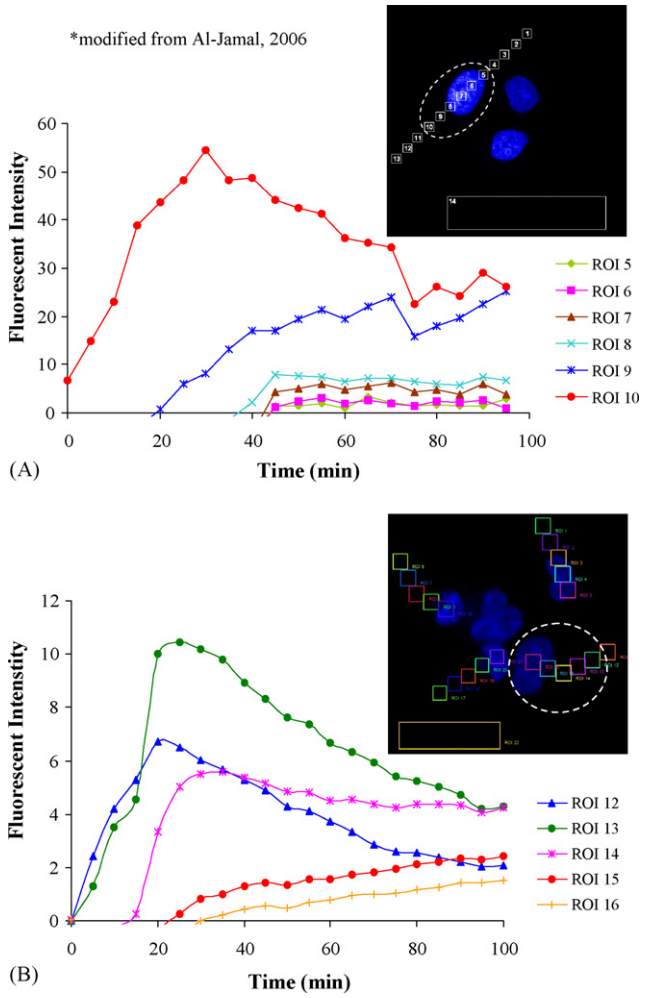


Fig. 4. The cumulative fluorescent intensity of the dendrimers inside Caco-2 cell (A) and SKMES-1 cell (B) as a function of time; ROI represents the region of interest, drawn on the cell (circled) in the inset pictures.

resents the mean distance between the plasma membrane and nucleus.

The cytoplasmic radius was found to be 12.09 (±6.02 (S.D.)) and 8.01 (±3.21 (S.D.)) μm for Caco-2 cells and SKMES cells, respectively. The diffusion coefficients (D_{cyto}) of the dendrimer in both cell lines were calculated, using the experimental t_L value. D_{cyto} values of the dendrimer in the cell cytoplasm of Caco-2 and SKMES-1 cells were found to be 9.82 (±0.98) × 10^{-11} and 5.99 (±0.16) × 10^{-11} $cm^2\ s^{-1}$, respectively. Cellular components such as secretory granules have diffusion coefficients, 1.9 × 10^{-11} $cm^2\ s^{-1}$, even lower than that of the dendrimers (Kaether and Gerdes, 1999) as might be expected from their larger dimensions. The diffusion coefficients (D_{cyto}) relative to those in water (79.35 × 10^{-8} $cm^2\ s^{-1}$ (D_{water})) are, respectively, 1.24 (±0.12) × 10^{-4} and 0.76 (±0.05) × 10^{-4}. These values implied that macromolecular crowding and obstacles in the cytoplasm provide a formidable barrier to dendrimer transport. The cytoplasm hinders the mobility of the dendrimer by 1000-fold compared to that in water. This may be explained in loose terms by exclusion and obstruction effects (Ruenraroengsak and Florence, 2005), the former due to the loss of free water by hydration of macromolecules and the latter due to the tortuous pathway that the dendrimer must travel in the cytoplasm avoiding organelles and other structural features, such as actin fibres. The diffusion of globular proteins (Arrio-Dupont et al., 2000) in muscle cells approximates to zero when the hydrodynamic radius of the protein approaches 7 nm (Tseng et al., 2004). This implies that the cytosol may behave like a gel which can restrict movement of the particles by a sieving effect. This is in accordance with the cell model proposed by Luby-Phelps et al. (1993) which describes the cytoplasm as a densely entangled filament network of actin bundles (insoluble phase) interpenetrated by a fluid phase crowded with globular macromolecules.

Furthermore, we have evidence of an interaction due to the electrostatic attraction between the dendrimer and the actin cytoskeleton *in vitro*, and have determined the diffusion coefficient of the dendrimer in the presence of actin using fluorescent recovery after photobleaching (FRAP) technique (unpublished data). The ratio D_{actin}/D_{water} was found to be 0.26. The viscosity of the cytosol is said to be 2.6 to 10-fold higher than water; the variation found here is, perhaps due to the differences in cell types and different techniques (Luby-Phelps et al., 1986, 1987; Kao et al., 1993; Swaminathan et al., 1996; Seksek et al., 1997; Swaminathan et al., 1997; Partikian et al., 1998). These viscosity values contribute to the retarded diffusion although the medium in which particles diffuse approximates to that of pure water (Fushimi and Verkman, 1991; Kao et al., 1993). Therefore, the auxiliary reduction of the translation diffusion of the dendrimers in cytoplasm should be attributed to microscopic barriers. Further studies on this are needed.

In conclusion an understanding of movement of delivery vectors within cells and in the nucleus is essential for the development of effective systems. Good models of the physical structure of cell interiors are required along with probes such as the one described here to advance the design of new delivery vectors.

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