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Note

Transport of poly amidoamine dendrimers across Madin–Darby canine kidney cells

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Abstract

The objective of this study was to determine the permeability of a series of poly amidoamine (PAMAM) dendrimers of generations 0–4 (G0–G4) across MDCK (Madin–Darby Canine Kidney) cell line. PAMAM dendrimers with incremental increase in size and molecular weight were labeled by fluorescein isothiocyanate (FITC) and the least polydisperse fractions were collected by size exclusion chromatography. MDCK cells were grown on Transwell® filters for four days. The conjugates were detected by HPLC equipped with fluorescence detector. The permeability of the dendrimers across MDCK cells was determined in the apical to basolateral direction. The rank-order permeability of the PAMAM dendrimers was $G4 \gg G1 \approx G0 > G3 > G2$. The permeability of mannitol in the presence of G4 increased by nine-fold. Results suggest that the transepithelial transport of PAMAM dendrimers is effected by both the polymer size, and the modulation of the cell membrane by the cationic dendrimers.

Keywords: Transport; Poly amidoamine (PAMAM); MDCK cells; Drug delivery

Polymeric carriers are the main component of most novel drug delivery systems. Attachment of polymers to small molecular weight drugs allows for the design of controlled drug delivery systems that aim at lowering toxicity and maximizing efficacy (Kopecek et al., 2000).

One class of polymeric drug carriers that have shown potential in controlled drug delivery is poly amidoamine (PAMAM) dendrimers. PAMAM dendrimers are hydrophilic, highly branched macromolecules with repeating functional units and a well-defined structure (Tomalia et al., 1985, 1986, 1990). PAMAM dendrimers consist of an ethylenediamine core, from which poly amidoamine ($–CH_2CH_2CONHCH_2CH_2N–$) repeat monomer extends in all directions. This uniform expansion of geometry gives rise to generations

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(Tomalia et al., 1990), which provides incremental increase in molecular weight and size (Fig. 1). Applications of PAMAM dendrimers in drug delivery include carrying Cisplatin for tumor eradication (Malik et al., 1999), delivery of genes (Haensler and Szoka, 1993), and antisense oligonucleotide delivery (Yoo et al., 1999).

Unlike linear polymers such as poly ethylene glycol (PEG), PAMAM dendrimers posses a rigid spherical structure in solution with a fixed molecular diameter. PAMAM dendrimers can also serve as macromolecular models for studying the influence of geometry on transport across biological barriers. Recently, microvascular extravasation of PAMAM dendrimers was compared to a corresponding PEG molecule of similar molecular weight (El-Sayed et al., in press). Results of these studies and others (Ghandehari et al., 1997) suggest that in addition to the molecular weight, surface charge groups and geometry play a role in the transport of macromolecules across biological barriers.

Originally derived from the distal renal epithelium of a normal male cocker spaniel, the MDCK (Madin–Darby Canine Kidney) cell line has shown promising results for measuring membrane permeability (Irvine et al., 1999). For passively absorbed compounds, drug permeability across MDCK cells is similar to permeability across Caco-2 cells (Irvine et al., 1999). Confocal microscopy has indicated that, confluent MDCK monolayers form a tight junction network within 2–6 days (Rothen-Rutishauser et al., 1998). This short communication describes the results of the transepithelial transport of a series of PAMAM dendrimers (G0–G4) across MDCK monolayers.

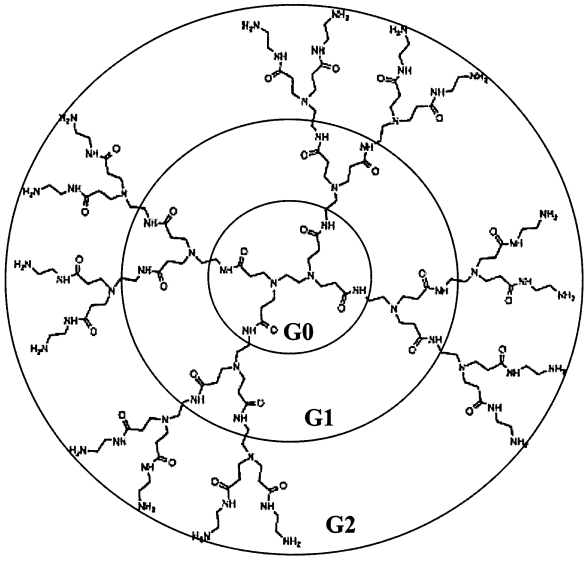

Fig. 1. Schematic structure of PAMAM. Successive incremental increases in size and molecular weight takes place in the same fashion as illustrated for G0–G2.

Aqueous solutions of G0–G4 of PAMAM dendrimers were purchased from Dendritech (Midland, MI). Fluorescein isothiocyanate (FITC), phosphate buffered saline (PBS) and HPLC-grade acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO). MDCK cells were obtained from ATCC (Rockville, MD). Six well cell cluster dishes with polycarbonate Transwell® filters were purchased from Corning-Costar (Cambridge, MA). Radiolabeled [^{14}C] mannitol (specific activity of 51.0 mCi/mmol) was obtained from Dupont NEN (Boston, MA). Hank's Balanced salt solution (HBSS), HEPES buffer, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma cell culture. Fetal bovine serum was purchased from Life Technologies, Inc. (Rockville, MD). The HPLC-equipment consisted of: Waters 510 pump (Milford, MA), Waters 717 Autosampler (Milford, MA), HP 3394 Integrator and Perkin-Elmer fluorescence detector LC 240 (UK). BDS Hypersil C18 column (120 Å, 3 μm, 150×4.6 mm ID) was purchased from Keystone Scientific (Pellefonte, PA).

PAMAM dendrimers (G0–G4) were labeled using fluorescein isothiocyanate (FITC) by procedure described previously (El-Sayed et al., in press). Briefly, PAMAM aqueous solutions were diluted with a dilution factor of 100 (v/v) in phosphate buffer saline (PBS) of pH = 7.4. The corresponding amount of FITC (i.e. polymer: FITC molar ratio = 1:1) was dissolved in acetone to give a solution of concentration < 5 mg/ml and added to regular unlabeled PAMAM solutions which were then allowed to stand overnight at room temperature with stirring.

Fluorescently-labeled PAMAM solutions were dialyzed against deionized water. The dialyzed

solutions were fractionated on a Superose 12 HR 16/50 preparative scale column using a Fast Protein Liquid Chromatography System (FPLC) (Amersham Pharmacia Biotech, Piscataway, NJ). The mobile phase was composed of (30:70 v/v) acetonitrile/Tris buffer (pH = 8) and the flow rate was adjusted to 1 ml/min. Detection of eluting molecules was done using an UV detector at a fixed wave length ($\lambda = 280$ nm). The fractions corresponding to the size and molecular weight of each probe were collected. The collected fractions of each probe were dialyzed against deionized water to remove the mobile phase salts. The dialyzed solutions were subsequently lyophilized and stored at 4°C for the permeability experiments.

To establish a detection methodology by HPLC for PAMAM dendrimers, previously developed analytical methods for FITC-labeled compounds with similar hydrophilicity to PAMAM dendrimers were considered. The detection methodology for PAMAM dendrimers was adapted from Sakai et al. (1998). Isocratic elution of the polymer–FITC conjugates was performed at a flow rate of 1 ml/min, using a mobile phase consisting of 88% phosphate buffer saline and 12% acetonitrile, adjusted to pH 7.25–7.30. The λ_{ex} and λ_{em} of the fluorescense detector was set to 494 and 518 nm, respectively. Standard curve regressions typically produced $< 5\%$ error. A linear correlation was obtained between the detector response and the concentration of the dendrimers with r^2 values higher than 0.99 (data not shown).

MDCK cells were maintained in DMEM, containing 10% fetal bovine serum. The cells were grown in T-75 flasks. At 80–90% confluency, the cells were trypsinized and split at 1:6 ratio. For transport studies, the cells were plated at a seeding density of 1×10^6 cells/4.71 cm^2 on Transwell® filters (0.4 μm pore diameter). The medium was changed 48 and 72 h post seeding. The cells were cultured for 4 days before use.

Transport studies were conducted using Transwell® filters in the apical to basolateral direction in HBSS containing 10 mM HEPES buffer (solution pH 6.8) at 37°C and 50 oscillations per minute. Monolayer integrity was monitored by determining [^{14}C] mannitol permeability. The volume of the donor and receiver compartments were 1.5 ml and 2.6 ml, respectively. After incubating the cells in HBSS, 1.5 ml of given polymer concentration in HBSS was added to the donor compartment (time = 0 min). At 30 and 60 min, the filters were moved to adjacent wells containing fresh HBSS (2.6 ml) in order to maintain sink conditions. The receiver samples were obtained at two time points, 30 and 60 min. At the end of each study, the receiver and donor samples were analyzed by HPLC. [^{14}C] mannitol was quantified using scintillation counter and the PAMAM samples were quantified by HPLC. Permeability coefficients (P_{app}) of the dendrimers were calculated according to equation 1:

$$P_{app} = \frac{\Delta Q/\Delta t}{A C_d}, \quad (1)$$

where P_{app} is the apparent permeability (cm/s) of the studied probe, $\Delta Q/\Delta t$ is the appearance rate in the receiver compartment (μg/s), A is the cross-sectional area (i.e. 4.71 cm^2), and C_d expresses the donor substance concentration (μg/ml). The permeability of the dendrimers was measured in triplicate. Results are presented as mean ± standard error of mean (SEM).

Permeability of PAMAM dendrimers G0–G4 was examined at a concentration of 100 μg/ml. Permeability of mannitol was studied in the presence and absence of G4. In addition, permeability of G2 was studied at a concentration range of 50–300 μg/ml.

Table 1 illustrates important physicochemical properties of the studied dendrimers and their respective permeabilities. P_{app} of PAMAM dendrimers was in the order of $G4 \gg G1 \approx G0 > G3 > G2$ (Fig. 2). P_{app} for G0 and G1 were similar, followed by a decrease for G2, and a subsequent increase for G3 and G4. These results suggest that in addition to molecular size, other factors are also involved in the permeability of dendrimers.

The high P_{app} of G4 can probably be attributed to the interaction of positively charged PAMAM dendrimers with the cell surface. Recent studies have pointed to the cytotoxicity of PAMAM dendrimers in a generation dependent fashion, i.e. higher generations showed a higher cytotoxicity (Roberts et al., 1996; Brazeau et al., 1998; Malik

Table 1
The molecular size, number of terminal amine groups and permeability values of PAMAM dendrimers

Probe	MW (Da)[a]	Diameter (Å)[a]	No. surface NH_2 groups per molecule[a]	$P_{app} \times 10^6$ (cm/s)[b]
G_0	517	15	4	1.02 ($\pm$0.084)
G_1	1430	22	8	1.08 ($\pm$0.064)
G_2	3256	29	16	0.076 ($\pm$0.0056)
G_3	6909	36	32	0.55 ($\pm$0.031)
G_4	14215	45	64	16.1 ($\pm$0.58)

[a] Reported by Tomalia et al. (1990).
[b] Permeability is the mean $\pm$ SEM of three analyzed MDCK monolayers.

et al., 2000). It has been shown that PAMAM G4 dendrimers are myotoxic in a concentration dependent fashion (Brazeau et al., 1998). Scanning electron microscopy of red blood cells, when exposed to PAMAM dendrimers have shown a 'clumping' behavior, where cells were brought into contact, probably by cell crosslinking in a generation-dependent fashion (Malik et al., 2000). Studies using other cell types (e.g. lung fibroblasts) have also shown a generation- and concentration-dependent cytotoxity when exposed to PAMAM dendrimers (Roberts et al., 1996). Results of these studies are consistent with the higher permeability of G4 observed in our experiments, suggesting that PAMAM permeability across MDCK cells is governed by a balance between the size of the polymer and its interaction with the cells.

The primary amine groups on the surface of PAMAM dendrimers have a pK_a value of 6.85, indicating that $\sim$50% are positively charged since this p*K*a value is about the same as the pH 6.8 of HBSS. As the generation of PAMAM increases, the number of surface NH_2 groups also increase (Table 1). However the total concentration of primary charged amine groups in the apical side for the permeability experiments was approximately the same ($\sim 2.3 \times 10^4$ mole/ml) regardless of the generation studied. This suggests that the cell modulation by PAMAM G4 appears to be not per se a function of the primary amine concentration in the apical solution. Rather, the larger molecular weight of PAMAM G4 relative to the lower generations may be responsible for influencing the integrity of MDCK monolayers.

To examine the influence of PAMAM G4 on cell integrity, the permeability of mannitol across the MDCK cells was determined in the presence and absence of G4 at 100 μg/ml. Mannitol permeability increased by nine-fold from 1.54 ($\pm$ 0.29) $\times 10^{-6}$ cm/s in the absence of G4 to 13.9×10^{-6} ($\pm$0.43) cm/s in the presence of G4, suggesting that cell integrity is compromised in the presence of G4.

The concentration dependency of the permeability of PAMAM G2 is illustrated in Fig. 3. As shown, the P_{app} of the concentrations 50 and 100 μg/ml are approximately the same with a tendency to increase at $\sim$130–200 μg/ml. The P_{app} increased by approximately three-fold from 200 to 300 μg/ml of G2 in the apical side. It has been shown that the cytotoxicity of PAMAM dendrimers in part depends on concentration, i.e. an increase in concentration generally leads to an increase in cytotoxicity (Roberts et al., 1996;

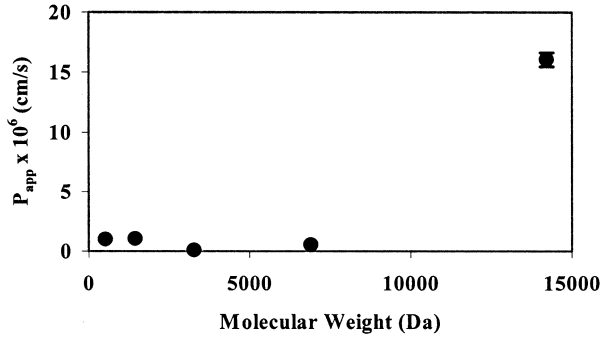

Fig. 2. P_{app} of PAMAM dendrimers G0–G4 at 100 μg/ml. For polymer characteristics, see Table 1. Results are in triplicate, mean value $\pm$ standard error of mean (SEM).

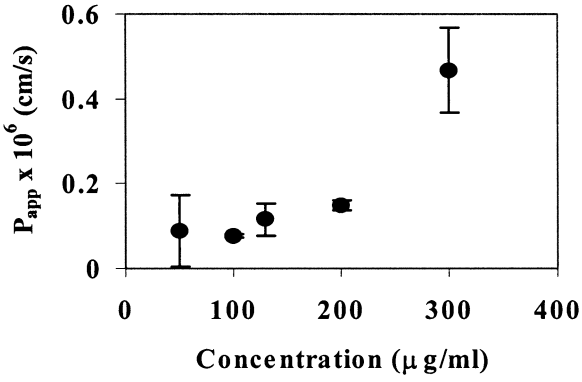

Fig. 3. P_{app} of PAMAM G2 at concentrations 50, 100, 130, 200 and 300 μg/ml. Results are in triplicate, mean value ± standard error of mean (SEM).

Brazeau et al., 1998). Consistent with reports in the literature, our results suggest that the cell modulation of permeability by PAMAM is concentration dependent.

In conclusion the results of this initial study suggest that the permeability of PAMAM dendrimers across MDCK cells appear to be a function of both the size of the dendrimers and their interaction with the cells. These studies pave the way for future detailed mechanistic and morphological studies that are necessary to elucidate the nature of the interaction of PAMAM dendrimers with epithelial cells.

References

Brazeau, G.A., Attia, S., Poxon, S., Hughes, J.A., 1998. In vitro myotoxicity of selected cationic macromolecules used in non-viral gene delivery. Pharm. Res. 15, 680–684.

El-Sayed, M., Kiani, M.F., Naimark, M.D., Hikal, A.H., Ghandehari, H., in press. The extravasation of polyamidoamine dendrimers across the microvascular network endothelium, Pharm. Res. (in press).

Ghandehari, H., Smith, P.L., Ellens, H., Yeh, P.-Y., Kopecek, J., 1997. Size-dependent permeability of hydrophilic probes across rabbit colonic epithelium. J. Pharmaco. Exp. Ther. 280, 747–753.

Haensler, J., Szoka, F.C., 1993. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjugate Chem. 4, 372–379.

Kopecek, J., Kopeckova, P., Minko, T., Lu, Z.-R., 2000. HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action. Eur. J. Pharmaceutics Biopharm. 50, 61–81.

Irvine, J.D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J.W., Selick, H.E., Russelo, G., 1999. MDCK (Madin–Darby Canine Kidney) cells: a tool for membrane permeability screening. J. Pharm. Sci. 88, 28–33.

Malik, N., Evagorou, E.G., Duncan, R., 1999. Dendrimer-platinate: a novel approach to cancer chemotherapy. Anti-Cancer Drugs 10, 767–776.

Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weener, J.W., Meijer, E.W., Paulus, W., Duncan, R., 2000. Dendrimers: relationship between structure and biocompatility in vitro, and preliminary studies on the biodistribution of ^{125}I-labelled polyamidoamine dendrimers in vivo. J. Control. Rel. 65, 133–148.

Roberts, J.C., Bhalgat, M.K., Zera, R.T., 1996. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst dendrimers. J. Biomed. Mater. Res. 30, 53–65.

Rothen-Rutishauser, B., Kreamer, S.D., Braun, A., Guenthert, M., Wunderli-Allenspach, H., 1998. MDCK cell cultures as an epithelial in vitro model: cytoskeleton and tight junctions as indicators for the definition of age-related stages by confocal microscopy. Pharm. Res. 15, 964–971.

Sakai, M., Imai, T., Ohtake, H., Azuma, H., Otagiri, M., 1998. Simultaneous use of sodium deoxycholate and dipotassium glycyrrhizinate enhances the cellular transport of poorly absorbed compounds across Caco-2 cell monolayers. J. Pharm. Pharmacol. 28, 27–33.

Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., Smith, P., 1985. A new class of polymers: starburst-dendritic macromolecules. Polym. J. 17, 117–132.

Tomalia, D.A., Baker, H., Dewald, J., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J., Smith, P., 1986. Dendritic macromolecules: synthesis of starburst dendrimers. Macromolecules 19, 2466–2468.

Tomalia, D.A., Naylor, A.M., Goddard, W.A., 1990. Starburst dendrimers, molecular-level of size, shape, surface chemistry, morphology, and flexibility from atoms to macroscopic matter. Angew. Chem. Int. Ed. Engl. 29, 138–175.

Yoo, H., Sazani, P., Juliano, R.L., 1999. PAMAM dendrimers as delivery agents for antisense oligonucleotides. Pharm. Res. 16, 1799–1804.