Anionic PAMAM Dendrimers Rapidly Cross Adult Rat Intestine *In Vitro*: A Potential Oral Delivery System?

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Received March 6, 2000; accepted May 4, 2000

Purpose. To investigate systematically the effect of polyamidoamine (PAMAM) dendrimer size, charge, and concentration on uptake and transport across the adult rat intestine *in vitro* using the everted rat intestinal sac system.

Methods. Cationic PAMAM dendrimers (generations 3 and 4) and anionic PAMAM dendrimers (generations 2.5, 3.5, and 5.5) that were modified to include on average a single pendant amino group were radioiodinated using the Bolton and Hunter Reagent. ^{125}I-Labelled dendrimers were incubated with everted sacs *in vitro* and the transfer of radioactivity into the tissue and serosal fluid was followed with time.

Results. The serosal transfer rates seen for all anionic generations were extremely high with Endocytic Indices (EI) in the range 3.4–4.4 μL/mg protein/h. The concentration-dependence of serosal transfer was linear over the dendrimer concentration range 10–100 μg/mL. For ^{125}I-labelled generation 5.5 the rate of tissue uptake was higher (EI = 2.48 ± 0.51 μL/mg protein/h) than seen for ^{125}I-labelled generations 2.5 and 3.5 (0.6–0.7 μL/mg protein/h) ($p < 0.05$). The ^{125}I-labelled cationic PAMAM dendrimers (generations 3 and 4) displayed a tissue uptake (EI = 3.3–4.8 μL/mg protein/h) which was higher ($p < 0.05$) than the rate of serosal transfer (EI = 2.3–2.7 μL/mg protein/h), probably due to nonspecific adsorption of cationic dendrimer to the mucosal surface.

Conclusions. As the anionic PAMAM dendrimers displayed serosal transfer rates that were faster than observed for other synthetic and natural macromolecules (including tomato lectin) studied in the everted sac system, these interesting nanoscale structures may have potential for further development as oral drug delivery systems.

KEY WORDS: PAMAM dendrimers; oral delivery.

INTRODUCTION

From the patient perspective, the oral route is by far the most convenient for drug administration. Nonetheless, for many drugs, novel delivery systems are still being sought that can reproducibly display one or more of the following features: optimal gastrointestinal (GI) transit, site-specific drug delivery (e.g., colonic delivery of anti-inflammatory drugs) and consistently high oral drug absorption. The latter is particularly important for oral administration of peptide and protein drugs that have notoriously poor oral bioavailablity (1,2). Nanometer-sized, highly branched macromolecules, commonly called dendrimers, arborols or cascade polymers (reviewed in 3), arising from innovative synthetic chemistry, offer potential advantages as drug carriers. They have narrow polydispersity and it is possible to tailor-make their surface chemistry by attachment of targeting/bioadhesive moieties, e.g., carbohydrate residues (4,5) or multiple peptidyl epitopes for vaccine development (6). Additionally the reduced structural density within the dendrimer core permits host-molecule entrapment with scope for subsequent controlled release. This is the so-called "dendritic box" (7).

Biocompatible polyamidoamine (PAMAM) dendrimers with a sodium carboxylate surface have already been described (8,9) that can target anticancer platinates after intravenous administration (10). The aim of this study was to determine whether small, 3–7 nm diameter PAMAM dendrimers have the ability to traverse the GI tract. (Preliminary results have already been presented in abstract form (11)). The improved everted rat intestinal sac method (12,13) was used as an *in vitro* model as this technique is routinely used to study the tissue uptake and serosal transfer of macromolecules and polymers (12,14,15). A series of PAMAM dendrimers with either an amine (generations 3 and 4) or sodium carboxylate (generations 2.5, 3.5 and 5.5) surface were chosen. Their characteristics are described in detail in Fig. 1 and Table I. The synthesis of these PAMAM dendrimers was pioneered by Tomalia and is described at length elsewhere (8). The PAMAM dendrimers used here were prepared using ethylenediamine as a core with further modification by sequential addition. First the core was reacted with methylacrylate (by Michael addition) to produce a methyl ester intermediate (designated the half generation). This was then reacted with ethylenediamine to produce the complete repeat unit (full generation) (8). To facilitate study of their transport, dendrimers were radioiodinated using the Bolton and Hunter Reagent. The amine-containing dendrimers were labelled directly whereas the dendrimers bearing a carboxylate surface were first modified to include (on average) a single pendant amino group.

Before transport studies were undertaken, inherent dendrimer toxicity towards intestinal tissue was carefully investigated by measuring the ability of the tissue to actively transport glucose in the presence of increasing concentrations of dendrimer. As a result, a concentration of ^{125}I-labelled PAMAM dendrimer known to be non-toxic (20 μg/mL) was chosen to study the effect of dendrimer generation (size) and charge on tissue uptake and serosal transport. Subsequently the concentration-dependency (0–100 μg/mL) of both processes was also studied using a 60 min incubation time.

MATERIALS AND METHODS

Materials

PAMAM dendrimers were obtained from Aldrich U.K. Ltd. They were synthesised from an ethylenediamine core with alternating sequential reaction of ethylenediamine and methylacrylate and characterised using methods described elsewhere (8). Ethylenediamine, medium 199 with Earle's salts, L-glutamine and sodium bicarbonate, *o*-dianisidine.HCl, peroxidase, glucose oxidase, copper sulphate pentahydrate, sodium potassium tartrate, monobasic potassium phosphate and bovine serum albumin, fraction V, (BSA) were obtained from Sigma Chemical Company Ltd. (U.K.). Sodium dodecyl sulphate was from Bio-Rad Laboratories. Folin-Ciocalteau's reagent was from Fluka.

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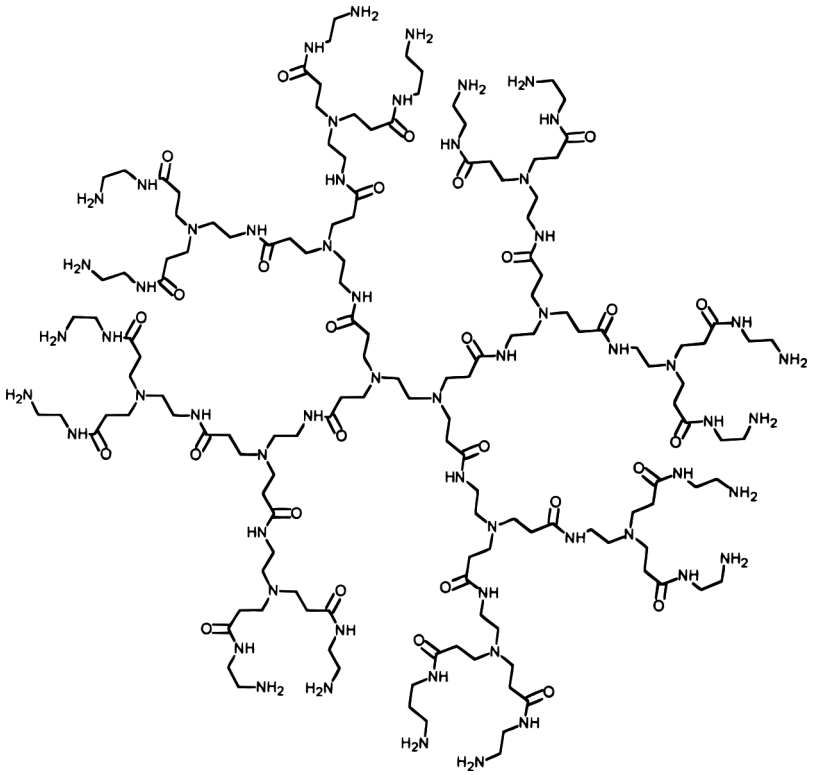


Fig. 1. Structure of PAMAM dendrimers. The example shown is generation 3. This is structurally similar to generation 2.5 which has the same number of terminal groups but in this case they are carboxylate (sodium salt) groups. The core and repeat units are the same for all dendrimers.

^{125}I-Labelled Bolton and Hunter reagent was from Amersham International (U.K.). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was obtained from Pierce-Warriner (U.K.). Wistar rats were from Banton & Kingman, U.K.

Preparation of Everted Rat Intestinal Sacs

Everted sacs were prepared according to the method of Woodley and colleagues (12,13). Briefly, adult male Wistar rats (250–350 g, 10–12 week old) were fasted overnight and killed by cervical dislocation. The small intestine was rapidly removed and placed in medium 199 oxygenated with a 95% O_2/5% CO_2 mixture and kept at 37°C. After washing through with saline (0.9% NaCl) the tissue was everted over a glass rod (2.5 mm in diameter) and was filled with oxygenated medium 199 (37°C). To prepare sacs, approximately 2.5 cm of small intestine without visibly containing Peyer's patches was tied off using surgical silk. Each sac was immediately placed in a sealed Erlenmeyer flask containing 10 mL of oxygenated medium 199 and placed in a shaking water bath at 37°C.

Table I. Characteristics of the PAMAM Dendrimers Used

Dendrimer	No. of surface groups	Mw Range (Da)	Approximate diameter (nm)	Termini
PAMAM dendrimers				
gen 3	32	6,909	3.1	$-NH_2$
gen 4	64	14,215	4.0	$-NH_2$
gen 2.5	32	6,011	3.1	COO^-Na^+
gen 3.5	64	12,419	4.0	COO^-Na^+
gen 5.5	256	50,865	6.7	COO^-Na^+

Intestinal Sac Viability

It was important to establish that the PAMAM dendrimers under investigation would not damage the intestinal tissue during the uptake studies and hence give spurious results. Tissue culture medium 199 contains glucose at a concentration of 1 mg/mL and thus active transport with time (medium to serosal) of glucose could be used as a measure of tissue viability. After incubating intestinal sacs (prepared as above) with PAMAM dendrimers (0–100 μg/mL) for 1 h, the serosal fluid and a sample of culture medium were collected and the glucose concentration in both samples determined using a modification of the method by Dahlqvist (16). Briefly, 20 μL of sample were incubated at 37°C with 1 mL containing glucose oxidase reagent (0.2% Triton-X100 (w/v in ethanol), 10 μg/mL *o*-dianisidine.HCl, 1 μg/mL peroxidase, 200 μg/mL glucose oxidase in 0.5 M Tris/HCl, pH 7.2). After 45 minutes, the reaction was terminated by the addition of 2 mL of 5 M HCl, and the absorbance was measured at 525 nm. Results were then calculated against a glucose standard curve that had been subjected to the same protocol.

Introduction of an Amine Group into Carboxylate PAMAM Dendrimers to Allow Radioiodination by the Bolton and Hunter Reagent

Anionic dendrimers (generations 2.5, 3.5 and 5.5) were supplied in methanol (10% w/v) as a sodium salt. Samples (10 mg) were first dried under a stream of nitrogen and then redissolved in double distilled water (DDW) to give a final concentration of 10 mg/mL. The pH was monitored and adjusted back to 6.5 with dilute HCl. EDC (a molar ratio sufficient to modify one carboxylate residue per dendrimer) was added and the reaction left for 30 min at room temperature. Ethylenediamine (also in a molar equivalence to modify one surface group) was then added slowly to prevent crosslinking. The reaction was left for 4 h and unreacted EDC (which hydrolyses off as urea) was removed by washing using a Centriprep® concentrator. The ninhydrin assay was used to verify the number of amino groups on the surface of the cationic and anionic-modified dendrimers (17).

^{125}I-Radiolabelling of PAMAM Dendrimers

The cationic PAMAM dendrimers gen 3 and 4 (10 mg) and the anionic dendrimers gen 2.5, 3.5 and 5.5 (20 mg) modified with ethylenediamine were dissolved in borate buffer 0.5 mL (pH 8.5, 0.1 M). ^{125}I-Labelled Bolton and Hunter reagent (0.5 mCi; 100 μL in benzene; Specific Activity 2000 Ci/mmol) was carefully dried under a stream of nitrogen. The dendrimer solution was then added and allowed to react for 15 min on ice, mixing periodically. A sample (5 μL) of the reaction mixture was removed for determination of the labelling efficiency and the remaining solution was carefully purified by dialysis against NaCl (1% w/v). The ^{125}I-labelled dendrimer preparations were then stored at 4°C until use. The labelling efficiency and percentage of free [^{125}I]iodine in each preparation was determined by paper electrophoresis. Samples of the crude reaction mixture and dendrimer preparation (4 μL) were loaded in the origin of a Whatman No 1 chromatography paper strip (5 × 30 cm). The strip was placed in a paper electrophoresis tank containing sodium barbitone buffer and run at 400 V for 30 min. After which, the paper was removed and 5 mm strips were cut and assayed for radioactivity. Results were plotted as counts per minute against the distance migrated and the amount of [^{125}I]iodide present as free and bound calculated. The labelling efficiency was in the range 80–95% and dendrimer Specific Activity in the range 1–12 μCi/mg (Table II).

Table II. Labelling Efficiency and Purity of ^{125}I-labelled PAMAM Dendrimers

Dendrimer	Labelling efficiency (% bound vs free)	Final purity (% free ^{125}I)	Specific activity (μCi/mg)
Carboxylate			
Gen 2.5	90.7	0.73	1.1
Gen 3.5	94.4	0.84	1.2
Gen 5.5	94.1	0.31	2.1
Amine			
Gen 3	75.6	0.71	9.4
Gen 4	80.5	0.98	11.8

Uptake of ^{125}I-Labelled Dendrimers by Everted Intestinal Sacs

Everted sacs (prepared as above) were incubated with ^{125}I-labelled PAMAM dendrimers for periods up to 2 h, at concentrations between 10–100 μg/mL (see legends to Figures). At each sample time, sacs were removed, washed 3 times with saline and blotted dry. Sacs were then weight and the serosal contents were carefully collected. Sacs were re-weighed after draining to accurately calculate the volume inside each sac. The individual sac tissues were digested in 5 M NaOH (5 mL) by incubation at 37°C overnight. Samples (1 mL) of the serosal fluid, incubation medium and the tissue digest were assayed for radioactivity. The tissue digest was also analysed for protein content using the Lowry method as modified by Peterson (18). The uptake of ^{125}I-labelled dendrimer by tissue and the serosal transfer was expressed either in terms of ng dendrimer/mg sac protein or as the clearance rate termed the "Endocytic Index" (EI). The EI expresses uptake as the equivalent volume of culture medium (μL) whose contained substrate is captured or transported /mg tissue protein/h. The EI is useful as it can be used to allow comparison of the rate of tissue uptake or transport of different substrates.

Gel Permeation Chromatography of Medium and Serosal Fluid

To determine the nature of radioactivity in samples, media and serosal fluid was routinely subjected to sephadex G-25 (PD-10 columns, Pharmacia) chromatography. Samples (1.0 mL) were applied to the column which was then eluted with phosphate buffered saline (0.5 mL fractions).

Statistics

A Student's t-test was used for all statistical analysis; p values of ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Uptake and Serosal Transfer of ^{125}I-Labelled Polymers with Time

The improved everted rat intestinal sac system developed by Woodley and colleagues (12–15) has been used for more than 20 years to measure polymer transport across the gut. Neutral hydrophilic polymers of molecular weight 10,000 to 40,000 Da such as ^{125}I-labelled polyvinylpyrrolidone (PVP), ^{125}I-labelled poly (N-vinylpyrrolidone-co-maleic anhydride) (NVPMA) (14) and ^{125}I-labelled N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers (15) are taken up by the intestinal tissue by fluid-phase endocytosis and transported across relatively slowly. Typically they display EI values in the range 0.6–1.6 μL/mg protein/h for tissue uptake and 0.1–0.3 μL/mg protein/h for serosal transfer respectively. Modification of ^{125}I-labelled poly (NVPMA) to introduce cationic or anionic pendant groups elevated tissue capture approximately 6 and 4.8-fold respectively, whilst serosal transfer was elevated 16 and 20-fold respectively. The anionic NVPMAs displayed the greatest rate of serosal transfer (14). Although tissue uptake and transfer of ^{125}I-labelled HPMA copolymers was shown to display molecular-weight dependance by (15), the higher tissue uptake (EI

= 3.2 μL/mg protein/h) and serosal transfer (EI = 0.5 μL/mg protein/h) seen for ^{125}I-labelled HPMA copolymers of Mw > 400,000 Da was probably due to capture and transfer by Peyer's patch tissue present in the everted sacs used for these studies.

As the cationic PAMAM dendrimer generation 4 showed some evidence of toxicity in the glucose-active transport assay at a concentration of 100 μg/mL (Fig. 2), a dendrimer concentration of 20 μg/mL was selected for the initial experiments designed to determine the effect of dendrimer generation and surface functionality on tissue uptake and serosal transfer. Tissue and serosal accumulation of all anionic dendrimers (Fig. 3a,b) increased linearly with time. The rate of tissue uptake of ^{125}I-labelled generation 5.5 was considerably higher (2.48 ± 0.51 μL/mg protein/h) than seen for ^{125}I-labelled generations 2.5 and 3.5 (0.6–0.7 μL/mg protein/h) ($p < 0.05$). In contrast, the serosal transfer rates for all anionic generations were similar and the EI values were extremely high (in the range 3.4–4.4 μL/mg protein/h). If the tissue and serosal radioactivity is expressed as a percentage of the total tissue uptake + serosal transfer, approximately 15–20% of dendrimer generation 2.5- and 3-associated radioactivity was found in the tissue whereas 80–85% was transferred to the serosal fluid. In the case of generation 5.5, approximately 30–35% of the radioactivity was recovered in the tissue and 65–70% in the serosal fluid and the serosal transfer seemed to plateau after the 60 min (Fig. 3).

The ^{125}I-labelled cationic PAMAM dendrimers showed a different pattern of accumulation (Fig. 4a,b). The tissue uptake was usually higher than serosal transfer at each time point ($p < 0.05$). Over the first 60 min, both generations 3 and 4 displayed the similar rate of tissue uptake (EI = 3.3–4.8 μL/mg protein/h) and serosal transfer (EI = 2.3–2.7 μL/mg protein/h). However, after 60 min, both the tissue and serosal accumulation of generation 3 plateaued. Generation 4 had a slower rate of tissue uptake and a constant rate of serosal transfer (Fig. 4a,b). Throughout approximately 55–60% of the radioactivity associated with generations 3 and 4 was located in the tissue and only 35–40% of the radioactivity was found in the serosal fluid.

For both cationic and anionic dendrimers gel permeation chromatography of serosal fluid samples showed that most radioactivity was macromolecular (always > 60% of the total radioactivity present) (Fig. 5a,b). This is consistent with transfer of dendrimer to the serosal compartment. Indeed the amount of free radiolabel in the serosal fluid was less than seen in the preparation or culture medium. All preparations contained <1%

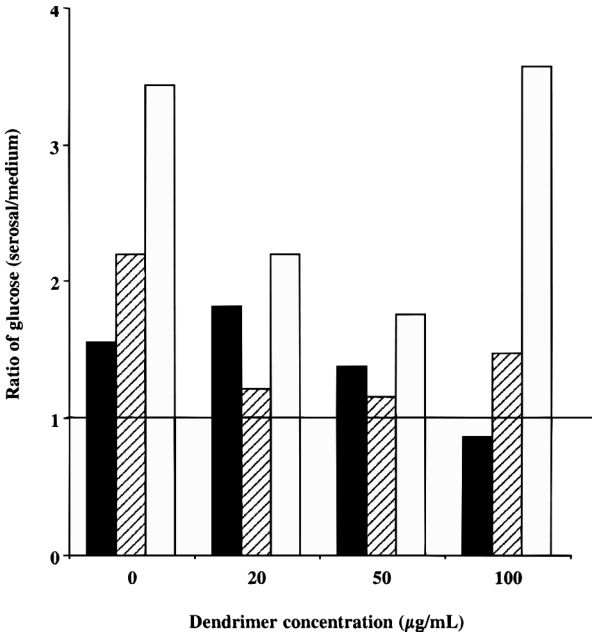


Fig. 2. Effect of PAMAM dendrimers on glucose active transport across evered sacs. The serosal:medium glucose ratio is shown after 90 min in the presence of increasing concentrations of dendrimer. The data show the ratio (n = 9) observed after incubation with PAMAM dendrimer generation 4 (■); generation 2.5 (▨); and generation 3.5 (□).

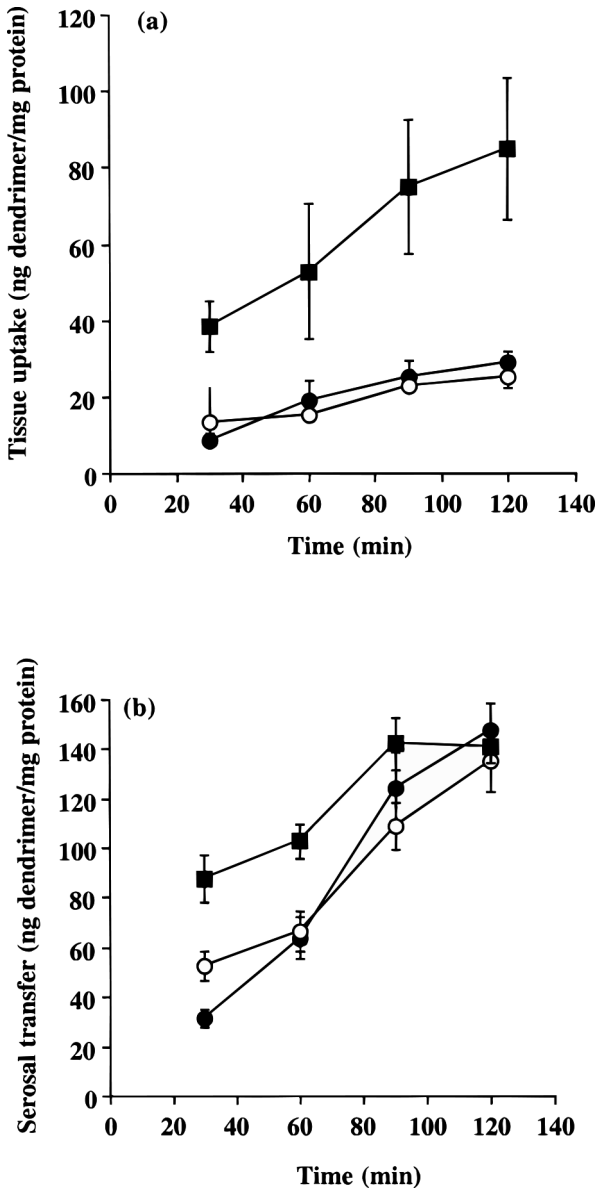


Fig. 3. Tissue uptake and serosal transfer of ^{125}I-labelled anionic PAMAM dendrimers incubated with everted rat intestinal sacs. Panel (a) tissue uptake and panel (b) serosal transfer. Data shown are the mean ± SD (n = 9). Key in both cases generation 2.5 (●—●); generation 3.5 (○—○); and generation 5.5 (■—■).

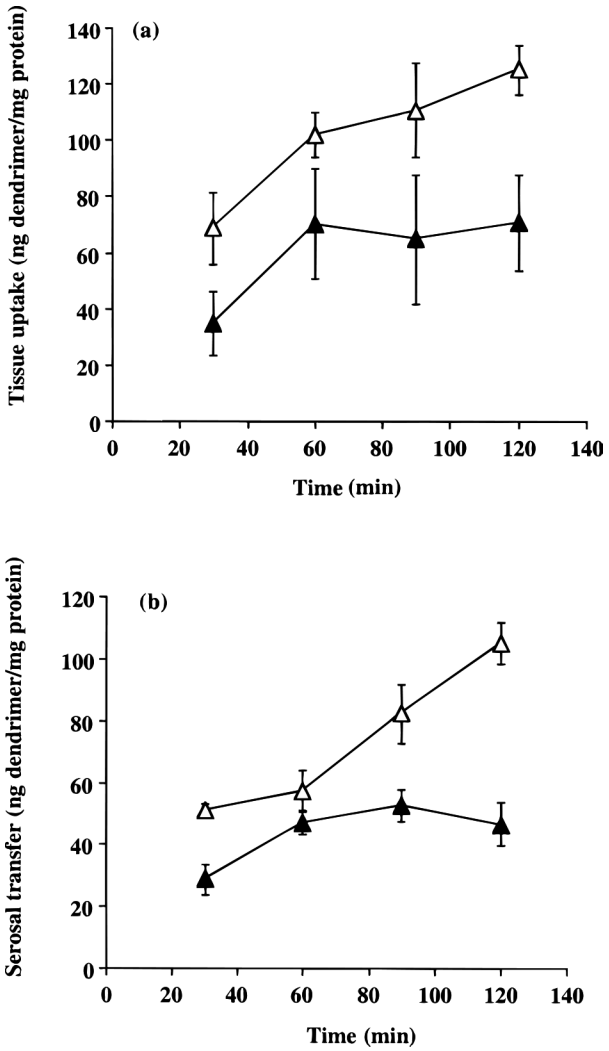


Fig. 4. Tissue uptake and serosal transfer of ^{125}I-labelled cationic PAMAM dendrimers incubated with everted rat intestinal sacs. Panel (a) tissue uptake and panel (b) serosal transfer. Data shown represent the mean ± SD (n = 9). Key in both cases generation 3 (▲—▲) and generation 4 (△—△).

free radiolabel at the time of use so it was interesting that the amount of "potentially contaminating free label" detected in the serosal samples was similar in all cases, irrespective of dendrimer generation and surface. This also indicates that the small amounts of free [^{125}I]iodide in the sample were a source for this serosal contamination.

Mechanism of Uptake

Dendrimers, like other macromolecules, could be transported across the intestine via either the transcellular or paracellular pathways. Small hydrophilic and charged drugs are often passively absorbed via the intercellular junctions. According to the literature, the approximate size limit for this pathway in rats is ~1.0–1.5 nm (19), but others have suggested that molecules with diameter up to 3 nm may pass via this route (20). Therefore, it is theoretically possible that generations 2.5 and 3.5 which have globular shape and small size (3.6 and 4.5 nm respectively) might use the paracellular pathway or indeed have the capacity to open transiently intercellular junctions. Markers such as [^{14}C]mannitol, [^{51}Cr]EDTA and radiolabelled poly(ethyleneglycol) (PEG) (400, 900 and 4000 Da) have all been routinely used as a markers for paracellular transport in Caco-2 cells (21–23) and rat small intestine (13,24). Using jejunal rat everted gut sacs (13) the rate of serosal uptake of [^{14}C]mannitol (182 Da) has been reported to be 8.9 μL/mg protein/h, passage through tight junctions being faster for low molecular weight compounds than the endocytotic route. Determination of the effect of dendrimers on the rate of paracellular transport of such markers would be interesting and might establish whether dendrimers have the capacity to open intercellular junctions.

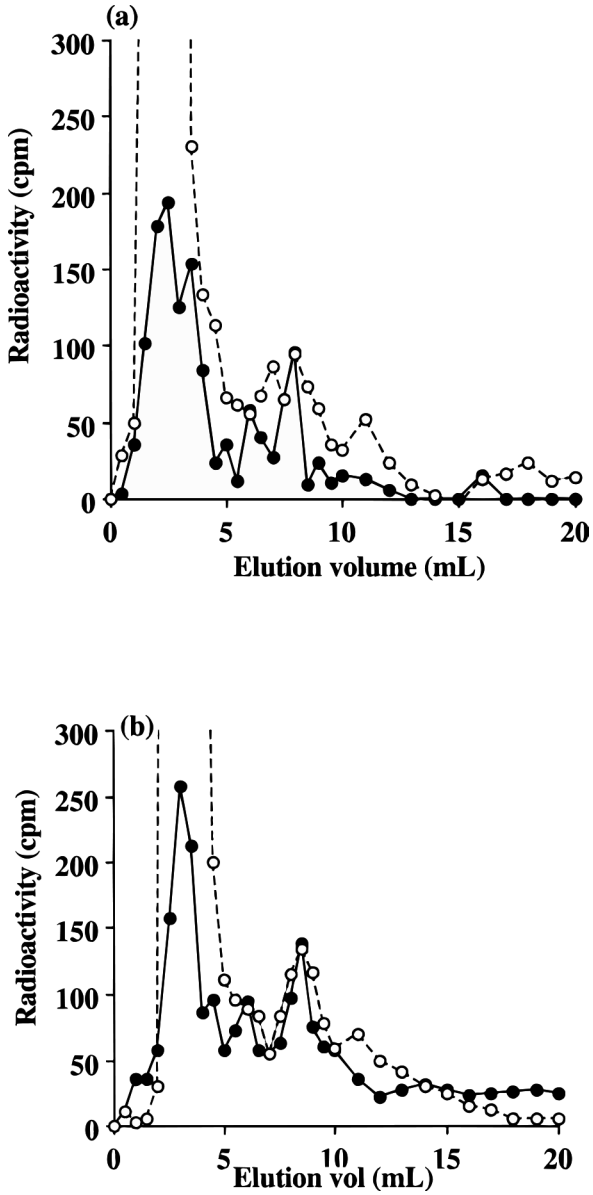
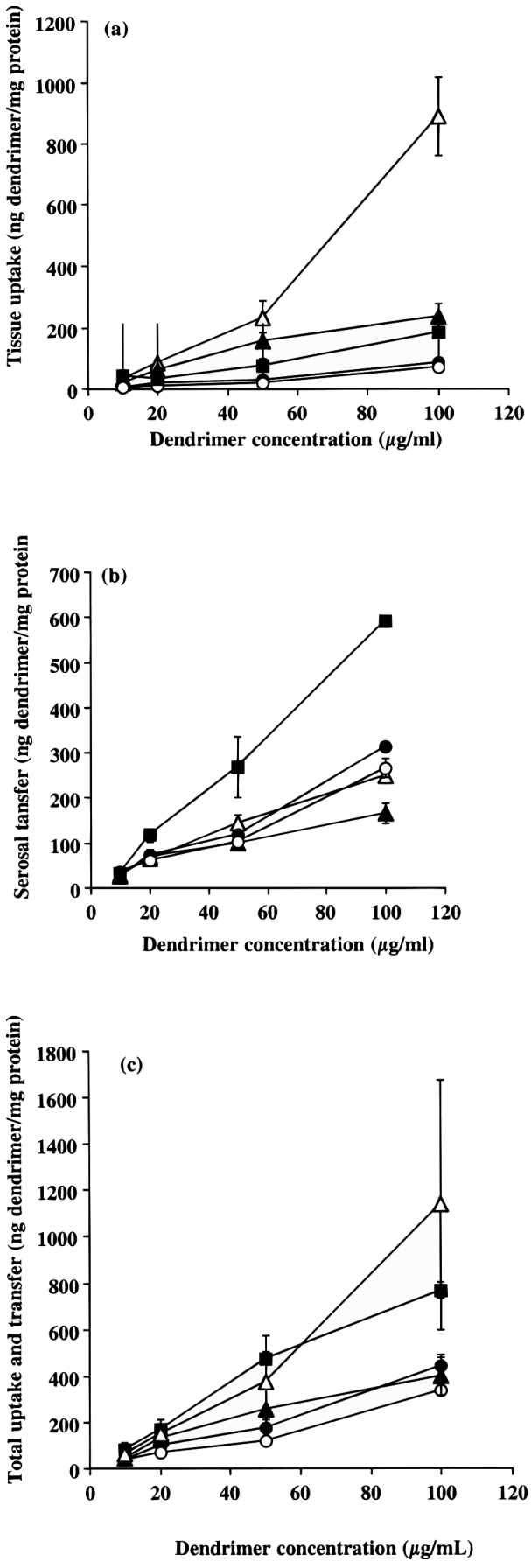


Fig. 5. Sephadex gel permeation chromatography (PD-10) of media and serosal fluid. As described in the Methods, samples of media (◯—◯) and serosal fluid (●—●) were subject to GPC analysis. The panels show typical results obtained for panel (a) generation 3 and panel (b) generation 3.5.



It is more likely that dendrimers, like the other polymers mentioned above, are taken across enterocytes by transcytosis. The upper size limit for particle transcytosis by enterocytes is approximately 100 nm (25), although recently it has been shown that 500 nm polystyrene particles coupled to Tomato lectin can transverse the rat intestine by this route (data from our laboratory, not shown). Additionally, we have shown previously that ^{125}I-labelled PVP of molecular weight up to ~100,000 Da can be taken up by epithelial cells by endocytosis (26). The rate of fluid-phase endocytosis in the everted sac system used here is already well established as 0.6–1.6 μL/mg protein/h for tissue uptake. Therefore, the EI values observed for ^{125}I-labelled generations 2.5 and 3.5 (0.6–0.7 μL/mg protein/h) is consistent with uptake via this mechanism. The observation that tissue uptake and serosal transfer of generations 2.5 and 3.5 increased linearly with substrate concentration (Fig. 6a,b) also indicates capture by fluid-phase endocytosis.

The high tissue EIs seen for the anionic PAMAM generation 5.5 (2.48 ± 0.51 μL/mg protein/h) and the cationic PAMAM dendrimers (3.3–4.8 μL/mg protein/h) would suggest that these molecules attach to the invaginating plasma membrane, and enter cells by specific or non-specific adsorptive endocytosis. As the uptake of generation 5.5 and cationic dendrimers also increased linearly with concentration (Fig. 6a–c) this would suggest that membrane binding is not saturable over the concentration range used. However, it is noteworthy that as the substrate concentration increased, the percentage radioactivity accumulating in tissue tended to increase. For example, ^{125}I-labelled generation 4 at a concentration of 20 μg/mL accumulated ~55–60% in tissue, whereas at the higher concentration of 100 μg/mL, the tissue level had increased up to 80% of the total capture. Although the tissue uptake rate of generation 4 at 100 μg/mL was very high, this is probably as a result of interference with the integrity of tissue as the cationic dendrimers are at their limit of biocompatibility at this concentration (Fig. 2).

The rate of serosal transfer of a fluid-phase marker like PVP or HPMA is typically low (0.1–0.3 μL/mg protein/h). Here the serosal transfer rates observed for the anionic PAMAM dendrimers (in the range 3.4–4.4 μL/mg protein/h) were much higher than previously measured for other substrates in this model, including the natural bioadhesive tomato lectin which has an extremely high tissue interaction (13.0 μL/mg protein/h) but relatively low rate of serosal transfer (0.85 μL/mg protein/h) (12) (Fig. 7). In future it will be important to understand better the mechanism of the rapid serosal transfer of dendrimers, but we have shown that the anionic PAMAM dendrimers are interesting hyperbranched, nanoscale architecture which provide a new opportunity for oral delivery.

CONCLUSIONS

Anionic PAMAM dendrimers generations 2.5 and 3.5 show particularly rapid serosal transfer rates and have low tissue

Fig. 6. Effect of dendrimer concentration on tissue uptake (panel a), serosal transfer (panel b), and total uptake (panel c) over 60 min. Data represent the mean ± SD (n = 9) and show generation 2.5 (●—●); generation 3.5 (○—○), generation 5.5 (■—■), generation 3 (▲—▲), and generation 4 (△—△).

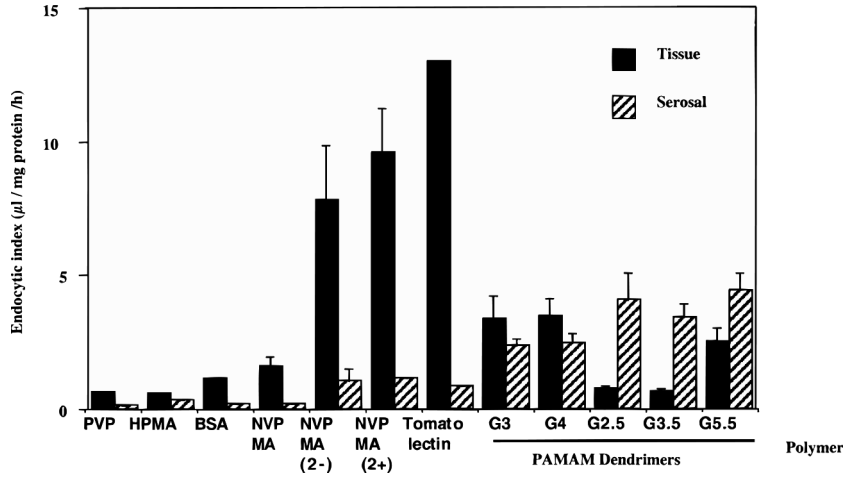


Fig. 7. Summary of the endocytic indices reported for tissue uptake and serosal transfer of various macromolecules (literature values: see text for references) and the dendrimers studied here.

deposition indicating a very efficient transport pathway. The rate of uptake and transfer was constant with time and did not show saturation over the substrate concentration range used. Generation 5.5 displayed higher tissue accumulation than 2.5 and 3.5 indicating size/conformation sensitivity of the transport mechanism. In contrast cationic PAMAM dendrimers showed much higher tissue association ($p < 0.05$ compared to anionic dendrimers), with lower transport rates. The plateau of tissue and serosal levels of the cationic dendrimers after 60 min suggested saturation of membrane binding sites; not surprising as the negatively charged cell membrane would interact strongly with these cationic molecules. Bioadhesion of cationic HPMA copolymers has been reported previously in the everted sac system and also other models (27,28). *In vivo* biodistribution studies are required to estimate the full potential of anionic PAMAM dendrimers for oral delivery and *in vitro* studies must be expanded to understand better their mechanism of transfer.

ACKNOWLEDGMENTS

RW would like to thank the Thai Government for supporting her postgraduate studentship.

REFERENCES

1. W. Wang. Oral protein drug delivery. *J. Drug Target.* **4**:195–232 (1996).
2. E. Allemann, J. C. Leroux, and R. Gurny. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. *Adv. Drug. Deliv. Rev.* **34**:171–189 (1998).
3. G. R. Newkome, C. N. Moorefield, and F. Vögtle. *Dendritic molecules*, VCH Publishers Inc. New York, 1996.
4. R. Roy. Recent developments in the rational design of multivalent glycoconjugates. *Topics in Current Chem.* **187**:241–274 (1997).
5. P. R. Ashton, S. E. Boyd, C. L. Bown, N. Jayaraman, S. A. Nepogodiev, and J. F. Stoddart. A convergent synthesis of carbohydrate-containing dendrimers. *Chem. Eur. J.* **2**:1115–1128 (1996).
6. J. P. Tam. Synthetic peptide vaccine design, synthesis and properties of a high density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA* **85**:5409–5413 (1998).
7. J. F. G. A. Jansen, E. M. M. de Brabander-van den Berg, and E. W. Meijer. Encapsulation of guest molecules into a dendritic box. *Science* **266**:1226–1229 (1994).
8. D. A. Tomalia, H. Baker, J. R. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, and P. Smith, A new class of polymers—starburst-dendritic macromolecules. *Polym. J.* **17**:117–132 (1985).
9. N. Malik, R. Wiwattanapatapee, R. Klopsch, K. Lorenz, H. Frey, J. W. Weener, E. W. Meijer, W. Paulus, and R. Duncan, Dendrimers: Relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of ^{125}I-labelled PAMAM dendrimers *in vivo. J. Control Release* **65**:133–148 (2000).
10. N. Malik, E. G. Evagorou, and R. Duncan. Dendrimer-platinate: A novel approach to cancer chemotherapy. *Anti-Cancer Drugs* **10**:767–776 (1999).
11. R. Wiwattanapatapee, B. Carreño-Gómez, N. Malik, and R. Duncan, PAMAM dendrimers as a potential oral drug delivery system: uptake by everted rat intestinal sacs *in vitro. J. Pharm. Pharmacol.* **50**:99 (1998).
12. B. Naisbett and J. F. Woodley. The potential use of tomato lectin for oral drug delivery: 2. Mechanism of uptake *in vitro. Int. J. Pharm.* **110**:127–136 (1994).
13. L. Barthe, J. F. Woodley, S. Kenworthy, and G. Houin. An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. *Eur. J. Drug Metab. Pharmacokin.* **23**:313–323 (1998).
14. J. Pató, M. Mora, B. Naisbett, J. F. Woodley, and R. Duncan. Uptake and transport of poly(N-vinylpyrrolidone-co-maleic acid) by adult rat small intestine cultured *in vitro*: Effect of chemical structure. *Int. J. Pharm.* **104**:227–237 (1994).
15. S. A. Cartlidge, R. Duncan, J. B. Lloyd, P. Rejmanova, and J. Kopeček. Soluble crosslinked N-(2-hydroxypropyl)methacrylamide copolymers as potential drug carriers. 1. Pinocytosis by rat visceral yolk sacs and rat intestinal cultured *in vitro*. Effect of molecular weight on uptake and intracellular degradation. *J. Control. Release* **3**:55–66 (1986).
16. A. Dahlqvist. Assay of intestinal disaccharidases. *Anal. Biochem.* **22**:99–107 (1968).
17. D. T. Plummer. *Introduction to practical biochemistry.* McGraw Hill, London, 1978.
18. G. L. Peterson. Determination of total protein. *Methods Enzymol.* **91**:95–119 (1983).
19. H. N. Nellans. Paracellular intestinal transport: Modulation of absorption. *Adv. Drug. Deliv. Rev.* **7**:339–364 (1991).
20. N. Pantzar, L. Lundin, L. Wester, and B. R. Westrom. Bidirectional

small-intestinal permeability in the rat to some common marker molecules *in vitro. Scand. J. Gastroenterol*. **29**:703–709 (1994).
21. S. Yee. *In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestine) absorption in man-Fact or myth. *Pharm. Res.* **14**:763–766 (1997).
22. G. Knipp, N. Ho, C. Barsuhn, and R. Borchardt. Paracellular diffusion in Caco-2 cell monolayers: Effect of perturbation on the transport of hydrophilic compounds that vary in charge and size. *J. Pharm. Sci.* **86**:1105–1110 (1997).
23. H. Yu, T. Cook, and P. Sinko. Evidence for diminished functional expression of intestinal transporters in Caco-2 cell monolayer at high passages. *Pharm. Res.* **14**:757–762 (1997).
24. M. Dowty and C. Dietsch. Improved prediction of *in vivo* per oral absorption from *in vitro* intestinal permeability using an internal standard to control for intra and inter-rat variability. *Pharm. Res.* **14**:1792–1797 (1997).
25. P. U. Jani, A. T. Florence, and D. E. McCarthy. Further histological evidence of the gastrointestinal absorption of polystyrene nanospheres in rat. *Int. J. Pharm.* **84**:245–252 (1992).
26. R. Duncan, M. K. Pratten, H. C. Cable, H. Ringsdorf, and J. B. Lloyd. Effect of molecular size of ^{125}I-labelled poly(vinylpyrrolidone) on its pinocytosis by rat visceral yolk sacs and rat peritoneal macrophages *Biochem. J.* **196**:49–55 (1980).
27. J. F. Bridges, J. F. Woodley, R. Duncan, and J. Kopeček. Soluble N-(2-hydroxypropyl)methacrylamide copolymers as a potential oral, controlled release, drug delivery system. 1. Bioadhesion to the rat intestine *in vitro. Int. J. Pharm.* **44**:213–223 (1988).
28. L. A. McCormick, L. C. W. Seymour, R. Duncan, and J. Kopeček. Interaction of a cationic N-(2-hydroxypropyl)methacrylamide copolymer with rat visceral yolk sacs cultured *in vitro* and rat liver *in vivo. J. Bioact. Compat. Polymers* **1**:4–19 (1986).