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Dendrimers as potential drug carriers; encapsulation of acidic hydrophobes within water soluble PAMAM derivatives

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Abstract—This paper describes the synthesis of three neutral water soluble poly(amidoamine) (PAMAM) dendrimer derivatives. The ability of the two larger dendrimers to bind small acidic hydrophobic molecules is reported. Spectroscopic data and pH behaviour suggested that the acidic hydrophobes were forming stable ion pairs with the dendrimer's internal, basic tertiary nitrogens. With respect to forming 1:1 and 2:1 substrate/dendrimer complexes, both of the larger dendrimers were equally efficient at binding. All dendrimer/substrate complexes were completely miscible with water in all proportions (i.e. infinitely water soluble). When the bound substrates are drug moieties, then the resulting complexes could be considered as potential drug delivery systems. Flow calorimetry demonstrated that the dendrimers were able to release their hydrophobic guests when in contact with a biological cell. © 2003 Published by Elsevier Science Ltd.

1. Introduction

We have been interested in applying dendritic molecules (dendrimers and hyperbranched polymers) towards a variety of applications, including catalysis,[1] drug and gene delivery,[2] biomimetics[3] and as solution phase supports for combinatorial chemistry.[4] However, this article will concentrate on our efforts towards the synthesis and application of water-soluble dendrimers for drug delivery.[5]

The therapeutic effectiveness of any drug is often diminished by its inability to gain access to the site of action in an appropriate dose. This is often due to the poor solubility of the drug in the body's aqueous environment. Medicinal chemists initially attempted to address this problem by synthesizing a water-soluble derivative of the drug moiety; unfortunately, even small structural changes can often reduce the efficacy of a drug. Another method of aiding solubilization is to encapsulate the drug within the hydrophobic domains of a colloidal or surfactant based system (i.e. emulsions, liquid crystals or micelles). However, the unstable dynamics of these systems, as well as their sensitivity to other functionality and pH, can lead to uncontrolled and premature release of the bound drug moieties, once again rendering this approach ineffectual. Ideally, a static or covalent micellar system is desirable. Micelle or surfactant-based delivery systems in effect dissolve drugs within their hydrophobic interior[6] and water solubility is then provided by the hydrophilic exterior of the micelle or surfactant based assembly. Dendrimers are spherical, branched macromolecules possessing a large number of terminal groups.[7] When these terminal groups are either charged or polar, then these macromolecules can indeed be considered as static, covalent micelles.[8] We therefore initiated a program of research directed towards the synthesis of water-soluble dendrimers, and an initial study into their host/guest properties. This paper describes the straightforward synthesis of a series of neutral water soluble dendrimers (based on simple poly(amidoamine) (PAMAM) dendrimers). The results from a preliminary investigation into the drug delivery potential of these static covalent micelles, are also presented.

2. Synthesis of the water soluble dendrimers

Although simple amine terminated PAMAM dendrimers are water soluble,[9] they are not well suited towards medicinal applications. Duncan et al. recently published a seminal paper describing the toxicity of amine terminated PAMAMs.[10] At physiological pH the terminal amines are protonated, these positive dendritic balls then attract and bind a number of negatively charged cells resulting in haemolysis. These results cast doubts with respect to any future clinical application of amine-terminated dendrimers and their derivatives (i.e. the activated dendrimers[11]). The paper by Duncan et al. therefore, serves as potent reminder that simple toxicological experiments should be performed before launching into a series of more complicated, expensive and perhaps unnecessary biological experiments (the plethora of papers reporting on the potential of

Keywords: dendrimers; PAMAM; drug delivery; static micelles.

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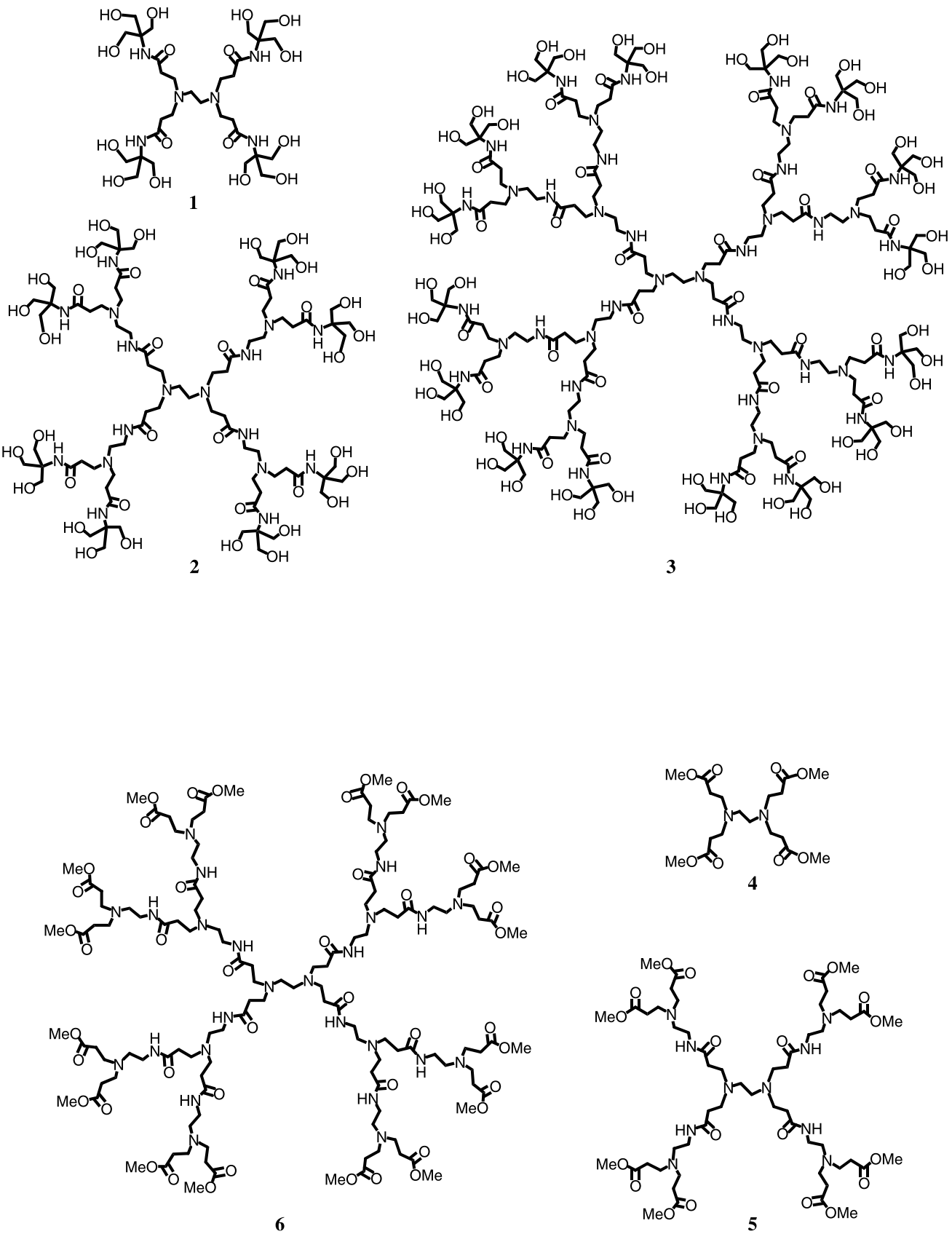


Figure 1. Hydroxyl terminated PAMAM dendrimers and their ester terminated precursors.

PAMAM dendrimers for clinical gene delivery is an excellent example). Although negatively charged carboxyl terminated dendrimers are less toxic,[10] they are less suited to drug delivery as they are unlikely to interact or bind to the negative surface of cells (ideally there should be an interaction between a cell and a drug delivery system). We therefore, decided to synthesise a series of neutral water soluble dendrimers. Rather than developing a new synthesis, our initial target molecules were simple derivatives of commercially available PAMAM dendrimers (Fig. 1).

Newkome has previously described how the TRIS group (tris(hydroxymethyl) amino methane) can be used to convert terminal esters into terminal hydroxyls.[12] Furthermore, the addition of TRIS converts one ester into three terminal hydroxyls, thus dramatically increasing the number of terminal groups in a single and final step. Using this procedure three neutral water soluble dendrimers, **1**–**3** were synthesized. One example is shown in Scheme 1, the ester terminated PAMAM dendrimer **5** (G 1.5), possessing 8 terminal esters, was reacted with 12 M equiv. of TRIS (original procedure as described by Newkome in 1990).[12]

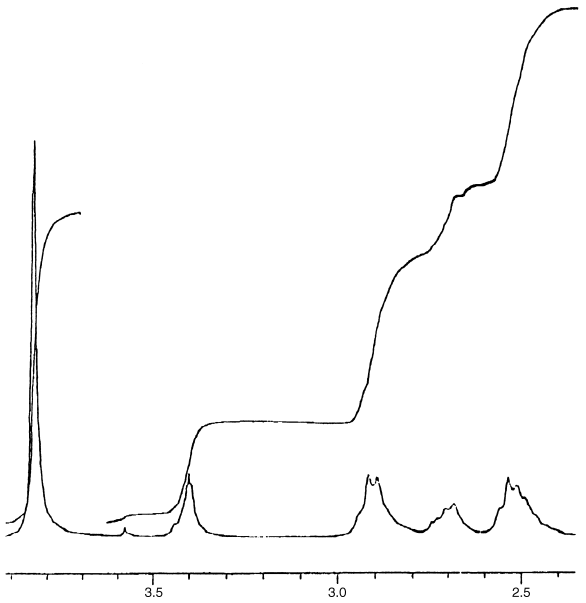
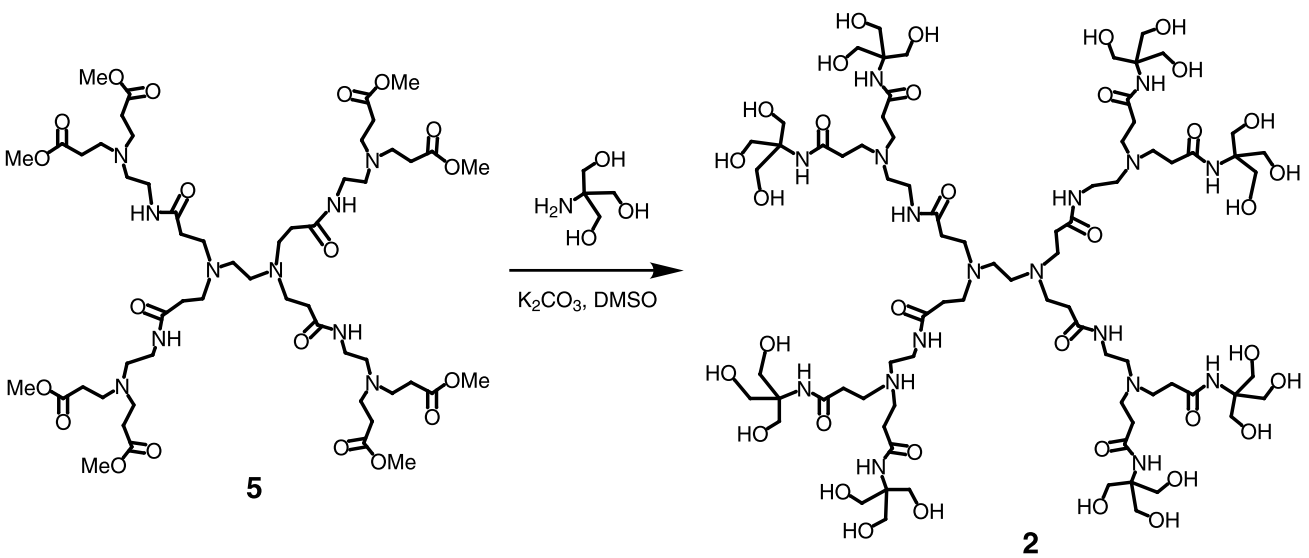


Figure 2. ^{1}H NMR spectrum of dendrimer **1** in D_2O.

The water soluble dendrimers could easily be characterized via ^{1}H NMR and ^{13}C NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectrum (in D_2O) of **1** is shown in Figure 2, all the expected signals at the correct intensity can be seen (albeit broad peaks). The resonances from the methyl ester at 3.5 ppm are no longer visible, confirming complete conversion of the ester groups. Furthermore, a new singlet at 3.82 ppm, resulting from resonances of the methylene protons adjacent to the terminal hydroxyl groups, is clearly evident. The ^{13}C NMR spectrum of **2** shows the correct number of carbon signals, for example, two clearly defined carbonyl peaks at 174 ppm (interior amides) and 176 ppm (exterior amides) can be seen. As in the ^{1}H NMR spectrum, the strong resonance corresponding to the methyl group from the terminal ester is no longer visible in the ^{13}C NMR, therefore, giving a good indication of purity. Absolute proof of purity was established using reversed phase HPLC; both of these dendrimers eluted to give single peaks, thus, indicating a high level of purity. A similar synthetic, purification and characterization procedure was used for the remaining dendrimers **1** and **3**; yields of 85 and 45% were obtained, respectively. As expected, all dendrimers proved to be extremely water soluble. In fact, the dendrimers were found to be completely miscible with water in all proportions (i.e. infinitely water soluble), so much so, that the solid dendrimers were extremely hydroscopic and required storage in a desiccator.

3. Complex formation

Benzoic acid is essentially insoluble in water at neutral pH,[13] and was therefore, an ideal substrate for an initial study of the dendrimer's solubilising and complex forming ability. A number of procedures for complex formation were tried, including the addition of benzoic acid to a stirred solution of dendrimer in phosphate buffered water (pH 7,



Scheme 1. Synthesis of dendrimer **2** with 24 terminal hydroxyl groups.

0.1 M), or formation of a dendrimer/benzoic acid paste, followed by the addition of phosphate buffered water (pH 7, 0.1 M). However, the most successful procedure for complex formation first involved the dissolution of both substances in methanol, this ensured that the crystal lattice of both substrates was broken. The methanol was then removed under vacuum giving a dendrimer/benzoic acid co-precipitate; on addition of phosphate buffered water (pH 7, 0.1 M), a complex of infinite water solubility was formed. These complexes remained stable at neutral pH, even after storage for several weeks. At this stage only 1:1 or 2:1 complexes were being assembled,[13] even so, we had increased the solubility of benzoic acid from 2.9 to 305 mg/mL via complex formation.[14] Measurement of the UV spectrum of the complexes confirmed both the presence and concentration of benzoic acid. Only the larger two dendrimers (**2** and **3**) were able to form stable inclusion complexes; however, as both dendrimers were equally capable of 'dissolving' benzoic acid when 1:1 or 2:1 complexes were formed, there seemed to be no real advantage (at this stage) in utilizing the larger, more expensive and synthetically less accessible dendrimer **3**. All further investigations therefore concentrated on the water soluble dendrimer **2** with 24 terminal OH groups.

At this stage we postulated that complexation might be due to a combination of hydrophobic binding, and hydrogen bonding (between the guest's carboxyl groups and the dendrimer's internal amides: **A** in Figure 3). In attempting to prove or disprove this hypothesis, ^{1}H NMR spectra of (i) the dendrimer, (ii) free benzoic acid and (iii) the dendrimer/benzoic acid complex, were recorded. If hydrogen bonding were an important factor with respect to binding, then we would expect to see differences in the amide and carbonyl resonances; these differences were not observed. Furthermore, on comparing the UV spectra of benzoic acid, before and after complexation, no changes in the spectra could be detected.

We also observed that all drug/dendrimer complexes were unstable at pHs less than pH 7 and the bound substrate begins to precipitate after only 10 min at pH 6. This suggests that it is the nitrogens within the dendrimer that are important with respect to binding, for as they are protonated (at pH 6 and below), the ability of the dendrimer to bind its guest is lost. This implied that the binding was probably due to a simple ion-pairing mechanism. The internal tertiary nitrogens are strongly basic, the pK_a of an aqueous solution of dendrimer **1** was measured as 9.5, and are therefore capable of deprotonating the acidic guest molecules. The ensuing quaternised nitrogens can then bind to the resulting carboxylate (or phenolate) counter-ions, **B** in Figure 3.[15]

Attempts to form inclusion complexes with other aqueous insoluble substrates and dendrimer **2** were also attempted; the solubility of the resulting complexes is displayed in Table 1. These substrates were chosen because of their antifungal or antibacterial properties. This would enable us to use microcalorimetric techniques to evaluate the drug delivery potential of these drug/dendrimer complexes. From the data in Table 1 and the observed pH dependence of binding, we concluded that it was only possible for acidic guest molecules to bind within the dendrimers interior, as tioconazole and other small non-polar molecules could not be retained within the dendrimer.

4. Microcalorimetric screening of the conjugates

In terms of drug delivery, the release of a substrate is just as important as its inclusion. There is little advantage in dissolving a drug if the host/guest interactions are so strong that they inhibit the delivery of the bound drug. In order to fully ascertain the drug delivery potential of these molecules we needed to demonstrate that the dendrimers could release their captive molecules when in contact with a biological cell. The drug molecules selected for our study were all antifungal or antibacterial agents, these were chosen because of their ease of screening using modern flow microcalorimetry.[16] When a small number of suspended

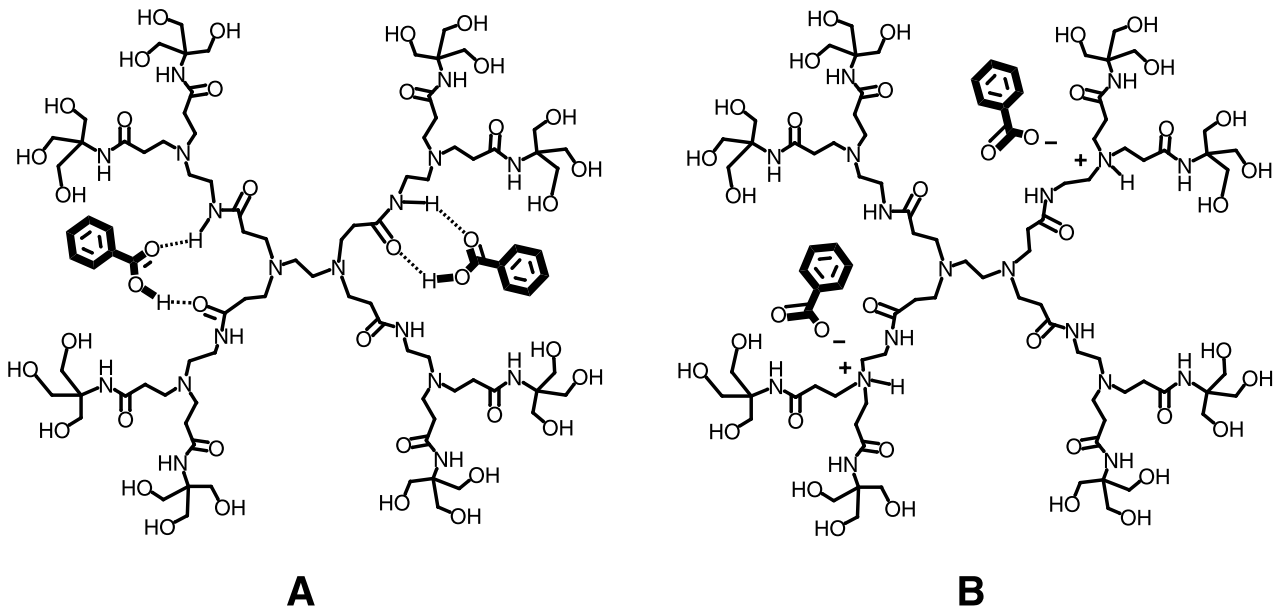
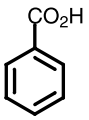
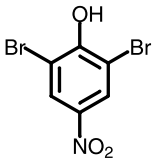
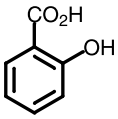
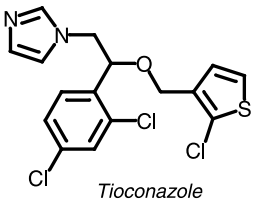


Figure 3. The unlikely hydrogen bonding interaction (**A**); the probable ion pairing interaction (**B**).

Table 1. Solubility of some small hydrophobic molecules bound within the hydroxyl terminated PAMAM dendrimer **2**

Substrate				 *Tioconazole*
Solubility	Infinitely soluble complex	Infinitely soluble complex	Infinitely soluble complex	No complex formed

bacteria flow through a microcalorimeter in a suitable growth medium, the subsequent growth can be detected by the production of heat, which can be recorded by modern instruments as power vs time curves ($p-t$ curves). Antibacterial agents would therefore, be expected to have significant effects on the $p-t$ curves obtained, and indeed such effects have been observed.[17] A schematic diagram of a flow calorimeter is shown in Figure 4. A solution of yeast in growth media (i.e. buffered glucose solution) is pumped through the calorimeter and as growth occurs, heat is produced (and detected). On addition of an antibacterial agent to the growth medium, cells are killed or growth prevented, this has a consequent effect on the heat produced and therefore, the magnitude of $p-t$ curve will be reduced. Flow microcalorimetry will enable us to make a qualitative study of the dendrimers capability to deliver antibacterial agents.

10 mg/mL stock solutions of the antifungal agents, as 1:1 drug-dendrimer complexes, were prepared in a buffered solution of glucose (phosphate buffer, pH 7). Various dilutions of these solutions, along with the controls (a solution of buffered glucose and a solution of dendrimer in buffered glucose), were pumped through the calorimeter. Each of these solutions was inoculated with a 1 mL suspension of *Saccharomycees s.p*. An overlay of the calorimetric traces for the dendrimer/2,6-dibromo-4-nitro phenol complex is shown in Figure 5. Clearly as the concentration of drug rises, from 1 to 4 mg/mL, then the biological response is reduced, until at 4 mg/mL no respiration can be detected. These results conclusively demonstrate that the dendrimer is capable of acting as a drug carrier, that is, without being toxic itself (i.e. the result from the control experiments). It can bind and deliver an antifungal drug and then deliver it after contact with a biological cell. A similar plot for the dendrimer/iodine complexes is obtained for a 2:1 iodine/dendrimer complex. Iodine is a powerful antibacterial and antiseptic agent used in hospitals throughout the world). On this occasion a 5 mg/mL stock solution of iodine, as a 2:1 drug-dendrimer complex, was prepared in a buffered solution of glucose (phosphate buffer pH 7). Various dilutions of the stock solution were made up and 24 mL of each were pumped through the calorimeter. Again, as the concentration of iodine rises, from 0.5 to 2 mg/mL, then the biological response is reduced until, at 2 mg/mL very little growth was detected. These two results clearly demonstrate that the dendrimer is capable of acting as a drug carrier, without being toxic itself.

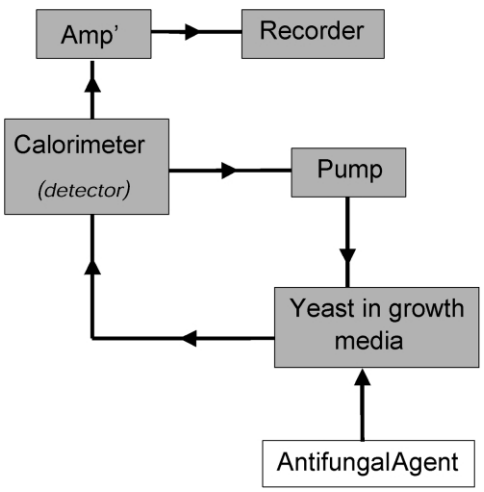


Figure 4. Schematic representation of a flow calorimeter.

The shape of these plots suggested that a direct kill was not occurring, and that populations of differently aged yeast cells were being prevented from growth (until at a high enough substrate concentration, no growth at all is possible). This was confirmed by a conventional microbiological tube assay, for the iodine complexes, which indicated that no kill occurred in the first 24 h. However, after 24 h varying degrees of kill could be observed, with 100% obtained at the maximum concentration used in the calorimetric study, 2 mg/mL (i.e. the same concentration at which a zero biological growth was obtained). The delayed response observed during the tube assay, could possibly indicate a delayed release mechanism for these dendrimeric delivery systems; this furthers broadens their potential use.

5. Conclusions

In conclusion, these results clearly demonstrate that simple water soluble dendrimers are capable of binding and solubilising small acidic hydrophobic molecules. Additionally, and by virtue of the infinite solubility of the complexes, the solubility of the bound hydrophobic guests can also be considered infinite. When these bound guests are drug molecules then the resulting complexes can be considered as potential drug delivery systems. The drug delivery potential of these systems was assessed using flow calorimetry and a series of hydrophobic antibacterial agents complexed within the hydrophobic interior of the water soluble dendrimers. The results from these experiments confirm that the dendrimers are able to release the drugs on contact with a biological cell. The plateau-like shape of the calorimetric

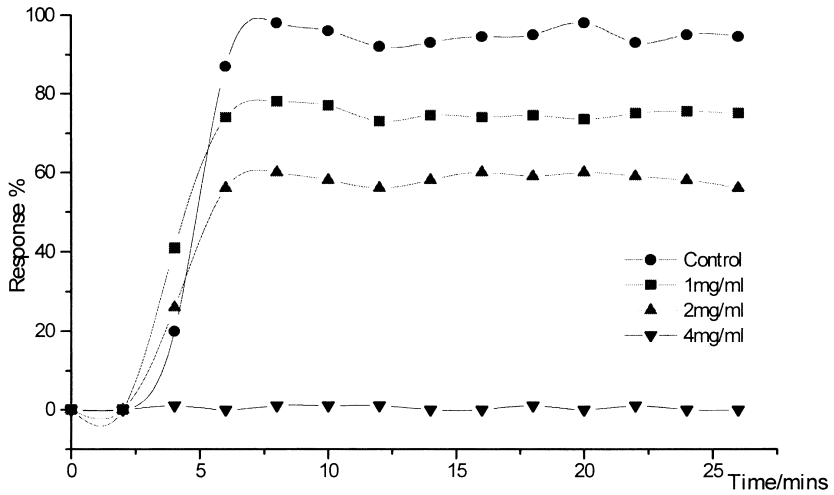


Figure 5. $p-t$ curves for the calorimetric output of *Saccharomycees s.p.* in the presence of dendrimer and drug/dendrimer complexes (traces above are for 2-6 dibromo-4-nitrophenol). The curve is the same for the buffer and dendrimer only in buffer (i.e. the control consists of two lines that are superimposed).

traces suggests that cells of different ages are being progressively killed as the concentration of drug increases, this suggests that a slow release mechanism may be occurring. Further work is in progress in our laboratory to develop these dendrimeric hosts as new and controlled drug delivery systems.

6. Experimental

6.1. Instrumentation

Infrared spectra were recorded on a Perkin–Elmer 983 instrument as thin films on KBr disks. ^{1}H NMR spectra were recorded on a JOEL FMX270FT or a Bruker AC250 spectrometer. ^{13}C NMR spectra were recorded on a JOEL FMX270FT or a Bruker AC250 spectrometer. Chemical shifts are reported in ppm and are referenced to residual solvent peaks. FAB mass spectra were obtained on a VG autospec mass spectrometer with ionisation in a matrix of 3-nitrobenzyl alcohol. HPLC was carried out on a C18 reverse phase analytical column, using a Pye Unicam PU40/0 HPLC pump and an LpC Refractometer III refractometer as detector.

6.1.1. PAMAM dendrimer with 4 terminal ester groups (4). Methyl acrylate (14.3 g, 0.166 mol) was added to a solution of ethylenediamine (2.0 g, 33.3 mmol) in methanol (30 mL). The reaction was stirred overnight and the excess reagents and solvent were then removed under vacuum. Yield 100%. IR (KBr, cm^{-1}) 3320 (broad), 1667, and 1642. ^{1}H NMR ($CDCl_3$) δ: 2.47–2.93 (20H, series of multiplets, all remaining CH_2) 3.83 (12H, s, CH_3O). ^{13}C NMR ($CDCl_3$) δ: 32.23, 49.41, 51.33, 51.85, 172.80. MS (FAB) m/z 405 $(M+H)^+$.

6.1.2. PAMAM dendrimer with 8 terminal ester groups (5). The ester terminated dendrimer **4** (2.0 g 4.95 mmol) was taken up in methanol (20 mL) and added dropwise to a stirred solution of ethylenediamine (22.3 g, 0.371 mol) in methanol (50 mL). The resulting solution was stirred at room temperature for 5 days. The excess ethylenediamine and solvent were then removed under vacuum. Final traces of ethylenediamine were removed by dissolving the dendrimer in 50 mL of butanol (a competitive hydrogen bonding solvent), the butanol was then removed under vacuum. This procedure was repeated (addition/removal of butanol) until no more ethylenediamine could be detected by GCMS. The crude tetraamine product was then used directly in the next step without further purification (final traces of butanol remained).[†]

Methyl acrylate (3.3 g, 38.7 mmol) was then added to a solution of the tetraamine (2.0 g, 3.87 mmol) in methanol (30 mL). The reaction was stirred overnight and the excess reagents and solvent were then removed under vacuum. Yield 98%. IR (KBr, cm^{-1}) 3320 (broad), 1667, and 1642. ^{1}H NMR ($CDCl_3$) δ: 2.47–2.93 (68H, series of multiplets, all remaining CH_2) 3.83 (24H, s, CH_3O). ^{13}C NMR ($CDCl_3$) δ: 32.67, 33.76, 37.31, 49.33, 49.96, 51.62. 52.55, 52.86, 172.76, 173.12. MS (FAB) m/z 1206 $(M+H)^+$.

6.1.3. PAMAM dendrimer with 16 terminal ester groups (6). The ester terminated dendrimer **5** with 8 terminal groups (2.0 g, 1.67 mmol) was dissolved in methanol (20 mL) and added dropwise to a stirred solution of ethylenediamine (24.0 g, 0.398 mol) in methanol (50 mL). The resulting solution was stirred at room temperature for 7 days. The excess ethylenediamine and solvent were then removed under vacuum. Final traces of ethylenediamine were removed using butanol (as described above for **5**) and the crude octa-amine used without further purification.[†]

Methyl acrylate (1.6 g, 18.5 mmol) was then added to a solution of the tetra amine (1.5 g, 1.05 mmol) in methanol (30 mL). The reaction was stirred overnight and the excess reagents and solvent were then removed under vacuum. Yield 94%. IR (KBr, cm^{-1}) 3320 (broad), 1667, and 1642. ^{1}H NMR ($CDCl_3$) δ: 2.47–2.93 (164H, series of multiplets,

[†] No traces of ethylenediamine could be detected at this stage, however, small amounts of butanol remained, even after persistent exposure to vacuum. After reaction with methyl acrylate these butanol peaks were no longer evident, suggesting that they had been hydrogen bonding to the terminal amine units.

all remaining CH_2) 3.83 (48H, s, CH_3O). ^{13}C NMR ($CDCl_3$) δ: 31.63, 32.94, 33.76, 36.66, 37.31, 48.47, 49.33, 49.86, 50.10, 51.62, 52.55, 52.89, 172.76, 172.94, 173.12. MS (FAB) *m/z* 2805 $(M+H)^+$, 2828 $(M+Na)^+$.

6.2. General procedure for the addition of TRIS groups to ester terminated PAMAM dendrimers

A solution of ester terminated dendrimer in anhydrous DMSO was added to a stirred suspension of TRIS (1.5 M equiv. per terminal ester) and anhydrous potassium carbonate (1.5 M equiv. per terminal ester) in dry DMSO. The reaction was stirred at 50°C under an atmosphere of nitrogen. The reaction mixture was filtered to remove excess solid reagents and the filtrate collected. The solvent was then removed by distillation using a Kugelrohr apparatus (0.5 mm Hg, 50°C). The resulting thick opaque oils were dissolved in the minimum quantity of water and precipitated with acetone. This precipitation procedure was repeated until the analytical HPLC trace showed just a single peak (reverse phase using methanol as the eluant). The product was collected and dried in a vacuum oven overnight to give the hydroxyl terminated dendrimers as extremely hydroscopic pale yellow solids.

6.2.1. PAMAM dendrimer with 12 OH groups (1). A solution of the PAMAM dendrimer with 4 terminal ester groups **4** (1.0 g, 2.47 mmol) in dry DMSO (5 mL) was added to a stirred suspension of TRIS (1.79 g, 14.9 mmol) and anhydrous potassium carbonate (2.08 g, 14.9 mmol) in dry DMSO (10 mL). The reactants were stirred at 50°C for 48 h before being purified as described above. Yield 88%. IR (KBr, cm^{-1}) 3312 (broad) and 1656. ^{1}H NMR (D_2O) δ: 2.47 (12H, m, NCH_2CH_2N+NCH$_2$CH_2CO) 3.63 (8H, br t, NCH_2CH$_2$CO). 3.94 (24H, bs, CH_2OH). MS (FAB) *m/z* 762 $(M+H)^+$.

6.2.2. PAMAM dendrimer with 24 OH groups (2). A solution of the PAMAM dendrimer with 8 terminal ester groups **5** (2.0 g, 1.67 mmol) in dry DMSO (10 mL) was added to a stirred suspension of TRIS (1.8 g, 15 mmol) and anhydrous potassium carbonate (2.1 g, 15 mmol) in dry DMSO (10 mL). The reactants were stirred at 50°C for 48 h before being purified as described above. Yield 78%. IR (KBr, cm^{-1}) 3320 (broad), 1667, and 1642. ^{1}H NMR (D_2O) δ: 2.51 (24H, broad multiplet, NCH$_2$CH_2CO), 2.70 (8H, broad multiplet, NHCH$_2$CH_2N), 2.91 (24H, broad multiplet, NCH_2CH$_2$CO), 3.41 (12H, broad multiplet, CH_2N (core)+NHCH_2), 3.84 (48H, s, CH_2OH). ^{13}C NMR (D_2O) δ: 34.5, 34.7, 38.4, 48.1, 50.0, 51.1, 53.5, 62.6, 63.6, 174.9, 176.0. MS (FAB) *m/z* 1919 M+H, and 1941 M+Na.

6.2.3. PAMAM dendrimer with 48 OH groups (3). A solution of the PAMAM dendrimer with 16 terminal ester groups **6** (1.0 g, 0.37 mmol) in dry DMSO (5 mL) was added to a stirred suspension of TRIS (750 mg, 6.05 mmol) and anhydrous potassium carbonate (850 mg, 6.05 mmol) in dry DMSO (15 mL). The reactants were stirred at 50°C for 72 h before being purified as described above. Yield 54%.

IR (KBr, cm^{-1}) 3318, broad, 1669 and 1642 (broad with a shoulder at ~1650). ^{1}H NMR (D_2O) δ: 2.42–3.08 (164H, broad series of multiplets, remaining CH_2) 3.95 (96H, s, CH_2OH). MS (FAB) *m/z* FAB 4233 $(M+H)^+$.

6.3. Complex formation

A typical procedure is described below: 2,6-dibromo-4-nitrophenol (250 mg, 0.89 mmol) and PAMAM dendrimer **2** with 24 OH groups (1.71 g, 0.89 mmol) were dissolved in methanol (50 mL). The solvent was slowly removed on a rotary evaporator to give a thick paste. 250 mL of water (pH 7.0, 0.1 M phosphate buffer) was added to give a 10 mg/mL stock solution of 2,6-dibromo-4-nitrophenol. At this stage the pH was checked to confirm that it remained at pH 7.0.

6.4. Antifungal calorimetric assay

Yeast cells, *Saccharomyces S.P*, were grown according to the procedure described by Beezer et al.[18] once grown these cells were collected and stored in 2 mL ampoules, these were then frozen (in liquid nitrogen), to provide a consistent and reproducible source of yeast cells. The samples could subsequently be recovered by placing the frozen ampoules in a water bath at 40°C for 3 min, and then held at room temperature for a further 2 min before use.

The calorimeter used in this study was a multichannel conduction calorimeter, operated in the flow-through mode at a flow rate of approximately 40 mL h^{-1}. The calorimeter was maintained at 37±0.2°C in a room maintained at 20±0.5°C. The input tube of the calorimeter was placed into a conical flask held at 37°C, which contained 24 mL of the respiration medium (a 0.1 M solution of phosphate buffered glucose at pH 7.0), this solution was then pumped through the calorimeter. The output tube of the calorimeter was returned to the original flask, therefore, giving a continuous looped volume. 1 mL of thawed yeast suspension was then added, giving a total volume in the loop of 25 mL. The peak height was measured as a percentage deflection on the recorder; this deflection was reproducible for a series of 1 mL inoculations (±5%). This deflection was subsequently normalized and made equivalent to a 100% biological response. The effect of various substrates could then be assayed as a measure of biological response (i.e. equal to or less than 100%).

6.5. Antifungal tube assay

1 mL of thawed yeast suspension was added to a tube containing 24 mL of the respiration medium (0.1 M solution of phosphate buffered glucose at pH 7.0). Various concentrations of dendrimer/drug complex were added (0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mg/mL). The tubes were visually examined periodically over the next 48 h (by qualitatively assessing the suspension).

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