Pergamon

SCIENCE DIRECT®

Tetrahedron 59 (2003) 3799–3813

TETRAHEDRON

Structure control within poly(amidoamine) dendrimers: size, shape and regio-chemical mimicry of globular proteins

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Received 29 November 2002; revised 21 February 2003; accepted 27 February 2003

Abstract—This work describes the syntheses of a new poly(amidoamine) (PAMAM) dendrimer family possessing a disulfide function (cystamine) in its core. Traditional redox-chemistry associated with the disulfide core in these dendrimer structures, provides a versatile strategy for designing unique sizes, shapes and controlling the regio-disposition of chemical groups on the surface of these dendrimers. Various single site, sulfhydryl functionalized dendron reactants may be generated in situ, under standard reducing conditions (i.e. dithiothreitol (DTT)). Facile control of size, shape and chemical functionality placement involves covalent hybridization of these single point, sulfhydryl reactive dendron components. This is accomplished by re-oxidation in the presence of air, to yield generation/surface chemistry differentiated cross-over products which may be isolated by preparative thin layer or column chromatography. Differentiated cystamine core dendrimers derived from combination and permutation of lower generation (i.e. Gen.=0–3) sulfhydryl functionalized dendrons possessing amino, hydroxyl, acetamido or dansyl surface groups, were synthesized and isolated. They were characterized by a variety of methods including; ^{13}C NMR, capillary electrophoresis (CE), gel electrophoresis (PAGE), thin layer chromatography (TLC) and electrospray (ES) or matrix assisted laser desorption ionization (MALDI-TOF) mass spectrometry. This general strategy has broad implications for the systematic size, shape and regio-chemical control of a wide range of dendritic nanostructures, many of which may be designed to mimic the sizes, shapes and regio specific chemo-domains observed for globular proteins.

1. Introduction

All living organisms in our universe are dependent on a variety of chemical, physical and biological strategies that have evolved over the past several billion years. These strategies led to the synthesis of a wide variety of precise nanoscale biostructures. This is evident in the routine biological production of proteins, viruses, cellular organelles, and other assemblies that possess dimensions that transcend the entire nanoscale region.[1,2] These unique structures such as DNA and proteins manifest the minimal level of 'ordered complexity' necessary to support, sustain and amplify the many familiar life forms of higher complexity.[3] Proteins are three-dimensional nanostructures with critical roles as both structural and functional components, that support the complexities of life. From the perspective of a chemist, such key strategies include: (a) 'genealogically directed,' covalent monomer sequencing (i.e. protein and DNA synthesis), (b) size/shape/chemical surface driven self assembly (i.e. supra and supramacromolecular chemistry) and (c) sequence induced, positional cross-linking within protein assemblies to produce persistent three dimensional shapes (i.e. cysteine related disulfide redox chemistry). Each of these strategies is part of an overall objective to produce nano-modules that are structure controlled as a function of size, shape and disposition of chemical functionality.[4]

Size is determined by the linear sequencing length (degree of polymerization) of the polypeptide chain. Appropriate sequencing of all twenty types of side chains found in the natural repertoire of amino acids, defines the secondary and tertiary structure of proteins. Shape, design, disposition of chemical functionality and flexibility is determined by the amino acid sequencing motif which in turn drives the folding process. Most notable, however, is the critical role that cysteine, a sulfhydryl functionalized amino acid plays in defining and stabilizing persistent dimensional shapes in proteins. Its sequence position in the polypeptide main chain determines the dominant quarternary structure (i.e. persistent three-dimensional shape) in both single chain and multi-chain proteins. Beginning with the first report by Anfinsen et al.,[5] considerable progress has been made in understanding the importance of 'disulfide redox chemistry' and its role in the determination of quarternary protein structure. These issues are reviewed elsewhere.[4]

Since the emergence of dendrimer technology in the early 1980s, the assembly of reactive monomers,[6] branch cells,[7,8]

Keywords: dendrimer; dendron; cystamine; disulfide; differentiated dendrimers; nanoscale modules; poly(amidoamine); protein mimicry.

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0040–4020/03/$ - see front matter
doi:10.1016/S0040-4020(03)00430-7

or dendrons[9] around atomic or molecular cores to produce dendrimers with adherence to either divergent or convergent, dendritic branching principles has become well recognized.[10,11] Such a systematic occupation of space around cores with monomers or branch cells as a function of generational growth stages (i.e. monomer shells) to give discrete quantized bundles of mass has been well demonstrated.[12] These parameters have been shown to be mathematically predictable[12,13] and exhaustively confirmed by mass spectrometry[14–17] gel electrophoresis[18,19] and other analytical methods.[18,19,29] Access to this level of macromolecular structure control has created substantial interest in the use of dendrimer structures as unimolecular mimics of globular proteins[20,21] micelles,[22–24] and a variety of other biological self-assemblies.[25,26]

The mimicry and comparison of spherical dendrimers to proteins was made as early as 1990.[26] The exquisite control of size, shape and surface chemistry that is possible with dendrimers has generally allowed their use as replacements for proteins in a variety of applications. Such uses include diagnostics,[27] gene delivery,[28] molecular weight calibrators,[29] presentation of antigens,[30] enzyme mimics, site isolation,[21] globular protein mimics, etc.[31]

Using strictly abiotic methods, it has been widely demonstrated over the past decade that dendrimers[32] can be routinely constructed with control that rivals the structural regulation found in biological systems. The close scaling of size,[20] shape and quasi-equivalency comparison of dendrimer surfaces[33–35] to nanoscale biostructures is both striking and provocative (Fig. 1).

These remarkable similarities suggest a broad strategy based on rational biomimicry as a means for creating a repertoire of structure-controlled, size and shape-variable dendrimer assemblies. Successful demonstration of critical chemistry to support such a biomimetic approach could provide a versatile and powerful synthetic strategy for systematically accessing virtually any desired combination of size, shape and surface in the nanoscale region. This would be possible by combinatorial variation of dendrimer module parameters such as: (i) families (interior compositions), (ii) surfaces, (iii) generational levels, or (iv) architectural shapes (i.e. spheroids, rods, etc.).

As early as 1990–1994, we proposed the use of focal point functionalized dendrons as a strategy for producing surface differentiated dendrimers.[12,26,36] Such intermediates are produced routinely during the course of practicing the 'convergent strategies' to various dendrimer targets. Fréchet subsequently reported the use of such intermediates for the synthesis of several differentiated *dendri*-poly(ethers). However, the primary focus since that time has been to use these intermediates for the hybridization of dendritic architecture with other polymeric architectural classes.[37,47]

This present work focuses on the use of 'divergent strategies' to produce focal point functionalized dendrons/di-dendrons. Specifically, we describe the use of disulfide redox chemistry found in cystamine core dendrimers to produce 'single site reactive' di-dendrons. Hybridization of these regio-specific sulfhydryl dendron components provides a very versatile strategy for shape designing and chemo-differentiation of dendrimer surfaces. In addition to globular protein mimicry, this methodology offers broad implications for the systematic shape and regiochemical control of organic nanostructures in general.

2. Results and discussions

2.1. Synthesis

Seven generations of a new family of tetra-dendron; cystamine core; poly(amidoamine) dendrimers were synthesized by the 'divergent, in situ branch cell' method.[32] A slight modification of the standard procedure[38] was used. This method involved a two-step iterative sequence to produce either ester or amine terminated structures (Scheme 1). Iterative sequencing involved (a) alkylation with methyl acrylate followed by (b) amidation with excess ethylenediamine. The alkylation step produces ester terminated (sub-shells) that are referred to as 'half-generations' and are designated (Gen.=0.5*n*). The second step involved amidation of the ester terminated (Gen.=0.5*n*) intermediates with large excesses of ethylenediamine to produce amine terminated, full generations, referred to as (Gen.=*n*). These iteration sequences define shells of the core-shell architecture associated with dendrimers and are catalogued in Figure 2. A shorthand designation for these structures is as follows: [Core]; *dendri*-PAMAM-$(-CO_2Me/-NH_2)_{Z(0.5n \text{ or } n)}$, where Z is the theoretical number of surface groups for generation (n) as determined by Eq. (1). The initial structures obtained from the first two reaction sequences are tetra-armed 'starbranched' PAMAMs. As such they are designated: [CYS]; *star*-PAMAM-$(CO_2Me)_{4(-0.5)}$ and [CYS]; *star*-PAMAM-$(NH_2)_{4(0)}$, respectively. The next iteration produces dendritic structures that are designated; [CYS] *dendri*-PAMAM$-(CO_2Me)_{8(0.5)}$ and [CYS] *dendri*-PAMAM$-(NH_2)_{8(1)}$, respectively.

Sixteen cystamine core PAMAM dendrimer products (i.e. G=0–7) in this series were obtained in crude isolated yields ranging from nearly quantitative to 77%. Ultra-filtration separation procedures were used to isolate the products which were generally obtained as pale-yellow syrups. In most cases, these syrups could be lyophilized to yield hygroscopic, light yellow solids.

The cystamine core appeared to undergo divergent dendronization reactions more slowly and produced more structure defects, under standard conditions, than the corresponding hydrocarbon cores (i.e. α,ω-(ethylene, butylene and dodecylene) diamine cores). The reasons for this behavior are not entirely clear at this time. Acquisition of pure, defect-free dendrimers generally required fractionation of the crude products on a silica gel column. Recovered yields of pure mono-disperse dendrimers from the crude products generally averaged 30–65%. The side products consisted primarily of missing arms, loops and higher dimeric/oligomeric megamers as determined by MALDI-TOF and electrospray mass spectrometry.

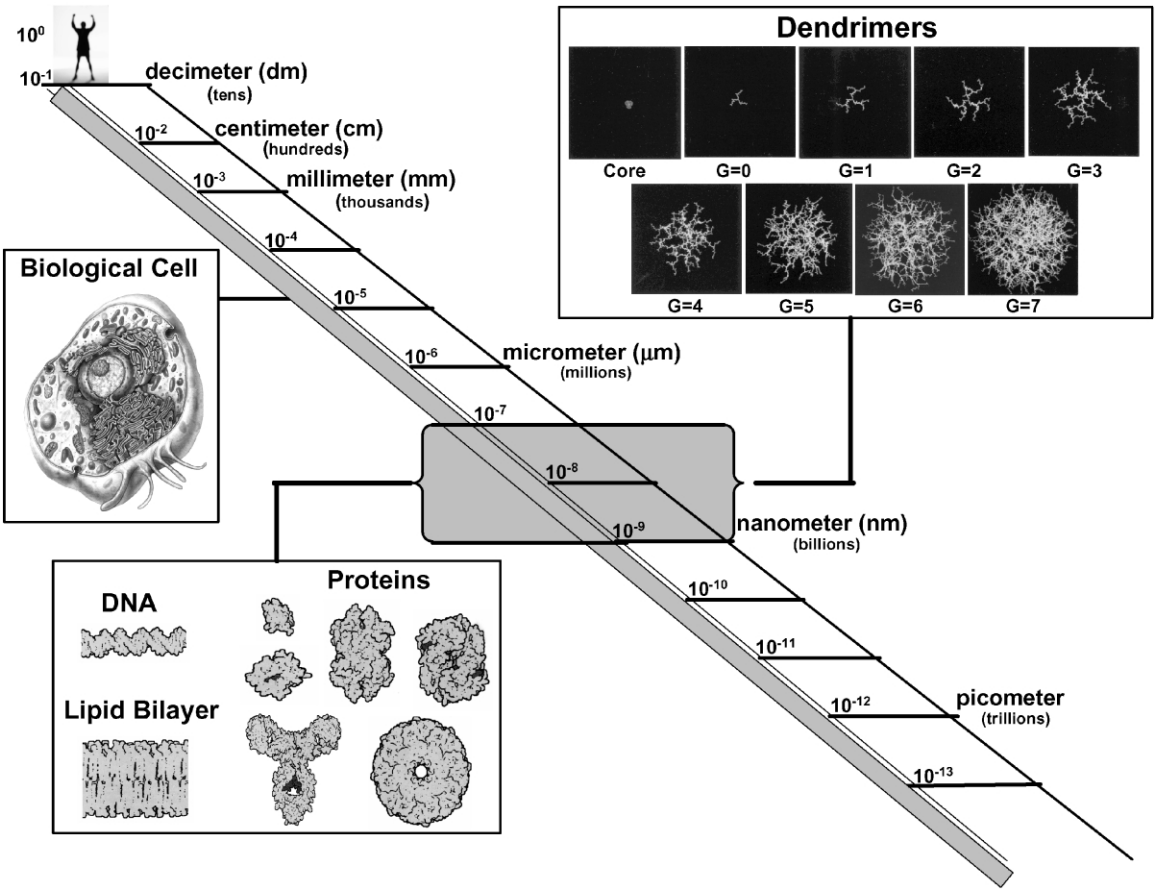
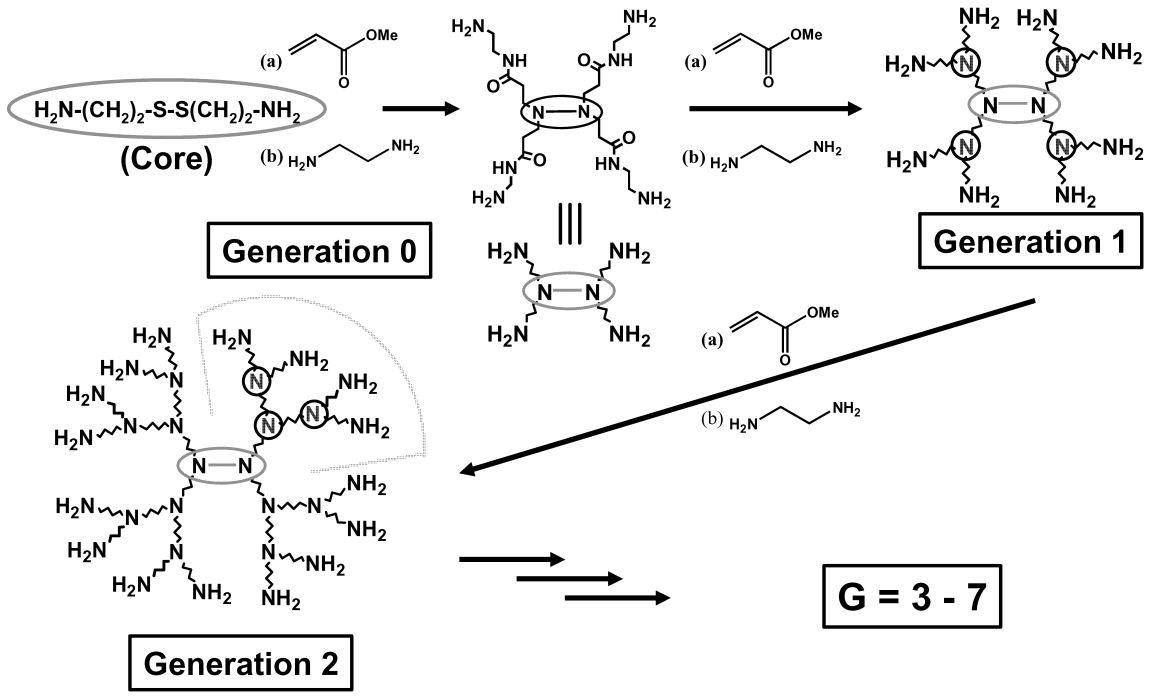
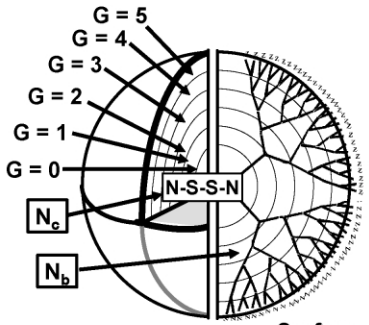


Figure 1. Nanoscale dimensional comparison of poly(amidoamine) dendrimers [NH_3 core] (Gen.=0–7) with proteins, DNA, lipid bilayer and the polio viruses.



Scheme 1. Divergent synthesis of [cystamine]; *dendri*-poly(amidoamine) dendrimers utilizing the iterative sequence (a) alkylation with methyl acrylate and (b) amidation with excess ethylenediamine to produce generations=3–7.



Number of Surface Groups : $Z = N_c N_b^G$ = Surface Group Amplification/Gen.

Number of Branch Cells : $BC = N_c \left[\frac{N_b^G - 1}{N_b - 1}\right]$ = Number of Covalent Bonds Formed/Generation

Molecular Weights : $MW = M_c + N_c \left[M_{RU}\left(\frac{N_b^G - 1}{N_b - 1}\right) + M_t N_b^G\right]$

Compound	Gen.	Surface Groups (Z) = -NH_2	Molecular Formula	MW	Diameter (nm)*
[2]	0	4	$C_{24}H_{52}N_{10}O_4S_2$	609	(>1.5)
[4]	1	8	$C_{64}H_{132}N_{26}O_{12}S_2$	1,522	(>2.2)
[6]	2	16	$C_{144}H_{292}N_{58}O_{28}S_2$	3,348	(>2.9)
[8]	3	32	$C_{304}H_{612}N_{122}O_{60}S_2$	7,001	(>3.6)
[10]	4	64	$C_{624}H_{1252}N_{250}O_{124}S_2$	14,307	(>4.5)
[12]	5	128	$C_{1264}H_{2532}N_{506}O_{252}S_2$	28,918	(>5.4)
[14]	6	256	$C_{2544}H_{5092}N_{1018}O_{508}S_2$	58,140	(>6.7)
[16]	7	512	$C_{5104}H_{10212}N_{2042}O_{1020}S_2$	116,585	(>8.1)

Figure 2. Mathematical expressions for calculating theoretical number of surface groups (*Z*), branch cells (BC) and molecular weights (MWt) for [cystamine core]; poly(amidoamine) (PAMAM) dendrimers. Approximate hydrodynamic diameters (Gen.=0–7) based on gel electrophoretic comparison to corresponding [ethylene diamine core]; poly(amidaomine) dendrimers.

The theoretical number of surface groups (*Z*), branch cells (BC) and molecular weights for this dendrimer series may be calculated using the expressions shown in Figure 2. These parameters, as well as approximate hydrodynamic diameters for the series are presented. These approximations were determined by direct comparison of the [CYS]; *dendri*-PAMAM–$(NH_2)_{Z(n)}$ series with the [EDA]; *dendri*-PAMAM–$(NH_2)_{Z(n)}$ series under identical conditions using gradient, poly(acrylamide) gel electrophoresis (PAGE). The slightly larger cystamine core produced a series that migrated consistently slower than the corresponding ethylenediamine core PAMAMs. Thus it can be assumed that corresponding members of this series are slightly larger nanoscale structures than those in the EDA core series.

2.2. Effect of core on dendritic purity and polydispersity

The most compelling evidence for the extraordinary structure control observed with divergent dendrimer synthesis is that based on mass spectroscopy[14,15] and gel electrophoresis.[18,19] These methods have gained wide acceptance and are routinely used to assess both dendritic purity (structure ideality), as well as mono-dendrimer polydispersity.[17] Systematic mass spectral analysis of both ammonia core and ethylenediamine core PAMAM dendrimers[32,39] has shown that nearly theoretical masses are obtained for the earlier and intermediate generations (i.e. Gen.=0–5). However, serious digression from ideal structures (i.e. mass defects) will occur as de Gennes dense packing effects begin to influence shell saturation levels at higher generations (i.e. ≥Gen.=7).

In view of these predictable generation dependent, shell saturation patterns, it was surprising to find that subtle, yet significant differences in dendritic purity and mono-dendrimer polydispersity were noted with the cystamine core. Using standard procedures with comparable reagent excesses and conditions, the cystamine core series invariably produced lower dendritic purities and hence more highly polydisperse products than the widely studied ammonia and ethylenediamine core series. Direct comparison of the cystamine to a 1,4-diaminobutane core series clearly indicated that the disulfide linkage is responsible for these aberrations. In this regard, suitable silica gel purification procedures were developed, which allowed us to obtain the desired cystamine core mono-dendrimers with high dendritic purity. Generally, the crude products could be fractionated on a silica gel column to produce gram quantities of desirable purity.

Electrospray mass spectrometry analyses of various early elution fractions obtained by silica gel separation proved to contain terminally looped and missing arm type structural defects. Isolation of the ideal structures (i.e. Gen.=1.0) was confirmed by single, well resolved TLC bands, capillary electrophoresis, gel slab electrophoresis (PAGE) and electrospray mass spectrometry (ES-MS). A rational explanation for this unusual core effect is not apparent at this time.

2.3. Thiol substituted, core reactive dendron reagents

The disulfide core found in cystamine-core PAMAM dendrimers is a relatively inert moiety under oxidizing or neutral conditions.[40] Cleavage of disulfide core products under reducing conditions were shown to give unique, focal point reactive di-dendron intermediates (Fig. 3). The lower generation (i.e. Gen.=0–3) mercapto-functionalized dendrons were readily recoupled under oxidative conditions.

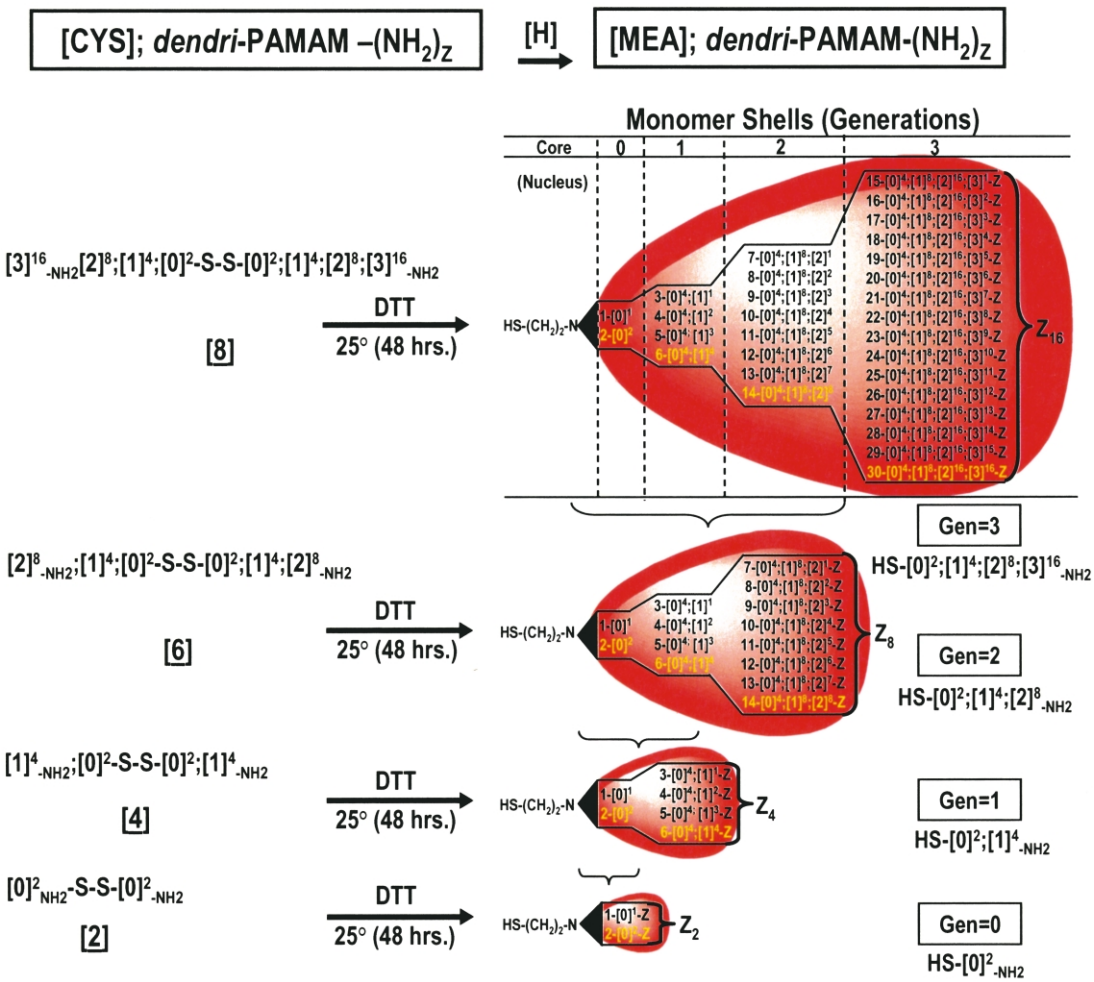


Figure 3. Conversion of [cystamine core]; *dendri*-poly(amidoamine) dendrimers to [2-(mercaptoethyl) amine core] [MEA]; *dendri*-poly(amidoamine) di-dendrons by reduction with dithiothreitol (DTT). [MEA]; *dendri*-PAMAM–$(NH_2)_Z$ are presented using space filling monomer shell configurations; wherein, sequential bold numbers indicate degree of polymerization (DP_{RU}) and highlighted configurations at the bottom of each shell (generation) indicate theoretical shell saturation limits.

Higher generation, mercapto-functionalized di-dendron analogues, however, tended to be more resistant to recoupling. This may be due to a 'nanoscale steric effect' imposed by the dendritic assembly surrounding the mercapto functionalized focal point.

Upon reduction, the reactive thiol group formed at the focal point of the resulting di-dendron fragments may be utilized as a distinct, singly reactive point group to which other dendrons or other substituents (e.g. proteins, oligonucleotides, peptides, hormones, organics, inorganics, or linear polymers) may be attached.[41] The remaining orthogonally reactive surface groups (which are commonly, but are not limited to, amines) may be used for the purposes of signal amplification, attachment to surfaces, analyte interaction, further conjugations, etc. The single, reactive functional group at the core allows the design of well-defined, homogenous conjugates which are unencumbered by the typical statistical distribution and degrees of substitution found in conjugates prepared via the surface groups of dendrimer molecules. A significant advantage of the present system is that a discrete regio-specific conjugate is formed, which has a narrow molecular weight distribution. This allows resolution with separation techniques such as chromatography or electrophoresis and facile characterization by various mass spectrometry methods. However, conjugates with classical polymers or with dendrimers, exhibiting varying degrees of substitution, may yield unacceptably broad peaks or bands when subjected to these analytical methods.

2.4. Core-shell architectural features of cystamine core PAMAM dendrimers

The core-shell architectural features of dendrimers have been described earlier in great detail.[12,36] Certain features of these dendritic architectures were shown to be quantized as a function of core (N_c) and branch cell (N_b) multiplicity. The concentric monomer shell (generations) surrounding the nucleus (core) of the dendrimer were shown to have well defined monomer shell saturation levels analogous to that observed for electrons at the atomic level, however, at a Newtonian dimension scale. By analogy to electron shells in atoms, certain quantized monomer shell parameters surrounding a dendrimer core (i.e. nucleus) can be mathematically predicted. The maximum monomer content per generation is defined by the simple expression shown below:

$$\text{Monomer Shell Saturation Level}: Z = N_c N_b^G \quad (1)$$

More specifically, the divergent strategy involving the 'in situ branch cell' approach to PAMAM dendrimers may be described as a series of quantized, molecular level 'aufbau events.' Formally, such construction involves the covalent, self assembly of *N*-(2-aminoethyl) acrylamide (2-AEA) monomer units. These structure controlled, building events are completed by appropriate iterations of the familiar two-step sequence involving (a) alkylation of amino precursors with methyl acrylate and (b) amidation of amplified ester terminated intermediates using excess ethylenediamine. These amine (nucleophilic) and acrylate (electrophilic) reagents assembled to produce dendritic covalent connectivity consisting of β-alanine units. The *N*-(2-aminoethyl) acrylamide monomer degree of polymerization (DP_{RU}) for each generation (monomer shell level) of a perfect structure is discrete and quantized according to the following expression:

$$DP_{RU} = N_c\left[\frac{N_b^{G+1} - 1}{N_b - 1}\right] \quad (2)$$

As such, the monomer shell level (G) and degree of polymerization (DP_{RU}) for the cleaved [cystamine core]; PAMAM dendrimer can be described relative to the new core or sulfhydryl focal point. This focal point resides at a terminus opposite to the surface groups on the hemi-ellipsoid as shown in Figure 3. Such a two-dimensional display illustrates the core, monomer shells and crude coordinates for specific monomer units or terminal groups relative to the core. An abbreviated notation for these coordinates, lists the monomer unit degree of polymerization (DP_{RU}) in bold sequential numbers as they appear in each principle shell or generation. These monomer units are associated with generation (monomer shell) levels and are designated by bold numbers in brackets. The superscript associated with each bracket indicates the number of monomer units in that shell. This serves as a monomer accounting system. In this manner, the monomer content is audited within a particular shell as each sequentially introduced monomer unit advances the shell toward a maximum quantized value. This saturation limit is defined by Eq. (1). This monomer accounting system demonstrates how the monomer content per shell (generation) is quantized as a maximum value for each generational level. The total accumulation DP_{RU} of monomer units around the core can be predictable as a function of generation by Eq. (2).

2.5. A comparison of quantized, sub-nanoscale and nanoscale modules

Earlier we compared the core-shell architecture of dendrimer based nanoscale modules to the core-shell architecture of sub-nanoscale atom modules.[12,36] This comparison was inspired by Niels Bohr's non-traditional organization of the elements in a unique periodic table presentation in his Nobel lecture of 1922.[42] Bohr's representation provides the familiar electron configuration accounting system, as well as, a facile visualization of several important periodic and quantized features associated with atoms as shown in Figure 4.

Bohr's unique periodic display of the quantized electron space filling features of atoms as a function of their atomic number and electron shell level, allows a very crude but relative size comparison of respective elements (atoms) in the sub-nanoscale region (i.e. 0.1–1 nm). Similarly, our two dimensional display of quantized, monomer space filling features observed for dendrimers allows a crude, but nevertheless, relative comparison of module size in the nanoscale region (i.e. 1–100 nm).

2.6. Size and shape designing features of the single site mercapto-core functionalized dendrons

The enormous variety of sizes, shapes and chemically differentiated surfaces that are possible by the combination and permutation of atoms to form molecular orbitals found in all small molecules is staggering. A small sampling may be visualized, to a crude first approximation, as space filling objects represented by Corey–Pauling models. Such models are arranged in ascending complexity from right to left, as shown in Figure 5. The importance of these parameters in defining the central dogma of traditional chemistry cannot be overstated. A scaled comparison of these small molecule parameters with buckminsterfullerene, a reference structure that defines entry into the nanoscale region, reveals several interesting features. Glucose has a diameter of approximately 0.5 nm. Although it is about half the size of a bucky ball, it possesses surfaces which are richly decorated with chemo-differentiated primary and secondary hydroxyl as well as ether domains whose molecular orbitals defined sub-nanoscale cusps and clefts in space. In contrast, the bucky ball symmetry presents an undifferentiated spheroidal surface with a singular dimension of approximately 1 nm.

Cystamine core PAMAM dendrimers (Gen. 0–3) are displayed vertically as coupled spheres above the bucky ball. They are represented with abbreviated notations in brackets to designate the generation level of the respective sulfur bonded di-dendrons attached to the core before cleavage (i.e. [CYS]; Gen.=3; PAMAM is designated as [3]:[3]). By cleaving these homo-dimers and performing subsequent oxidative coupling reactions on these mercapto-core functionalized didendrons (i.e. [G]-SH) utilizing various generation levels and surfaces, it should be apparent that a wide variety of size, shape and chemo differentiated homo and heterodimer type nanoscale modules are possible. A brief catalogue of size and shape differentiated products that are possible by hybridizing various combinations of homo-functionalized (Gen. 0–3) cystamine precursors are shown in Figure 5. Two experimentally demonstrated examples of size, shape and regio-chemical differentiated hybridization product are illustrated by structures **20** and **22**. To the right of these hybridized dendrimers are two well known globular proteins, namely insulin (dia. 3.0 nm) and cytochrome C (dia. 4.0 nm). It should be noted that not only do the overall dimensions of these proteins scale closely to these dendrimers, but the nanoscale clefts and cusps defined in the hybridized dendrimer architectures are a step toward mimicking these unique and important shapes found in these biostructures.

On the other hand, buckminsterfullerenes (bucky balls) are precise, quantized nanostructures consisting of 60 or 70

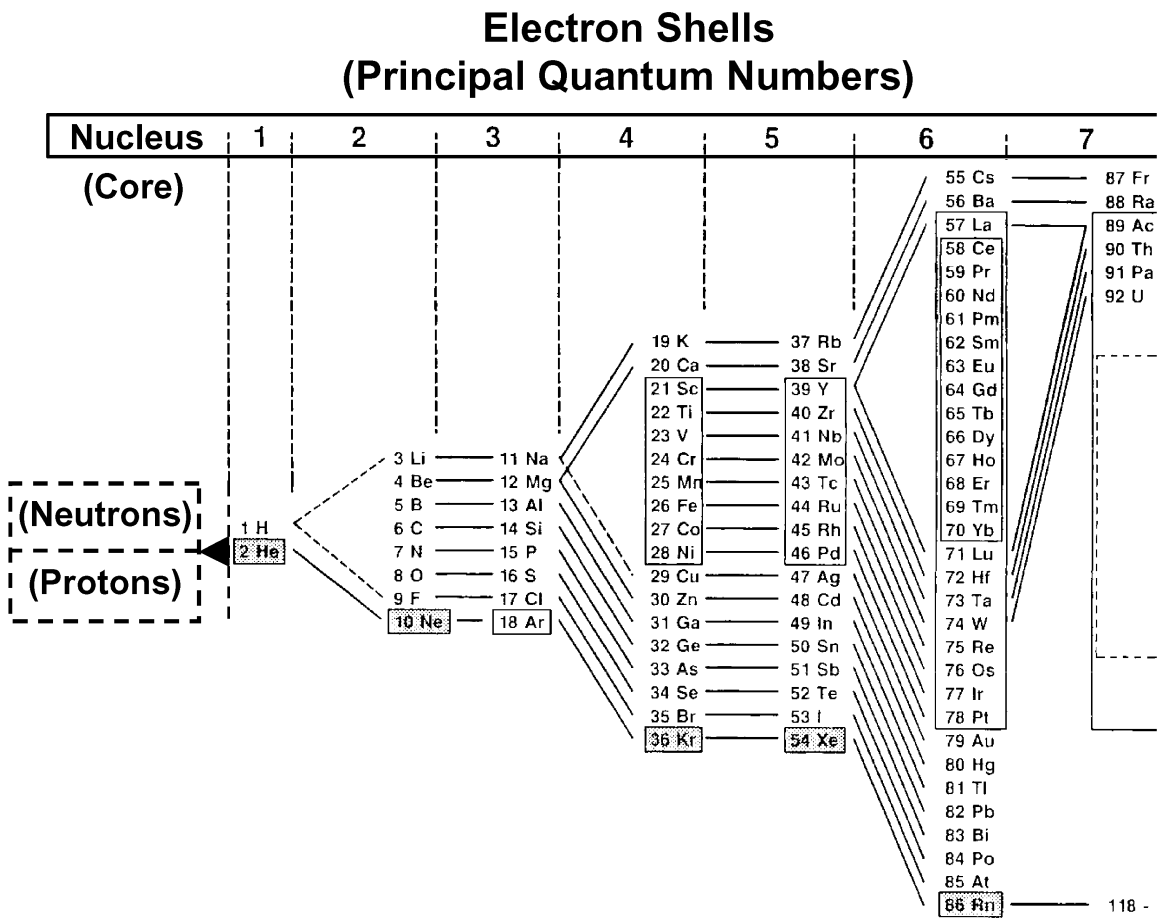


Figure 4. Core-shell representation of elements as a function of principle quantum numbers (electron shells) according to Niels Bohr (1922).

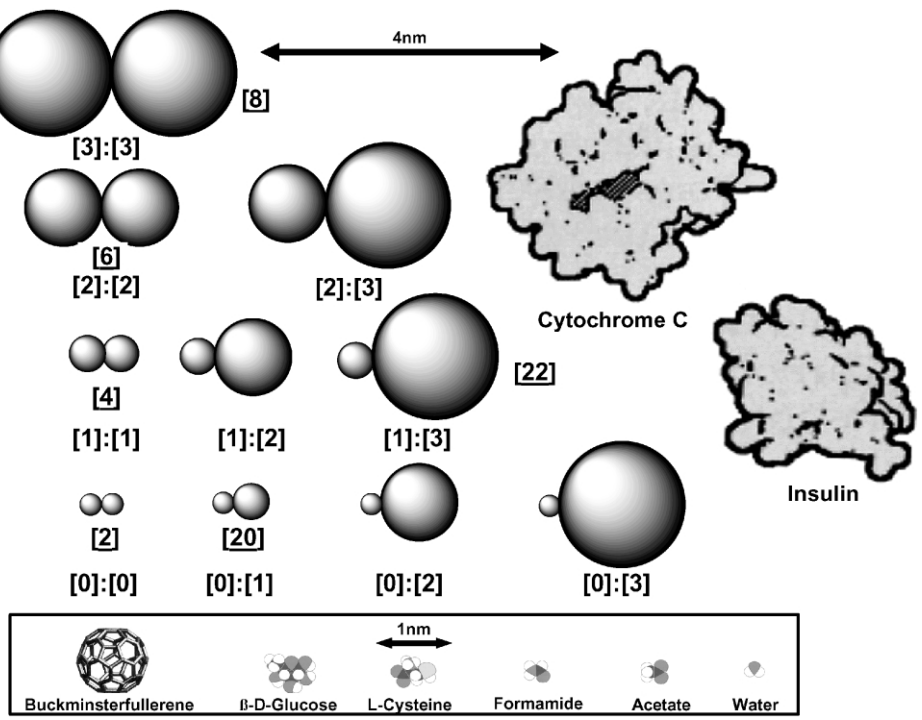


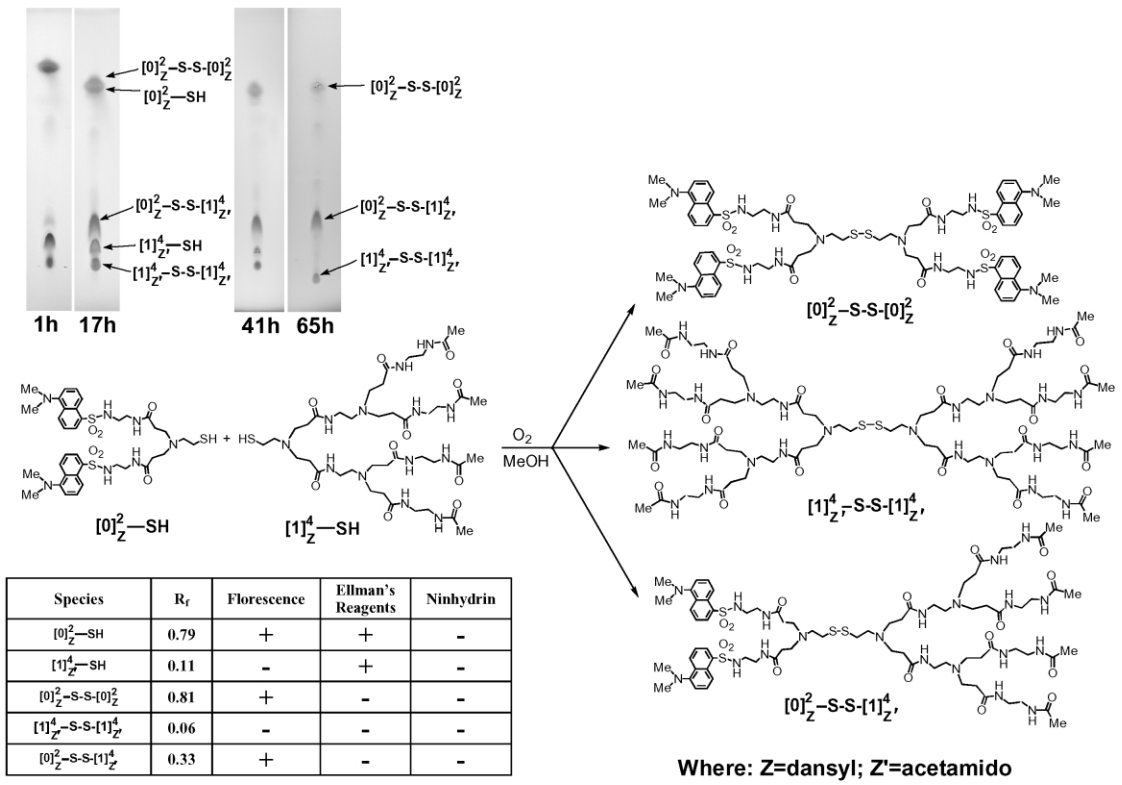
Figure 5. Scaled space filling models comparing small molecules (Corey–Pauling) to buckminsterfullerene and various [cystamine core]; poly(amidoamine) dendrimers (represented as spheres); wherein, bold numbers in brackets indicate generation level of respective dendrimer hemi-ellipsoids. These size scaled synthetic structures are compared to two globular proteins; insulin and cytochrome C.

carbon atoms which have been polymerized into the familiar soccer ball type structures. They possess diameters of approximately 1 nm; whereas, nanotubes derived from carbon and other elements[43] are available in various lengths, but with only several discrete diameters. Bucky balls and carbon nanotubes are some of the most intensely studied modules for abiotic nanoscale device design.[44,45] As nanoscale building blocks, these modules allow very limited opportunity to control structure relative to size, shape, compositional or functional group design. It should be apparent that the above dendrimer based strategies offer promising new alternatives for controlling these parameters.

2.7. Hybridization of mercapto substituted, core reactive PAMAM dendrons to produce generation and surface group differentiated PAMAM dendrimers

2.7.1. Hybridization monitored by fluorescent tagged reactants on thin layer chromatography. A general concept for the facile synthesis of differentiated PAMAM dendrimers was demonstrated by hybridizing two different core functionalized, mercapto-dendron reagents. Firstly, a cystamine core dendrimer (i.e. $[0]^2_{\text{dansyl}}$-S-S-$[0]^2_{\text{dansyl}}$) of a desired generation level is exhaustively dansylated. Secondly, another cystamine core dendrimer suitably advanced to a different generation level (i.e. $[1]^4_{-\text{NHCOMe}}$-S-S-$[1]^4_{-\text{NHCOMe}}$) is surface functionalized (i.e. acetylated) with appropriate substituents that allow it to be chromatographed by TLC on a silica gel plate. Each reagent is reduced with DTT (dithiothreitol) and chromatographed by TLC to determine the R_f values of the reduced and oxidized forms. The bands for the reduced forms of both the dansylated and acetylated mercapto dendron reagents were identified with Ellman's reagent. The reduced and oxidized forms of the per-dansylated derivatives were confirmed by observing fluorescent bands using a UV lamp (Fig. 6).

Specifically, [CYS]; *dendri*-PAMAM; $-(NH_2)_{4(0)}$ was per-dansylated overnight with dansyl chloride in the presence of triethylamine. Pure product $[0]^2_{\text{dansyl}}$-S-S-$[0]^2_{\text{dansyl}}$ was obtained by preparative TLC. Treatment of the TLC band with Ellman's reagent produced no color change, however, examination with a UV lamp revealed a strong fluorescent band. Next a [CYS]; *dendri*-PAMAM; $-(NH_2)_{(8)(1)}$ sample was per-acetylated with acetic anhydride overnight in the presence of triethylamine to give pure product **19** by TLC. Using the abbreviated nomenclature described in Figure 5, each of the above products (i.e. $[0]^2_Z$-S-S-$[0]^2_Z$ and $[1]^4_{Z'}$S-S-$[1]^4_{Z'}$ was reduced with DTT and chromatographed by TLC to produce new single bands that tested positive (i.e. yellow color) with Ellman's reagent. This demonstrated the conversions to $[0]^2_Z$-SH and $[1]^4_{Z'}$-SH, respectively. Hybridization of $[0]^2_Z$-SH and $[1]^4_{Z'}$-SH was accomplished by combining equivalent accounts of each and bubbling in air. This process was monitored by TLC and found to be complete in 65 h at room temperature. As noted in Figure 6, only three bands were observed, none of which were positive to Ellman's reagent. The new band assigned to $[0]^2_Z$-S-S-$[1]^4_{Z'}$ and $[0]^2_Z$-S-S-$[0]^2_Z$ exhibited fluorescence; whereas, $[1]^4_{Z'}$S-S-$[1]^4_{Z'}$ did not. Preparative TLC of $[0]^2_Z$-S-S-$[1]^4_{Z'}$ yielded a product which was consistent with the proposed structure by ^{13}C NMR and gave an appropriate mass of 1701 Da by electrospray mass spectrometry for the hybridized product (see Section 4).



Species	R_f	Florescence	Ellman's Reagents	Ninhydrin
$[0]^2_Z$—SH	0.79	+	+	-
$[1]^4_{Z'}$—SH	0.11	-	+	-
$[0]^2_Z$–S-S-$[0]^2_Z$	0.81	+	-	-
$[1]^4_{Z'}$–S-S-$[1]^4_{Z'}$	0.06	-	-	-
$[0]^2_Z$–S-S-$[1]^4_{Z'}$	0.33	+	-	-

Figure 6. Thin layer chromatography (TLC) monitoring the reduction of $[0]^2_Z$-S-S-$[0]^2_Z$ and $[1]^4_{Z'}$-S-S-$[1]^4_{Z'}$, followed by oxidation to produce the homo-dimers and the hybridized hetero-dimer product; $[0]^2_Z$-S-S-$[1]^4_{Z'}$.

2.7.2. Hybridization monitored by capillary electrophoresis. Extension of this concept to higher generation (i.e. Gen.=1 and 3) mercapto core reactive, dendron components, bearing orthogonally reactive surface groups (i.e. hydroxyl or primary amines), was demonstrated using capillary electrophoresis (CE). A cystamine core, perhydroxylated PAMAM dendrimer (Gen.=3) was prepared by the reaction of [CYS]; *dendri*-PAMAM–$(CO_2Me)_{32(2.5)}$, reagent **7** with 2-aminoethanol to give *N*-(2-hydroxyethylamido) terminated product **21**. This characterized product was combined in equivalent amounts with [CYS]; *dendri*-PAMAM–$(NH_2)_{8(1)}$, **4** and characterized by capillary electrophoresis (CE). See capillary electropherogram (A) (Fig. 7). In situ reduction with dithiothreitol (DTT) gave the corresponding mercapto-core reactive dendron reagents; [1]-SH and [3]-SH as determined by CE. Bubbling air through this mixture overnight led to re-oxidation and formation of the desired hybridization product [1]:[3], **22** accompanied by the corresponding oxidation products; [1]:[1], **4** and [3]:[3], **21**. This conversion was readily monitored by CE as noted in Figure 7B. Isolation of the hybridized product; [1]:[3], **22** by ultrafiltration gave a

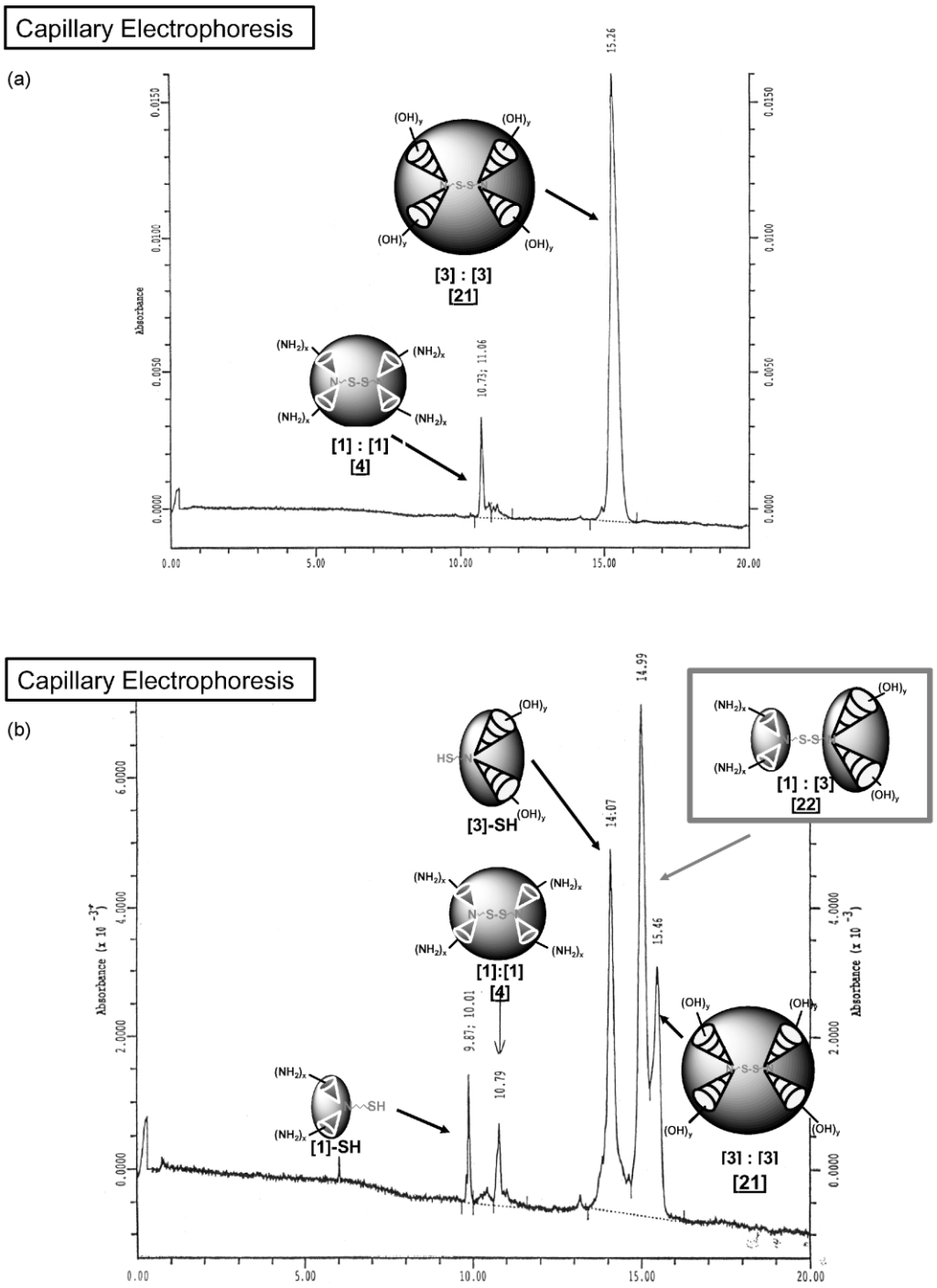


Figure 7. (A) Capillary electropherogram displaying signals for [CYS]; *dendri*-PAMAM–$(NH_2)_{(8(1))}$ and [CYS]; *dendri*-PAMAM–$(CONH–CH_2–CH_2–OH)_{(32)(3)}$; (B) capillary electropherogram displaying signals for the two homo-dimers **4** and **21**, their mercapto-core functionalized reduced forms [4]-SH and [21]-SH, as well as the hybridized, hetero-dimer product **22**.

product which was analyzed by MALDI-TOF mass spectrometry. Experimental mass: 4281 Da; theoretical mass: 4277 Da.

3. Conclusions

Synthetic procedures are presented for the 'divergent synthesis' of disulfide containing, cystamine core PAMAM dendrimers; Gen.=0–7. Surprisingly, conversions to 'monomer shell saturated' products (i.e. ideal mono-dendrimers) under standard conditions were generally less efficient than previously observed for all hydrocarbon α,ω-alkylenediamine cores.[38] Hence, 'dendritic species' possessing an unsaturated monomer shell were often encountered that exhibited 'autoreactive' behavior. These species which included missing branch structures led to the formation of mono-dendrimers containing macrocylic terminal groups, as well as moderate amounts of megamers (i.e. dimeric, trimeric, etc. species). Ideal dendrimer structure (i.e. monomer shell saturated products) could, however, be separated from these side products by column silica gel and preparative TLC isolation techniques. Ideal dendrimer structures that exhibited mathematically predictable masses, as well as 'unsaturated monomer shell' products exhibiting mass defects were readily characterized by electrospray (ESI) and mass spectrometry (MALDI-TOF).

This present work provides additional evidence to show that 'unfilled monomer shell' species are 'autoreactive intermediates' and do indeed lead to megamer formation. In general, 'saturated shell' PAMAM dendrimers are very robust species (i.e. analogous to inert gas configurations observed at the atomic level). In that regard, they do not exhibit autoreactive characteristics. Such samples may be stored for months or years without change. PAMAM dendrimer samples possessing unfilled monomer shells on the other hand are notorious for exhibiting autoreactive properties leading to terminal looping (i.e. macrocycle) and megamer formation.[12]

Remarkably, these autoreactivity patterns are also observed for core-shell (tecto) dendrimer architectures. For example, saturated shell,[46] core-shell (tecto)dendrimer architectures exhibit 'no autoreactivity'; whereas, partial shell filled,[39] core-shell tecto(dendrimers) exhibited 'profound autoreactivity,' unless pacified by reagents possessing orthogonally reactive functionality.

In summary, cleavage of ideal disulfide dendrimer products under reducing conditions have been shown to give unique, focal point reactive di-dendron intermediates. The lower generation (i.e. Gen.=0–3) mercapto-functionalized di-dendrons were readily recoupled under oxidative conditions. Higher generation mercapto functionalized di-dendron analogues tended to be more resistant to recoupling. In any case, oxidative hybridization of these size, shape and surface differentiated mercapto-functionalized di-dendrons to give a variety of size, shape and surface differentiated [CYS]; *dendri*-PAMAM dendrimers has been demonstrated. This provides an important step toward synthetically mimicking analogous parameters that are found in globular proteins.[50]

4. Experimental

4.1. Materials and equipment

All chemicals and solvents were obtained from Acros Organics or Sigma-Aldrich and used as received with the exception of cystamine hydrochloride which was recrystallized from a 2:1 mixture (v:v) isopropanol and methanol. Silica gel 60, particle size 0.040–0.063 mm, 230–400 mesh ASTM was obtained from EM Sciences. Separations of amine terminated dendrimers were performed by column chromatography using a 3.8 cm×55 cm column. Thin layer chromatography (TLC) was performed using Whatman Adsorption plates, 60 Å silica gel, 250 μm layer thickness.

The ^{13}C NMR spectra were obtained using either a Varian Unity 300 or a Bruker WM 360 SF instrument. Spectra for amine terminated dendrimers were recorded in D_2O or CD_3OD using 1,4-dioxane as an internal standard. Spectra for ester terminated dendrimers were recorded in $CDCl_3$. FT-IR spectra were recorded on a NTC Nicolet 20 DXB FT-IR spectrometer using a KBr plate. The FT-IR spectra were analyzed utilizing OMNIC FT-IR software. MALDI-TOF mass spectrometry was performed on a Thermo-Bioanalysis Vision Mass Spectrometer. Electrosrpay ionization mass spectrometry (ESI) was obtained using a Finnigan TSQ-700 spectrometer.

4.1.1. [CYS]; *star*-PAMAM–$(CO_2Me)_{4(-0.5)}$ (1). Cystamine dihydrochloride (10 g, 0.044 mol) and methyl acrylate (69 g; 0.80 mol) were mixed in 100 mL of methanol. To this stirred suspension, triethylamine (10 g, 0.10 mol) was added dropwise. After the addition of 1/4–1/2 of the triethylamine, the cloudy, white suspension became clear. The reaction mixture was allowed to stir at room temperature under a nitrogen atmosphere for 48 h, followed by heating at 40°C for 30 min. The volatiles of this mixture were removed using a rotoevaporator under vacuum. The residue remaining after removal of volatiles was a viscous liquid interspersed with white precipitate. The residue was dissolved in diethyl ether and extracted twice with water. Combined water layers were re-extracted with diethyl ether. The combined ether layers were dried over magnesium sulfate, filtered and evaporated. The product as a colorless liquid weighed 20 g (91% yield) after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3450 (w), 2950 (s), 2830 (s), 1725 (s), 1450 (s), 1120 (s), 1050 (s), 1000 (s), 850 (m), ^{13}C NMR ($CDCl_3$, 90 MHz) δ 32.5, 36.3, 49.1, 51.3, 53.1, 172.6. Electrospray mass spectrometry of product **1** gave a mass of 496 Da (theoretical mass: 496 Da).

4.1.2. [CYS]; *star*-PAMAM–$(NH_2)_{4(0)}$ (2). Ethylenediamine (240 g, 4 mol) was mixed with 60 mL of methanol and cooled to 5°C. To this cooled solution was added a 50% (w/w) methanol solution of [CYS]; *star*-PAMAM–$(CO_2Me)_{4(-0.5)}$, reagent **1** (20 g, 0.040 mol). The reaction was allowed to proceed under a nitrogen atmosphere at 5°C for 5 days. The reaction was then heated to 40°C for 30 min. Most of the excess ethylenediamine and methanol was

removed by rotary evaporation under vacuum. The remaining ethylenediamine was removed by azeotroping using 3/1 toluene/methanol. The product as a light yellow viscous oil weighed 24 g (98% yield) removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3280 (s), 3075 (m), 2940 (m), 2850 (m), 1650 (s), 1550 (s), 1120 (w). ^{13}C NMR (D_2O, 90 MHz) δ 33.3, 33.5,35.9, 41.0, 41.4, 41.9, 49.2, 49.6, 52.2, 172.6. Electrospray mass spectrometry of product **2** gave a mass of 609 Da (theoretical mass: 609 Da).

4.1.3. [CYS]; *dendri*-PAMAM–$(CO_2Me)_{8(0.5)}$ (3). To a 50 wt% methanol solution of methyl acrylate (19 g, 0.2 mol) was added with stirring, [CYS]; *star*-PAMAM–$(NH_2)_{4(0)}$, reagent **2** (15 g, 0.025 mol) over a 30-min period. This reaction was allowed to stir at room temperature under a nitrogen atmosphere for 48 h. The reaction was then heated to 40°C for 30 min. Rotary evaporation under vacuum was used to remove methanol and excess methyl acrylate. The product as a light yellow viscous oil weighed 32.3 g (99% yield) after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3320 (m), 2950 (m), 2830 (m), 1740 (s), 1660 (s); 1530 (s), 1450 (s), 1050 (m). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 32.1, 33.2, 35.7, 36.8, 48.9, 49.2, 51.2, 52.2, 52.5, 171.9. 172.7. Electrospray mass spectrometry of product **3** gave a mass of 1298 Da (theoretical mass: 1298 Da).

4.1.4. [CYS]; *dendri*-PAMAM–$(NH_2)_{8(1)}$ (4). Ethylenediamine (421 g, 7 mol) was mixed with 106 mL of methanol and cooled to 5°C. To this cooled solution was added a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(CO_2Me)_{8(0.5)}$, reagent **3** (25 g, 0.019 mol). The reaction was allowed to proceed under a nitrogen atmosphere at 5°C for 5 days, followed by heating at 40°C for 30 min. Excess ethylenediamine and methanol were removed by rotary evaporation under vacuum. Remaining ethylenediamine was removed by azeotroping using a 3/1 toluene/methanol mixture. The product as a viscous light yellow oil weighed 28.5 g (99%) yield after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3275 (m), 3060 (m), 2925 (m), 2850 (m), 1650 (s), 1550 (s). ^{13}C NMR (D_2O, 90 MHz) δ 33.4, 33.9, 35.7, 37.5, 41.1, 41.9, 49.4, 50.1, 52.2, 52.4, 172.5, 173.1. Electrospray mass spectrometry of product **4** gave a mass of 1521 Da (theoretical mass: 1522 Da).

4.1.5. [CYS]; *dendri*-PAMAM–$(CO_2Me)_{16(1.5)}$ (5). To a 50 wt% methanol solution of methyl acrylate (20 g, 0.21 mol) was added with stirring [CYS]; *dendri*-PAMAM–$(NH_2)_{8(1)}$, reagent **4** (20 g, 0.013 mol) over a 30-min period. This reaction was allowed to stir at room temperature under a nitrogen atmosphere for 48 h. The reaction was then heated to 40°C for 30 min. Rotary evaporation under vacuum was used to remove any excess methyl acrylate and methanol. The product as a light yellow oil weighed 37.3 g (99% yield) after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3300 (m), 3075 (w), 2950 (m), 2825 (m), 1740 (s), 1650 (s), 1540 (s), 1450 (s), 1050 (m). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 32.3, 33.1, 33.4, 35.4, 36.9, 37.2, 48.9, 49.2, 49.5, 51.3, 52.1, 52.5, 172.3, 172.4, 172.8. Electrospray mass spectrometry of product **5** gave a mass of 3348 Da (theoretical mass: 3348 Da).

4.1.6. [CYS]; *dendri*-PAMAM–$(NH_2)_{16(2)}$ (6). Ethylenediamine (727 g, 12.1 mol) is mixed with 225 mL of methanol and cooled to 5°C. To this cooled solution was added a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(CO_2Me)_{16(1.5)}$, reagent **5** (25 g, 0.0086 mol). The reaction was allowed to proceed under a nitrogen atmosphere at 5°C for 5 days and then heated to 40°C for 30 min. Excess ethylenediamine and methanol was removed by rotary evaporate ion under vacuum. The remaining ethylenediamine was removed by azeotroping using a 3/1 toluene/methanol mixture. The purified product as a slightly yellow viscous oil weighed 28 g after removing residual volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3275 (s), 3070 (m), 2950 (m), 2850 (m), 1650 (s), 1550 (s), 1460 (m), 1440 (m), 1030 (w). ^{13}C NMR (D_2O, 90 MHz) δ 33.1, 33.4, 33.6, 35.4 37.2, 40.9, 41.6, 49.2, 49.6, 49.8, 51.9, 52.2, 172.6, 173.0. Electrospray mass spectrometry of the product **6** gave a mass of 3347.8 Da (theoretical mass: 3348 Da).

4.1.7. [CYS]; *dendri*-PAMAM–$(CO_2Me)_{32(2.5)}$ (7). To a 50 wt% methanol solution of methyl acrylate (18.2 g, 0.2 mol) was added with stirring, [CYS]; *dendri*-PAMAM–$(NH_2)_{16(2)}$, reagent **6** (20 g, 0.006 mol) over a 30-min period. This reaction mixture was allowed to stir at room temperature under a nitrogen atmosphere for 4 days followed by heating to 40°C for 30 min. Rotary evaporation under vacuum was used to remove excess methyl acrylate and methanol. The product as a slightly yellow viscous oil weighed 36 g (99% yield) after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3290 (m, br), 3080 (w), 2950 (m), 2830 (m) 1740 (s), 1650 (s), 1550 (s), 1450 (s), 1050 (m), 850 (w). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 32.3, 33.0, 33.3, 35.3. 36.9, 37.2, 48.9, 49.1, 49.4, 49.7, 51.3, 52.0, 52.1, 52.4, 172.3, 172.8. Electrospray mass spectrometry of this product gave a mass of 6100.6 Da (theoretical mass: 6103 Da).

4.1.8. [CYS]; *dendri*-PAMAM–$(NH_2)_{32(3)}$ (8). Ethylenediamine (1353 g, 22.5 mol) was mixed with 427 mL of methanol and cooled to 5°C. To this cooled solution was added a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(CO_2Me)_{32(2.5)}$, reagent **7** (25 g, 0.004 mol). The reaction was allowed to proceed under a nitrogen blanket at 5°C for 6 days followed by heating to 40°C for 30 min. Excess ethylenediamine was removed by rotary evaporation under vacuum. The remaining ethylenediamine was removed by ultrafiltration on a 3000 MWCO spiral membrane. All traces of water were removed by lyophilization. The dried light yellow solid product weighed 22.8 g (82% yield). IR (KBr, neat) cm^{-1}: 3300 (s); 3075 (m) 2940 (s); 2850 (m); 1650 (s); 1550 (s); 1475 (m); 1310 (m); 1025; 700 (w). ^{13}C NMR (75 MHz, D_2O) δ 32.67, 32.78, 36.73, 39.78, 441.54, 48.81, 49.06, 51.23, 174.13, 174.22, 174.31, 174.72. Electrospray mass spectrometry of product **8** gave a mass of 7001 Da (theoretical mass: 7001 Da).

4.1.9. [CYS]: *dendri*-PAMAM–$(CO_2Me)_{64(3.5)}$ (9). To a 50 wt% methanol solution of methyl acrylate (11 g, 0.12 mol) was added dropwise, with stirring, over a 30-min period, a 50–75 wt% methanol solution of [CYS]; *dendri*-PAMAM–-$(NH_2)_{32(3)}$, reagent **8** (12 g, 0.0017 mol). This reaction was stirred under a nitrogen atmosphere at room temperature for 4 days. The reaction was then heated to 40°C for 30 min. Rotary evaporation under vacuum was

used to remove excess methyl acrylate and methanol. The light yellow product weighed 21 g (98% yield) after removing volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3300 (m, br); 2950 (s); 1750 (s); 1650 (s); 1560 (s); 1400 (m); 1325 (m); 1125 (s); 1040 (s). ^{13}C NMR (75 MHz, $CDCl_3$) δ 32.27, 33.39, 36.86, 37.13, 48.87, 49.39, 49.68, 551.22, 52.09, 52.48, 172.13, 172.23, 172.68. MALDI-TOF mass spectrometry of product **9** gave a major mass peak of 6233 Da (theoretical mass: 12511 Da) indicating cleavage of the disulfide linkage has occurred during mass spectrometry analysis. Product **9** gave a negative Ellman's test before analysis.

4.1.10. [CYS]; *dendri*-PAMAM–$(NH_2)_{64(4)}$ (10). Ethylenediamine (1563 g, 26 mol) was mixed with 467 mL of methanol and cooled to 5°C. To this cooled EDA/methanol solution was added a 50% (w/w) methanol solution of [CYS]: *dendri*-PAMAM–$(CO_2Me)_{64(3.5)}$, reagent **9** (15 g, 0.0012 mol). The reaction was allowed to proceed under nitrogen at 5°C for 6 days. The reaction was then heated to 40°C for 30 min. Excess ethylenediamine was removed by rotary evaporation under vacuum. The remaining EDA was removed by ultrafiltration using a 3000 MWCO membrane. All traces of water were removed by lyophilization. The purified product weighed 15.3 g for a 90% yield. IR (KBr, neat) cm^{-1}: 3280 (m); 3075 (m); 2940 (m); 2840 (m); 1650 (s); 1550 (s); 1475 (m); 1440 (m); 1150 (w); 1050 (w). ^{13}C NMR (75 MHz, D_2O) δ 32.79, 32.91, 36.84, 39.91, 41.78, 48.90, 49.18, 51.36, 174.36, 174.44, 174.52, 174.99. MALDI-TOF mass spectrometry of this product gave a major mass peak at 7158.3 Da (theoretical mass for disulfide cleavage of **10** is 7154.5 Da). This indicates cleavage of the disulfide linkage occurred during analysis, since **10** gave a negative Ellman's test before analysis.

4.1.11. [CYS]; *dendri*-(PAMAM)–$(CO_2Me)_{128(4.5)}$ (11). To a 50 wt% methanol solution of methyl acrylate (4.3 g, 0.045 mol) was added dropwise with stirring a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(NH_2)_{64(4)}$, reagent **5** (5 g, 0.00035 mol) over a period of 30 min. This reaction was allowed to stir at room temperature under a nitrogen atmosphere for 4 days followed by heating to 40°C for 30 min. Rotary evaporation was used to remove excess methyl acrylate and methanol. The purified product weighed 8.5 g (96% yield) after removal of volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3000 (m); 3060 (w); 2950 (m); 2840 (m); 1740 (s); 1650 (s); 1540 (s); 1450 (m); 1360 (m); 1200 (s); 1050 (m); 840 (w). ^{13}C NMR (75 MHz, $CDCl_3$) δ 32.72, 33.82, 37.29, 37.56, 49.31, 55.50, 50.35, 51.65, 52.54, 52.92, 172.52, 172.61, 173.10.

4.1.12. [CYS]; *dendri*-PAMAM–$(NH_2)_{128(5)}$ (12). Ethylenediamine (1460 g; 24.3 mol) was mixed with 460 mL of methanol and cooled to 5°C. To this cooled EDA/methanol solution was added a 50% (w/w) methanol solution of [CYS]; *dendri*-(PAMAM)–$(CO_2Me)_{128(4.5)}$, reagent **11** (7 g, 0.00028 mol). The reaction was allowed to proceed under a nitrogen blanket at 5°C for 8 days followed by heating to 40°C for 30 min. Excess ethylenediamine was removed by ultrafiltration on a 10000 MWCO membrane. All traces of water were removed by lyophilization. The dried light yellow solid product weighed 6.2 g (77% yield). IR (KBr, neat) cm^{-1}: 3260 (m); 3050 (w); 2920 (w); 2825 (w); 1650 (s); 1550 (s); 1460 (w); 150 (w); 1030 (w). ^{13}C NMR (75 MHz, D_2O) δ 32.79, 32.91, 36.84, 39.91, 41.78, 48.90, 49.18, 51.36, 174.36, 174.44, 174.52, 174.94. (theoretical mass: Daltons).

4.1.13. [CYS]; *dendri*-PAMAM–$(CO_2Me)_{256(5.5)}$ (13). To a 50 wt% methanol solution of methyl acrylate (2.6 g, 0.027 mol) was added dropwise with stirring a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(NH_2)_{128(5)}$, reagent **12** (3 g, 0.0001 mol) over a 30-min time period. This reaction was allowed to stir at room temperature under a nitrogen atmosphere for 5 days followed by heating to 40°C for 30 min. Rotary evaporation was used to remove excess methyl acrylate and methanol. The purified product weighed 4.9 g (93% yield) after removal of volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3300 (m); 3075 (w); 2950 (m); 2830 (m); 1740 (s); 1650 (s); 1550 (s); 1440 (s); 1350 (m); 1260 (s); 1200 (s); 1050 (m); 850 (w).

4.1.14. [CYS]; *dendri*-PAMAM–$(NH_2)_{256(6.0)}$ (14). A methanol solution of ethylenediamine (2921 g, 34.5 mol) (29% by weight of methanol) was cooled to 5°C. To this cooled EDA/methanol solution was added a 50% (w/w) methanol solution of [CYS]; *dendri*-PAMAM–$(CO_2Me)_{256(5.5)}$, reagent **13** (5 g, 0.0001 mol). The reaction was allowed to proceed under a nitrogen blanket at 5°C for 9 days. The reaction was then heated to 40°C for 30 min. Excess ethylenediamine was removed by rotary evaporation under vacuum. The remaining EDA was removed by ultrafiltration on a 10000 MWCO membrane. All traces of water were removed by lyophilization. The light yellow dried product weighed 5.1 g (90% yield). IR (KBr, neat) cm^{-1}: 3275 (m); 3075 (w); 2940 (w); 2825 (w); 1650 (s); 1550 (s); 1475 (w); 1340 (w); 1175 (w); 1050 (w).

4.1.15. CYS]; *dendri*-PAMAM–$(CO_2Me)_{512(6.5)}$ (15). To a methanol solution (at least 50 wt% methanol) of methyl acrylate (2.65 g, 0.028 mol) was added dropwise with stirring [CYS]; *dendri*-PAMAM–$(NH_2)_{256(6.0)}$, reagent **14** (3.2 g, 0.000055 mol), diluted to at least 50% (w/w) with methanol, over a 30-min time period. This reaction was allowed to stir at room temperature under a nitrogen atmosphere for 5 days. The reaction was then heated to 40°C for 30 min. Rotary evaporation was used to remove excess methyl acrylate and methanol. The purified product weighed 4.8 g (86% yield) after removal of volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 3300 (m); 3100 (w); 3000 (m); 2875 (m); 1750 (s); 1650 (s); 1550 (s); 1450 (m); 1375 (m); 1220 (s); 1075 (w); 875 (w).

4.1.16. [CYS]; *dendri*-PAMAM–$(NH_2)_{512(7.0)}$ (16). A methanol solution (27 wt% methanol) of ethylenediamine (4141 g, 69 mol) was cooled to 5°C. To this cooled solution was added [CYS]; *dendri*-PAMAM–$(CO_2Me)_{512(6.5)}$, reagent **15** (5 g, 0.00005 mol) in 75 wt% methanol. This reaction was allowed to proceed under a nitrogen blanket at 5°C for 10 days, followed by heating to 40°C for 30 min. Excess ethylenediamine was removed by rotary evaporation under vacuum. The remaining EDA was removed by ultrafiltration using a 10000 MWCO membrane. All traces of water were removed by lyophilization. The dried light yellow solid product weighed 5.1 g (87% yield). IR (KBr,

neat) cm^{-1}: 3275 (s); 3075 (m); 2950 (m); 2850 (m); 1650 (s); 1550 (s); 1475 (m); 1350 (m); 1160 (w); 1050 (w).

4.1.17. [CYS]; *dendri*-PAMAM–$(CO_2Me)_{1,024(7.5)}$ (17). To a 50 wt% methanol solution of methyl acrylate (3.0 g, 0.031 mol) was added dropwise with stirring [CYS]; *dendri*-PAMAM–$(NH_2)_{512(7.0)}$, reagent **16** (3.5 g, 0.00003 mol), diluted to at least 50 wt% methanol, over a 30-min period. This reaction was allowed to stir at room temperature for 7 days. The reaction was then heated to 40°C for 30 min. Rotary evaporation under vacuum was used to remove excess methyl acrylate and methanol. The purified product weighed 4.2 g (69% yield) after removal of volatiles by high vacuum. IR (KBr, neat) cm^{-1}: 300 (s); 3075 (m); 2950 (s); 2830 (s); 1740 (s); 1650 (s); 1550 (s); 1450 (s); 1350 (m); 1200 (s); 1050 (m); 840 (w).

4.1.18. Preparation of tetra-dansyl terminated: PAMAM *star* branched product: [CYS]; *star*-PAMAM-$(dansyl)_{4(0)}$; compound (18). A solution of [CYS]; *star*-PAMAM–$(NH_2)_{4(0)}$, reagent **2** (95 mg, 0.156 mmol) and triethylamine (93 μL, 0.60 mmol) was formed by dissolving in 1 mL of anhydrous dichloromethane under nitrogen. While stirring, a solution of dansyl chloride (212 mg, 0.788 mmol) in 0.5 mL of anhydrous dichloromethane was added dropwise. The reaction was stirred overnight at room temperature and the devolatilized to reveal a yellow oily product. This product was purified on a preparative TLC plate (Whatman, 20×20 cm, 250 μm thickness, Gel 60 Å). The yield was 10 mg per TLC plate; wherein, a 20 mg yield of pure product was obtained as a yellow oil which was shown to be a single spot, pure product by subsequent TLC (R_f=0.81, $CHCl_3$:CH_3OH–NH_4OH=3:1:0.02). Ninhydrin reagent showed it was completely per-dansylated. ^1H NMR ($CDCl_3$, 300 MHz): δ ppm 2.33 (s, 8H), 2.66–2.71 (m, 12H), 2.81 (s, 24H), 2.93 (m, 12H), 3.28 (m, 8H), 6.80 (s, 4H), 7.06 (d, J=7.8 Hz, 4H), 7.41 (m, 8H), 7.69 (s, 4H), 8.14 (d, J=7.5 Hz, 4H), 8.27 (d, J=8.7 Hz, 4H), 8.46 (d, J=8.4 Hz, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): 33.5, 35.8, 39.2, 43.1, 45.4, 49.8, 52.0, 115.2, 118.9, 123.2, 128.3, 129.2, 129.5, 129.8, 130.3, 134.7, 151.8, 173.2 ppm. IR (neat) cm^{-1}: 3273 (s), 2937 (s), 2831 (s), 1651 (s), 1574 (s), 1450 (m), 1317 (s), 1140 (s). Electrospray mass spectrometry of **18** gave a mass of 1542 Da (theoretical mass: 1541 Da).

4.1.19. Reduction of [CYS]; *star*-PAMAM-$(dansyl)_{4(0)}$; compound (18)→(18)-SH. A sample of [CYS]; *star*-PAMAM-$(dansyl)_{4[0]}$ (20 mg, 0.0130 mmol) was dissolved in a mixed solvent consisting of 0.5 mL dichloromethane and 0.5 mL of methanol. All solvents were purged with nitrogen for 15 min prior to use. This was followed by the addition of dithiothreitol (DTT) (1.7 mg, 0.011 mmol) which is 0.85 equiv. relative to the dendrimer. The reaction mixture was stirred for two days at room temperature under nitrogen. The DTT was completely consumed according to TLC; whereas, the new reduced product band gave a positive test to Ellman's reagent on the TLC plate (R_f=0.79, $CHCl_3$–CH_3OH–NH_4OH=3:1:0.02). The mercapto-functionalized *star*-branched product was used directly for the hybridization reaction without further purification.

4.1.20. Preparation of octa-acetamido terminated PAMAM dendrimer: [CYS]; *dendri*-PAMAM–$(NHCOCH_3)_{8(1)}$; compound (19). A sample of [CYS]; *dendri*-PAMAM–$(NH_2)_{8(1)}$, reagent **4** (525 mg, 0.345 mmol) was dissolved in 2 mL of methanol, followed by the addition of triethylamine (450 μL, 3.17 mmol). This mixture was cooled to 0°C, as 300 μL of acetic anhydride was added. The reaction was allowed to warm to room temperature and stirred overnight. TLC showed that all starting material was consumed; whereas, a new TLC spot tested negative with both Ellman's reagent and ninhydrin reagent (R_f=0.79, $CHCl_3$–CH_3OH–NH_4OH=3:1:0.02). The solvent was removed and the crude product was used directly for the reduction step leading to hybridization.

4.1.21. Reduction of [CYS]; *dendri*-PAMAM–$(NHCOCH_3)_{8(1)}$; (19)→(19)-SH. A sample of [CYS]; *dendri*-PAMAM–$(NHCO_2CH_3)_{8(1)}$; **19** (30 mg, 0.0107 mmol) was dissolved in 1.0 of methanol. The solution was purged with dry nitrogen for 15 min prior to use. To this solution was added DTT (1.3 mg, 0.0084 mmol, 0.85 equiv. relative to dendrimer). The reaction mixture was stirred at room temperature under nitrogen for two days. TLC indicated that all the DTT had been consumed, whereas a new spot at (R_f=0.11, $CHCl_3$–CH_3OH–NH_4OH=3:1:0.02) tested positive to Ellman's reagent. This product was used as is for the hybridization reaction.

4.1.22. Hybridization of (18)-SH with (19)-SH→ compound (20). The two reduced forms of **18** and **19** were mixed in a 1:1 by weight ratio in methanol. While stirring, a stream of air was slowly bubbled through the reaction mixture. Methanol was added periodically as necessary to replace that lost due to solvent evaporation. The reaction was monitored by TLC and found to be completed after 65 h, as evidenced by a negative test with Ellman's reagent. The thin layer chromatogram consisted of three spots which were identified as **19**; R_f=0.06, **20**; R_f=0.33 and **18**; R_f=0.81 (solvent, $CHCl_3$–CH_3OH–NH_4OH=3:1:0.02). ^1H NMR(methanol-d^4, 300 MHz): δ ppm 1.92 (s, 12H), 2.28 (m, 4H), 2.42 (m, 16H), 2.74 (m, 8H), 2.78–2.98 (m, 24H), 3.12 (m, 4H), 3.27 (m, 24H), 4.88 (s, 12H), 7.23 (d, J=7.5 Hz, 2H), 7.55 (m, 4H), 8.15 (d, J=6.9 Hz, 2H), 8.29 (d, J=8.7 Hz, 2H), 8.53 (d, J=8.4 Hz, 2H). ^{13}C NMR (methanol-d^4, 75 MHz): 21.3, 32.3, 32.4, 34.9, 36.6, 38.6, 38.9, 41.8, 44.4, 49.2, 49.4, 49.7, 52.0, 115.0, 118.9, 122.9, 127.8, 128.8, 129.4, 129.7, 129.8, 135.2, 151.8, 172.1, 173.2, 173.5 ppm. IR (neat) cm^{-1}: 2940 (w), 2829 (w), 2359 (s), 1636 (s), 1466 (m). The hybridized product **20** was confirmed by ESI to have a mass of 1701 Da (theoretical: 1700 Da).

4.1.23. Preparation of *N*-(2-hydroxyethylamide) terminated PAMAM dendrimer, [CYS]; *dendri*-PAMAM–$(CONHCH_2CH_2–OH)_{32(3)}$; compound (21). To a 100 mL, one neck, round bottom flask containing a magnetic stir bar was added 2-aminoethanol (7.7 g, 6 equiv./ester) and methanol (10 mL). To this stirred mixture under N_2, cooled to 0–5°C, was added [CYS]; *dendri*-PAMAM–$(COMe)_{32(2.5)}$ **7** (4 g, 6.55×10^{-4} mol, 21 mmol ester) dissolved in 8 mL of MeOH. This mixture was warmed to 25°C and stirred for 3 days in a sealed vessel under N_2. This resulting mixture was diafiltered followed by

ultrafiltration to give recirculation of permeate using a tangential flow ultrafiltration device containing a 3K molecular weight cutoff, regenerated cellulose membrane. This mixture was filtered and devolatized under vacuum using a rotary evaporator to give 4.4 g (95% yield) of the desired product. ^{13}C NMR (D_2O, 75 MHz) δ 174.79, 174.33, 174.24, 174.13, 59.73, 51.14, 48.91, 48.67, 41.27, 36.62, 32.56.[48,49]

4.1.24. Reduction and re-oxidation of [CYS]; *dendri*-PAMAM–$(CONH–CH_2–CH_2–OH)_{32(3)}$ and [CYS]; *dendri*-PAMAM–$(NH_2)_{8(1)}$ to give hybridization product; compound (22). A 2 mg solution of **21** and a 0.5 mg solution of **4** were prepared independently in a pH 8.2 borate buffer. The respective dendrimer concentrations are 280 μM. l mL of each solution was withdrawn and mixed together. Analysis by CE clearly identified these two products as shown in Figure 7A. This sample was treated with 10 μL of a 0.21 M DTT solution in the same buffer and was then incubated at 40°C overnight followed by ultrafiltration through a 500 molecular weight cut-off membrane in a micropartition device to remove excess DTT. After ultrafiltration (1 mL down to 100 μL, repeated twice), the sample was analyzed by CE after one hour. At this time, some reoxidation to the mixed disulfide was observed. After shaking overnight in air, most of the mixture had become reoxidized to both homodimeric and heterodimeric disulfides (Fig. 7B). CE analysis clearly showed the presence of the hybridized product **22**. Analysis of this product by MALDI-TOF mass spectrometry gave a mass of 4281 Da (theoretical: 4277 Da).

Acknowledgements

This work was funded by the Army Research Laboratory (ARL) Dendritic Polymer Center of Excellence (Contract DAAL-01-1996-02-044). We are grateful to Dr G. L. Hagnauer and Dr Lars Piehler for valuable discussions and support. We express sincere appreciation to Ms L. S. Nixon for manuscript preparation.

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