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Heterogeneous Rupturing Dendrimers

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Supporting Information Placeholder

ABSTRACT: Utilizing macromolecular scaffolds as templates for the production of small molecules that are distinctively different to the original monomer feedstock has many potential uses. Herein, as a proof-of-concept, a family of dendrimers displaying internally queued disulfide bridges were synthesized, and exploited as flawless macromolecular templates that selectively rupture, into a set of monomeric mercaptans. Disassembly was accomplished in a reducing environment, using DTT as an external stimulus, and the thiol constituents were successfully isolated. Their composition was dictated by three dendritic regions i.e. i) the symmetrical tri-thiol of the core (C_3), ii) the interior-asymmetric tri-thiols (CD_2), and iii) the periphery-asymmetric monothiols (DB_2) in which B functionality is of an orthogonal nature. Taking into account the steady-state between disulfides and thiols in all living cells, the collapse of the dendrimers to a multitude of smaller thiols was intracellularly assessed and as a means to disrupt the balance of reactive oxygen species (ROS) often elevated in cancer cells. Indeed, the fragmentation induced a significant increase of ROS in human lung carcinoma A549 cells. These findings can potentially alter the perception of dendrimers limited as carriers to prodrugs for intracellularly delivery of ROS- with potential to fight cancer.

Introduction

Dendrimers are among the most structurally advanced synthetic macromolecules that modern chemistry has accomplished.[1] Their flawless nature is a consequence of iterative synthetic procedure which capitalizes on robust organic reactions that interconnect an exact number of AB_x building blocks in a confined and highly branched polymeric framework. The resulting macromolecules have structural perfection equivalent to proteins or peptides, a globular shape and monodispersity, as well as the possibility for molecular weights in the peptide/ protein region.[2-3] Such unique features along with the multiple representation of an exact number of functional groups have enabled researchers to conduct structure-to-property studies in an array of research fields.[4-5]

To further expand the application areas dendrimer chemists, fueled by the increased number of robust, efficient and orthogonal approaches,[6-7] are revisiting the synthetic drawing-board to tailor more sophisticated dendrimers for biomedical applications. Several synthetic improvements have successfully been identified for such dendrimers that greatly simplify the chemistry *i*) *via* accelerated orthogonal growth which reduces the number of reactions by half for the synthesis of dendrimers[2, 8]; and *ii*) that displays functional group heterogeneity *i.e.* the capacity to carry a large and exact number of distinctively different cargos.[2] The latter, denoted as heterofunctional dendrimers (HFDs), are often accomplished by introducing heterogeneous functionalities at the periphery, including the popular example of so called "bowtie" dendrimers with Janus conformations.[9]

It is apparent that the dominant number of dendrimers reported on, or assessed by scientists, is still focusing on the large number of reactive end-groups at peripheral position while the interior is considered redundant as a non-participating compartment.[10-11]A small number of reports have ventured in to the design of HFDs with functionalities not only located at the periphery but also incorporated as pendant functionalities along the interior framework.[12] These HFD frameworks with internal/external functionalities represent the state-of-art, and make use of the dendritic interior as an asset to incorporate desired functionalities as part of the internal skeleton. [2, 13] In this context, self-immolating dendrimers have emerged that can, through a single activation step and by an external stimuli such as pH [14-15], light [16-18] or heat,[19] cause a cascade-like de-polymerization releasing the original feedstock of small building blocks.[20-21] However, with the increasing pace and demand for more advanced scaffolds, especially towards biomedical applications, it is evident that the dendrimers need to reach new grounds of structural sophistication to meet current and future needs. More precisely, the next generation of internally functionalized dendrimers need to go beyond single purpose function *i.e.* the release of original building blocks. Consequently, we propose a new generation of templating soft materials based on internally functionalized dendrimers that can upon the presence of an external stimuli i) rupture to a multitude of isolatable building blocks, distinctively different from the original monomer feedstock, and ii) spontaneously collapse and releases biologically relevant species that can intracellularly tip the balance of reactive oxidative species (ROS).

RESULTS AND DISCUSSION

As a proof-of concept, dual-purpose and monodisperse polyester dendrimers based on 2,2-bismethylol propionic acid (bis-MPA) with large and exact number of dormant and internally queued disulfide bridges were synthesized. Bis-MPA based dendrimers were a natural choice of platform due to their synthetic versatility, as well as being benign with respect to biological applications.[22] Disulfide bridges were selected as the most promising dormant species on the basis that i) they can undergo selective cleavage in a single step based on the already established redox mechanism between dormant oxidized disulfide and reactive reduced thiol; ii) they are found in many important biological functions including the thioredoxin or glutaredoxin redox systems responsible for scission of disulfides in living organisms[23-26];

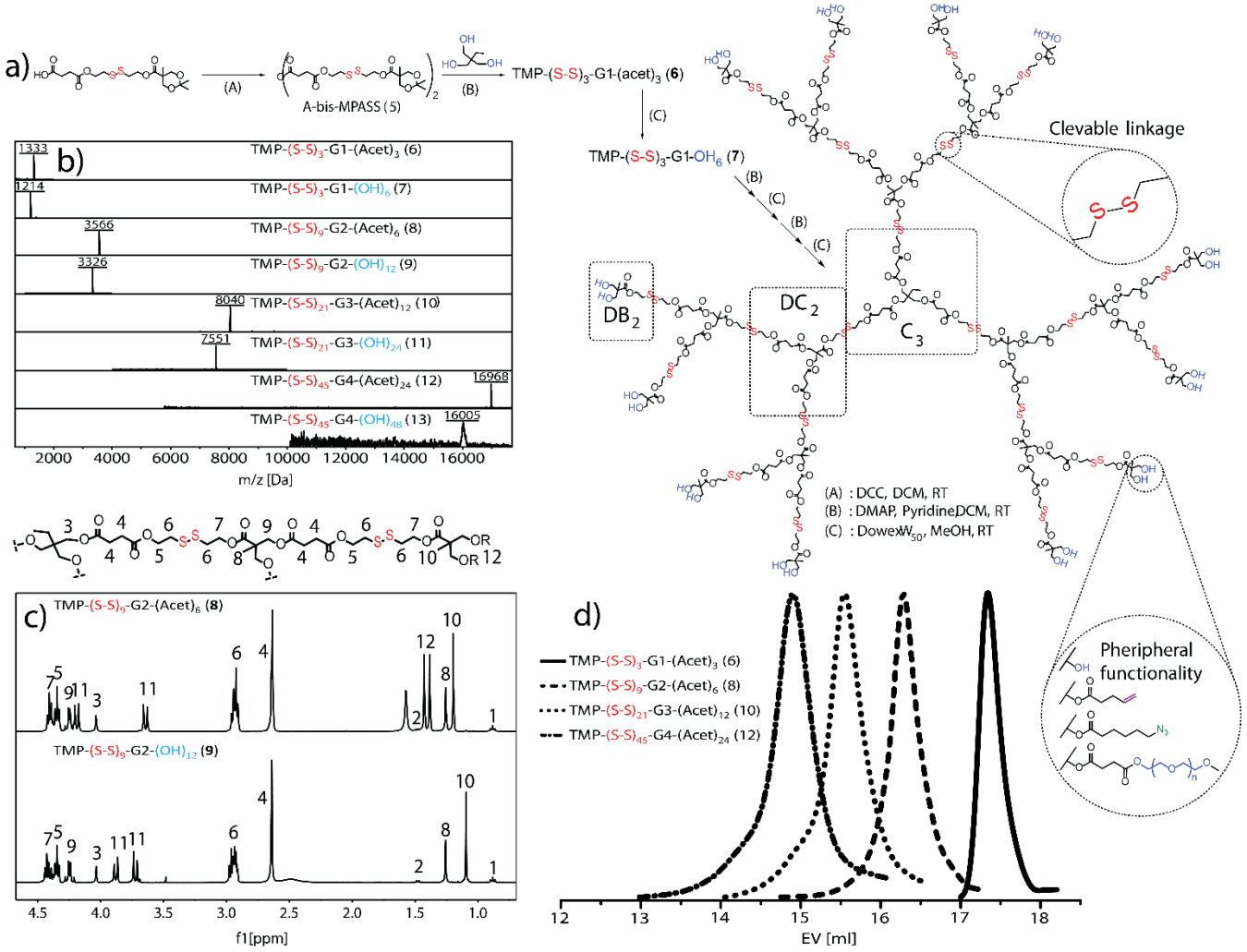

Figure 1 a) Synthetic strategy including monomer synthesis and divergent growth approach towards TMP-(S-S)$_{21}$-G3-(OH)$_{24}$ (**11**) as well as peripheral functionalities targeted in this work. **b)** MALDI-TOF-MS spectra of monodisperse dendritic scaffolds (**6-13**), attained masses are in agreement with calculated values. **c)** Structural analysis of TMP-(S-S)$_9$-G3-(Acet)$_6$ (**8**) (top (R = Acet)) and TMP-(S-S)$_9$-G2-(OH)$_{12}$ (**9**) (bottom (R = OH)) by ^{1}H NMR spectroscopy in $CDCl_3$ with spectra conforming to structure. **d)** THF SEC traces of acetonide protected dendrimers (**6**, **8**, **10** and **12**).

and iii) the collapse of these templating dendrimers would generate new highly reactive thiol functional monomers. These monomers are attractive building-blocks that can participate as key components in material science *via* a number of chemical transformation reactions including Michael addition and UV initiated thiol-ene or thiol-yne click reactions.[27-28]

Initially, the traditional AB$_2$ bis-MPA monomer required refinement to encompass asymmetric disulfides. This was accomplished in three consecutive synthetic steps, from commercial chemicals in >40 gram scale, resulting in anhydride activated carboxylic hydroxyethyl disulfide bis-MPA (A-bis-MPA^{SS}, **5**) with acetonide protected diols, **Figure 1a**. Dendrimers with heterogeneous bonds, *i.e.* internal disulfide bridges and ester bonds were accomplished *via* layer-by-layer divergent growth approach. The divergent route was chosen as it is the preferred growth strategy to generate flawless dendrimers in large scale with minimum loss of valuable dendritic feedstock.[29-31] The first generation [G] dendrimer based on a trimethylolpropane (TMP) core, TMP-(S-S)$_3$-G1-(Acet)$_3$ (**6**), with three dormant disulfide bonds was straightforwardly isolated in 95% yield through anhydride chemistry. From the various esterification conditions examined, a small excess of A-bis-MPA^{SS} (**5**) in CH_2Cl_2 and in the presence of pyridine and catalytic amounts of dimethylaminopyridine

(DMAP) with optimized molar ratios [$1_{TMP\text{-}OH}$][1.2_5][3_{Pyr}][0.2_{DMAP}] were found satisfactory.

Activation of the peripheral hydroxyl groups was achieved through simple acidic deprotection of TMP-(S-S)$_3$-G1-(Acet)$_3$ (**6**) in a 50:50 Vol% CH_2Cl_2: MeOH solvent mixture using Dowex W50^{TM} as an acidic resin. The resulting [G1] hydroxyl functional dendrimer TMP-(S-S)$_3$-G1-(OH)$_6$ (**7**) was isolated in 81 % yield. Iterative growth and activation steps yielded flawless dendrimers TMP-(S-S)$_9$-G2-(Acet)$_6$ (**8**), TMP-(S-S)$_9$-G2-(OH)$_{12}$ (**9**), TMP-(S-S)$_{21}$-G3-(Acet)$_{12}$ (**10**), TMP-(S-S)$_{21}$-G3-(OH)$_{24}$ (**11**), TMP-(S-S)$_{45}$-G4-(Acet)$_{24}$ (**12**) and finally TMP-(S-S)$_{45}$-G4-(OH)$_{48}$ (**13**) with a molecular weight of 15977 g/mol comprising 45 internal disulfide bonds as well as 48 peripheral hydroxides.

All reactions were monitored by NMR and MALDI-TOF MS and the final dendrimers were thoroughly characterized with respect to their structural perfection, **Figure 1** (**Figure S1-S13, S23** and **S25**). A typical ^{1}H-NMR of fully protected TMP-(S-S)$_{21}$-G3-(Acet)$_{12}$ (**10**) and its hydroxyl functional counterpart TMP-(S-S)$_{21}$-G3-(OH)$_{24}$ (**11**) can be seen in **Figure 1c**. To corroborate on the structural perfection of the dendrimers MALDI-TOF-MS was utilized, see **Figure 1b**.

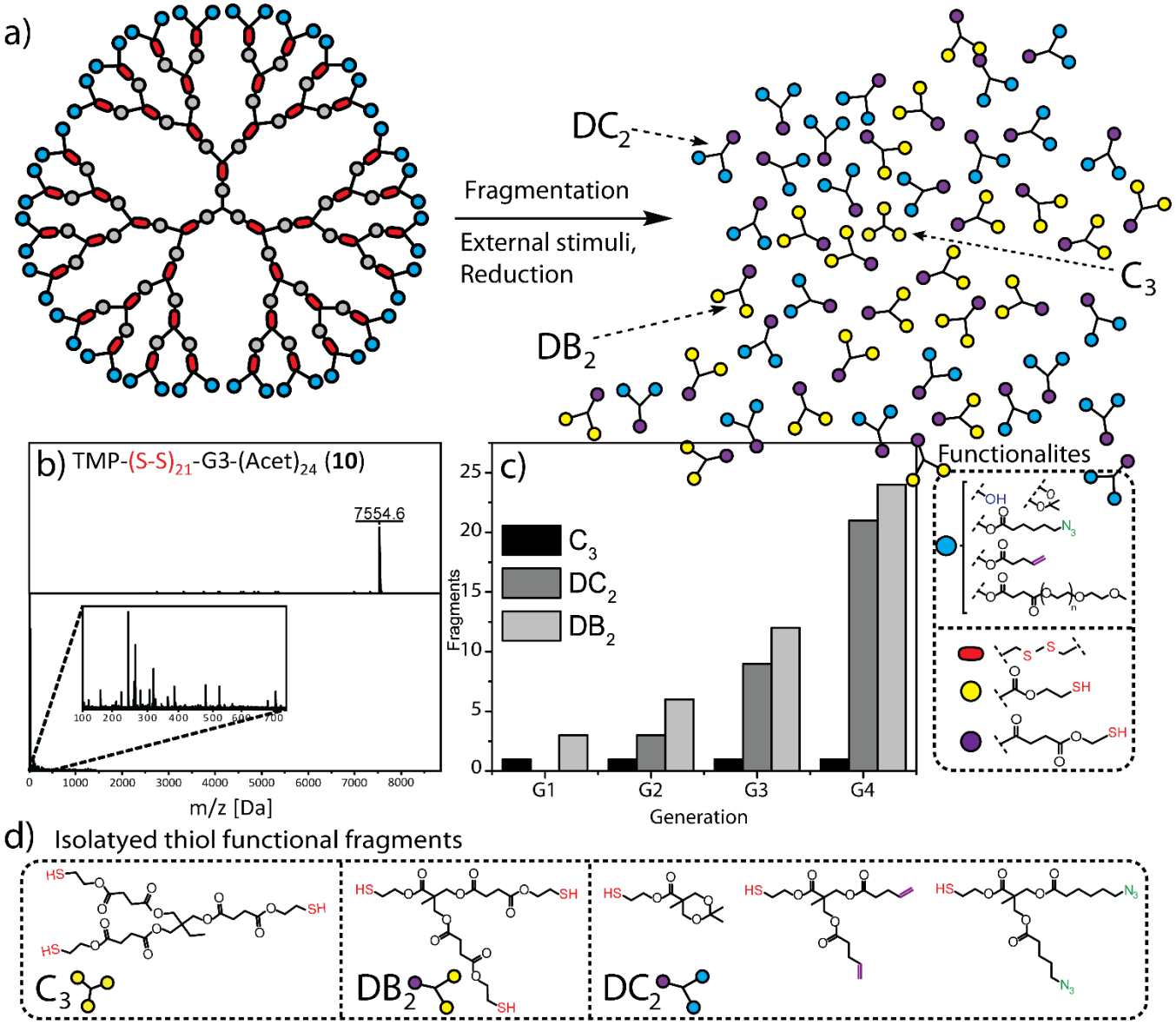

Figure 2 a) Illustration of dendrimer fragmentation with components gained upon fragmentation. **b)** MALDI-TOF-MS before and after degradation using DTT for TMP-(S-S)$_{21}$-G3-(Acet)$_{24}$ (**10**) displaying defined dendrimer peak before and complete disintegration after. **c)** Amount of thiols attained in proportion to dendritic generation as well as number of different fragments attained. **d)** All thiol functional fragments isolated in this work, analytical data can be found in supporting information.

SEC analysis further supported the molecular weight increase with generations in which all dendrimers exhibited consistently low dispersities (Đ) ranging from 1.02-1.13, see **Figure 1d**. Notably, no evidence of intramolecular sulphur rearrangements could be detected indicating that the dendritic architectures are stable during growth and activation steps as well as during storage. Subsequently, post-functionalization of TMP-(S-S)$_9$-G2-(OH)$_{12}$ (**9**) and TMP-(S-S)$_{21}$-G3-(OH)$_{24}$ (**11**) was undertaken to exhibit clickable reactive groups at the periphery, recognized for their robustness in various fields of

chemistry.[32-33] In this case, 12 and 24 allyls or azides respectively were introduced with the potential of further functionalization *via* thiol-ene coupling (TEC) and triazole forming click chemistries (CuAAC and SPAAC).[34-37] The resulting dendrimers with peripheral allyls TMP-(S-S)$_9$-G2-(Allyl)$_{12}$ (**14**), TMP-(S-S)$_{21}$-G3-(Allyl)$_{24}$ (**15**) and azides TMP-(S-S)$_9$-G2-(Azide)$_{12}$ (**16**), TMP-(S-S)$_{21}$-G3-(Azide)$_{24}$ (**17**) were isolated in 75%+ yields, analytical data can be found in **Figure S14-S17, S23 and S26**.

In contrast to the traditional dendrimers exhibiting only internal bonds of similar characteristics, these dendrimers display a heterogeneous combination of ester and disulfide bonds. In the presence of a reducing agent, an external stimulus, the dendrimers were found to undergo selective collapse, producing a burst of isolatable thiol-functional fragments, **Figure 2a**. The nature and number of the fragments produced is highly dependent on the generation and the constituents of the dendrimer. As seen in **Figure 2a** and **2c**, 1 mole of a fourth generation dendrimer with internally queued disulfide bridges would correspond to a total of 46 moles of three different potential fragments containing i) 1 symmetrical trithiol O,O'-(2-((2-mercaptoethoxy)carbonyl)-2-methylpropane-1,3-diyl) bis(2-mercaptoethyl) disuccinate (**C$_3$**) emanating from the core, ii) 21 internal unsymmetrical trithiols O,O'-(2-ethyl-2-(((4-(2-mercaptoethoxy)-4-oxobutanoyl)oxy)methyl)propane-1,3-diyl) bis(2-mercaptoethyl) disuccinate (**DC$_2$**) and iii) 24 unsymmetrical **DB$_2$** with 1 thiol unit and dual-functionality corresponding to the chosen peripheral groups, see **Figure 2d** for all fragments isolated.

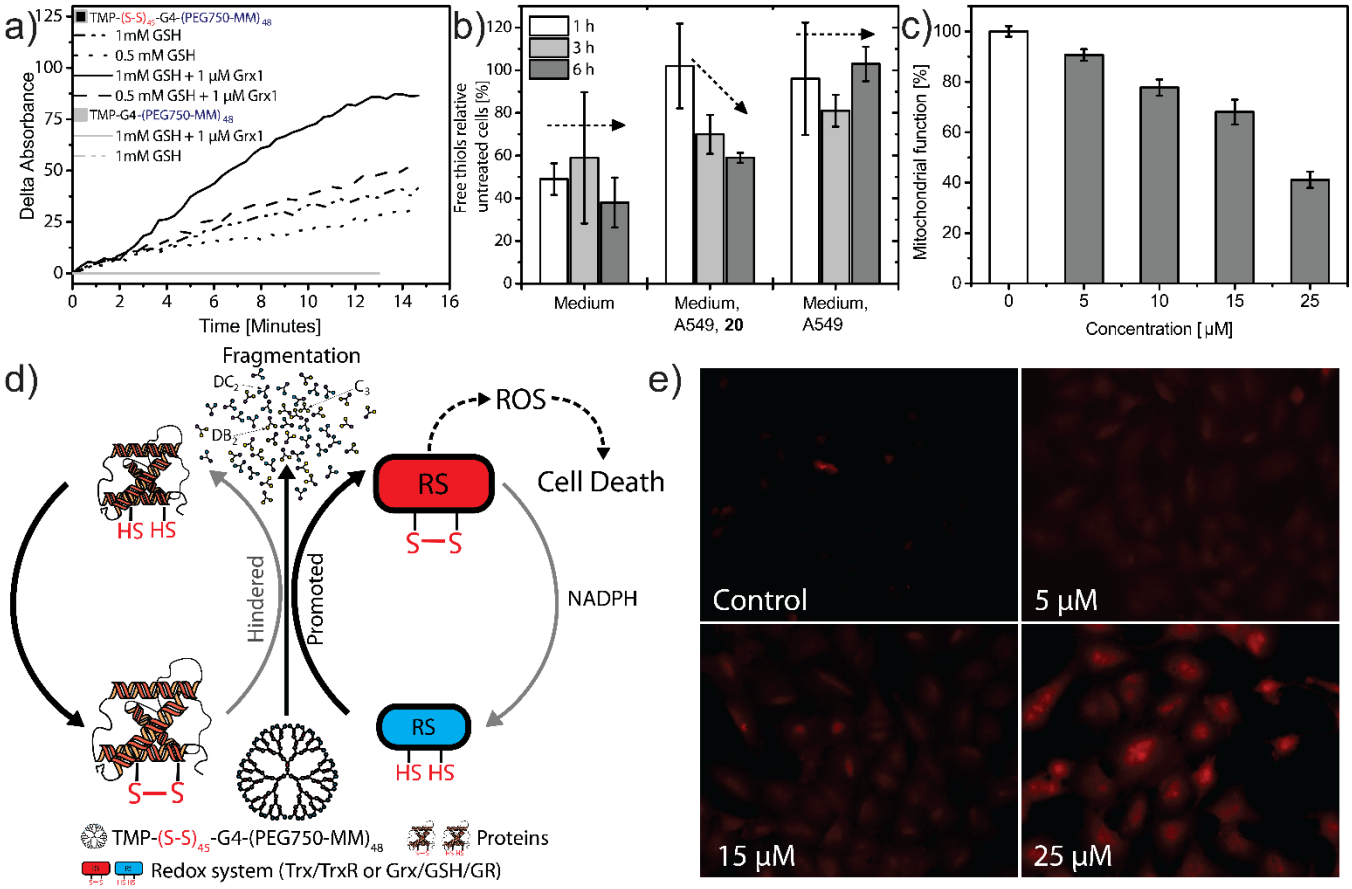

Figure 3 a) Degradation of TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**) in 50 mM Tris-HCl, pH 8.0, 1 mM, EDTA buffer using coupled enzyme reaction system comprising of nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH), glutaredoxin (Grx) and glutathione reductase (GR). **b**) Amount of free thiols generated in medium, from A549 cells, or by A549 cells combined with (**20**) determined by the DTNB assay. **c**) Cell viability by MTT assay with A549 cells treated with increasing concentrations of (**20**). **d**) Proposed intracellular mechanism of the rupturing dendrimers interfering with the thiol reductive systems (Trx/ TrxR or GSH/ Grx/ GR) causing thiol oxidation, generation of ROS and ultimately oxidative stress and cell death. **e**) ROS production illustrated by hydroethidine stained cells after treatment with in figure marked concentration of (**20**), control has no added (**20**).

This can be compared to a first generation dendrimer, the collapse of which would not generate any internal **DC$_2$** fragments, **Figure 2c**. For example, the TMP-(S-S)$_{21}$-G3-(Acet)$_{24}$ (**10**) with a molecular weight of ca 8 kDa and having 21 internally queued disulfides was exposed to dithiothreitol (DTT) in the presence of trimethylamine in chloroform at room temperature.

As seen in **Figure 2b and Figure S20**, the framework was fully cleaved within 15 minutes, from a dendritic template to its thiol functional fragments, using 2 equivalents of DTT for each internal disulfide bond. Upon the selective collapse, the fragments **C$_3$**, **DC$_2$** and **DB$_2$** were successfully isolated as pure compounds in 50-60% yields and characterized by NMR (**Figure S21**). Similarly, TMP-(S-S)$_9$-G2-(Allyl)$_{12}$ (**15**) and

TMP-(S-S)$_9$-G2-(Azide)$_{12}$ (**17**) were ruptured using identical conditions and resulted in isolatable **DB$_2$** fragments comprising one thiol as well as two allyls or azides, respectively, as well as **C$_3$** and **CD$_2$** fragments (**Figure 2d** and **Figure S22**). It should be emphasized that the yield of isolates was not subjected to further optimization but rather employed to validate the concept of using dendritic frameworks as precision polymeric templates to generate novel mercaptoethanol-functional building blocks that are difficult to access by conventional means.

These fragments have great potential for use as resin components to generated cross-linked networks via UV-initiated thiol-ene coupling chemistry, thiol-maleimide, Michael addition reactions etc. The concept illustrated in this work can be applied to a myriad of other polymeric scaffolds, such as hyper-branched or linear polymers. However, the structural perfection of dendrimers coupled with a large and exact number of peripheral reactive end-groups enables structural control that upon collapse generate new configurations of thiol-functional building-blocks.

From the large number of reports on monodisperse dendrimers and their use in biomedical applications, these scaffolds can be considered as next generation precision polymers in the field of nanomedicines. This is due not only to their flawless nature, large number of functional groups and fast fragmentation upon presence of external stimuli, but also the envisioned cellular mode-of-action. In an intracellular environment, and in contrast to the traditional bis-MPA dendrimers, the frameworks with internally queued disulfides are envisioned to undergo selective collapse catalyzed by endogenous disulfide reducing molecules, such as the glutathione and thioredoxin systems.[38-39] The intracellular reduction that disassembles the dendrimers to its individual fragments would require a substantial amount of electrons. The outcome of inducing an intracellular and cascade disassembling of the dendrimers would inevitably generate pronounced thiol oxidation, which would cause a highly artificial oxidizing environment that is toxic and potentially lethal to cells.[40-41] This is of greatest importance in the context of selective therapeutic treatment of cancerous cells: it is today well-known that cancerous cells have an increased basal production of ROS, owing to metabolic aberrations, making them more sensitive to additional ROS production.[42] Normal cells on the other hand, have a higher reserve capacity to buffer elevated ROS levels, and increasing the ROS level locally around cancerous tissue would hence suppress and induce cell death more effectively and specifically in the tumor. [43-47] To evaluate the intracellular performance of the dendrimers, further manipulation was required to introduce aqueous solubility as well as tune the size of the final dendrimer. The latter is a an vital variable to avoid the reticulo-endothelial system[48-51] and use a phenomena commonly known as the enhanced permeation and retention (EPR) effect.[52]

To this end, TMP-(S-S)$_{45}$-G4-(OH)$_{48}$ (**13**) was post functionalized with carboxylated poly(ethylene glycol) monomethyl ether (PEG750-MM) (**19**) yielding TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**). For comparison, PEGylation of a traditional bis-MPA dendrimer TMP-G4-(OH)$_{48}$ was also performed, TMP-G4-(PEG750-MM)$_{48}$ (**21**). The PEGylated dendrimers were water-soluble, with a molecular weight in the range of 57 kDa for TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**) and 47 kDa for TMP-G4-(PEG750-MM)$_{48}$ (**21**), for analytical data see **Figure S18-S19, S24, S27** and **S28.** Both scaffolds are above the threshold defined by Matsumura and Maeda as optimal for drug delivery vehicles.[48] The disassembly was investigated mimicking physiological conditions using the glutathione (GSH)/glutaredoxin (Grx)/glutathione reductase (GR) or the thioredoxin (Trx)/thioredoxin reductases (TrxR) systems as external stimuli on the disulfide containing dendrimer (**20**) and inactive (**21**) counterpart. The inactive dendrimer, with only ester bonds, displayed no disassembly in the presence of GSH in combination with Grx (**Figure 3a**). On the other hand, a dose dependent disassembly of (**20**) to its fragments was detected and over a time period of 15 minutes. The disassembly rate of (**20**) increased almost 4 times reaching a plateau at 1 mM of GSH and 1 μM of Grx. This may indicate that the dendrimer (**20**) is fully fragmented. A similar trend was observed with the Trx system, in which traditional bis-MPA dendrimer (**21**) displayed no physiological degradation while the active structure (**20**) disassembled rapidly. Trx was essential to the reaction as TrxR alone was unable to disrupt (**20**) (**Figure S29**).

Satisfied with observed disassembly of disulfide containing dendrimers in physiological condition, a preliminary *in vitro* investigation on their performance was conducted in the presence of human lung carcinoma A549 cells. The amount of free thiols generated in the media due to the disassembly of the dendrimers was determined through the DTNB assay, see **Figure 3b.** TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**) or A549 cells alone in media did not cause a significant change in free thiols. Notably, upon mixing both, a trend of thiol oxidation in the media could be observed which indirectly corroborates the disassembly mechanisms of the dendrimer through the consumption of the free thiols secreted by the cells. The amount of ROS created by TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**) is concentration dependent, and can be visualized in **Figure 3e,** where hydroethidine oxidation staining was used to visualize ROS as a red color. ROS has been argued to decrease cancer propagation by inducing senescence, disrupting their proliferation effectively halting the cancer growth.[53] **Figure 3c** displays the effect of TMP-(S-S)$_{45}$-G4-(PEG750-MM)$_{48}$ (**20**) as measured by MTT assay. A decrease below 80% cell viability was noted at a concentration of 10 μM, roughly corresponding to the ROS observed in **Figure 3b**. Further analysis of the cytotoxicity, signaling and programed cell death pathways, for deciphering the specific intracellular mechanism of (**20**) are required for future *in vivo* applications. Nonetheless, *in vivo* systems have been identified that can rupture (**20**) intracellularly, opening up new application potential for these types of dendrimers beyond the thiol oxidation caused in the cell, generating ROS, oxidative stress and cell death, **Figure 3d**. It should be emphasized that fragmentation strategy of the dendrimers is benign under biological conditions and may be an alternative avenue mode-of-action for precision polymers in the presence of cancerous cells.

CONCLUSIONS

A new family of polyester dendrimers with internally queued disulfide groups has successfully been synthesized and their

peripheral functionality manipulated to display highly desired reactive groups. These unique dendrimers exhibited a dual function as a consequence of their disassembly capability in a reductive environment. To one end, the dendrimers act as macromolecular scaffolds that fully collapses within 15 minutes upon exposure to the reducing reagent DTT into a novel set of thiol-functional building blocks suited for materials science. Further to this, the physiological environment with enzymatic redox systems, such as GSH-Grx and Trx present in all living cells, enabled selective rupturing of the dendrimers. Initial promising results detailed dendrimer collapse in short time periods, at relevant concentrations, and in the presence of human lung carcinoma A549 cells. Due to the intracellular disassembly of the dendrimers a cytotoxic amount of ROS was produced. To the authors' best knowledge, this is the first report of a dendrimer family with rupturing dual-capacity suited for both materials sciences as well as for nanomedicine.

ASSOCIATED CONTENT

Supporting Information

Experimental details, structure and synthetic procedure of small molecules and dendritic polymers, fragmentation conditions. Analytical data including ^{1}H, ^{13}C-NMR, SEC traces, MALDI-TOF-MS analysis and optimization, analysis of fragments. (PDF)

The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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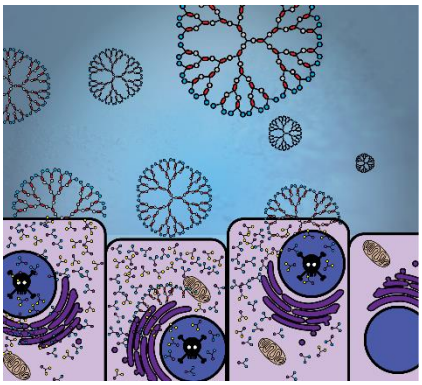

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