Low toxic, thermoresponsive dendrimers based on oligoethylene glycols with sharp and fully reversible phase transitions[†]

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Novel first (G1) and second (G2) generation dendrimers based on three-fold branched oligoethylene glycol dendrons are efficiently synthesized which show characteristic thermoresponsive behavior and negligible cytotoxicity (for G2).

A large variety of dendrimers¹ with different chemical structures have been developed in the last decades for applications in areas such as biomaterials, molecular devices, and catalysis.² Application-driven research also included the synthesis of representatives which are responsive to chemical or physical stimuli, such as light,³ solvent,⁴ and pH.⁵ Attention has further been paid to thermally responsive dendrimers.⁶ For example, in their pioneering work Kono and co-workers⁷ reported a series of PAMAM dendrimers which were rendered thermoresponsive (with lower critical solution temperature, LCST, in the range of 16-80 °C) by attaching short thermoresponsive units to the periphery. In a related approach, You, Hong et al.⁸ presented dendrimers with peripherally grafted long poly(N-isopropylacrylamide) (PNiPAM) chains. For application purposes it would be desirable to have materials with sharp, fast, and fully reversible phase transitions covering a broad temperature range.⁹ Unfortunately, these conditions are only partially met by the above cases. Either there is no mention of whether or not the transitions are reversible⁷ or the transitions take place in a relatively broad temperature range of ~ 5 °C.⁸ Also it seems that upon increased heating above the LCST, aggregates can disassemble.⁷ The cytotoxicity of thermally responsive dendrimers has not yet been investigated in great detail. It should be mentioned however that, for example, PAMAM exhibits considerable cytotoxicity.¹⁰

Linear oligo- and polyethylene glycols (OEG, PEG) are water-soluble macromolecules that exhibit low toxicity and excellent biocompatibility.11 This motivated us to decorate dendritic structures such as dendrons, dendrimers, and hyperbranched polymers with such units,¹² hoping that this surface decoration would render the products also watersoluble, non-toxic and bio-compatible.13 OEG and PEG are thermoresponsive. However, their LCSTs are in the range of 100–176 °C, which is rather high for many bio-related applications.¹⁴ This may perhaps be the reason why the thermoresponsive behavior of the decorated dendrimers was not investigated until recently, when Dai and Chang reported the thermoresponsiveness of G1 dendrimers. They however did not give information on the reversibility of their thermal transitions.¹⁵ It was discovered that dendronized polymers with specifically designed OEG-based dendrons exhibit unprecedentedly sharp, reversible and fast transitions in aqueous solution with LCSTs in the attractive range of 27-65 °C depending on the particular structure.¹⁶ In order to find out whether this rather unique behavior is associated with the structure of dendronized polymers, in which pendent dendrons are forced into a close packing by the backbone, or with that of the dendrons themselves, we sought for simpler and even easier to access macromolecules carrying exactly these OEG-based dendrons. We here report on the synthesis of dendrimers Me-G1, Et-G1, Me-G2, and Et-G2 (Fig. 1), which contain these dendrons as integral parts. These novel dendrimers' thermal transitions were studied in some detail by turbidity measurements. The aggregates that formed upon collapse above the LCST were characterized using optical and atomic force microscopy (AFM). Having such interesting compounds at hand, their cytotoxicity to both B16F1 and HaCat cell lines was also investigated and compared to linear



Fig. 1 Chemical structures of the dendrimers Me-G1, Et-G1, Me-G2 and Et-G2 discussed in this study.

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Scheme 1 Synthesis procedures for dendrimers Et-G1, Me-G1, Et-G2 and Me-G2. Reagents and conditions: (a) SOCl₂, DMAP, DCM, rt, 4 h (72–84%); (b) THPE, K_2CO_3 , KI, DMF, 80 °C, 24 h (88–90%); (c) THPE, Cs_2CO_3 , KI, DMF, 80 °C, 48 h (73–74%).

PEG in order to assess their potential applicability in the biomedical context.

The synthesis procedures for the dendrimers are outlined in Scheme 1. Starting from the known G1 and G2 dendron alcohols 1a, 1c, 2a and 2c,¹⁶ reaction with $SOCl_2$ in the presence of 4-dimethylaminopyridine (DMAP) afforded the corresponding benzyl chlorides 1b, 1d, 2b and 2d, respectively. The dendrimers were synthesized via Williamson etherification of corresponding dendron benzyl chlorides with the core molecule 1,1,1-tris(4-hydroxyphenyl)ethane, THPE. Et-G1 and Me-G1 were obtained with high yields (88-90%) by the reaction of **1b** and **1d** with THPE in the presence of K_2CO_3 and KI with the dendrons in an excess of 10% per phenolic function. Application of the same method furnished dendrimers Me-G2 and Et-G2 in very low yields only ($\sim 5\%$). Compounds in which THPE had reacted with one or two dendrons were the main products. Therefore, optimized conditions were applied: Cs₂CO₃ was used instead of K₂CO₃ and a larger excess (50%) of 2b or 2d applied. Additionally the reaction time was extended from 24 h to 48 h. Eventually, both Me-G2 and Et-G2 were obtained in yields of 73-74%. All new compounds were characterized by ¹H and ¹³C NMR spectroscopy, high resolution MALDI-TOF mass spectrometry, as well as elemental analysis to guarantee high purities. All dendrimers gave a single spot on TLC.

By visual inspection all dendrimers are water-soluble at room temperature; the clear aqueous solutions turn turbid however when heated. The temperatures at which these transitions occur (commonly, though not precisely referred to as LCST) and their course were investigated by turbidity measurements using UV/vis spectroscopy. The transmittance during the heating and cooling processes of aqueous solutions from **Et-G1**, **Et-G2**, **Me-G1** and **Me-G2** are plotted in Fig. 2a. The respective LCSTs are 27, 36, 51, and 65 °C. From these transition curves, the following conclusions can be drawn: (1) The transmittance below the LCST is close to 100%, while that above is close to zero. This suggests that for the concentrations used, the dendrimer solutions do not contain sizeable



Fig. 2 (a) Plots of transmittance *vs.* temperature (500 nm, $0.2 \,^{\circ}$ C min⁻¹; solid line: heating; dashed line: cooling) for 0.25 wt% aqueous solutions of dendrimers. (b) Dependence of LCSTs on concentrations of dendrimers and related polymers. Detailed plots of transmittance *vs.* temperature are shown in Fig. S1 in the ESI.†

amounts of aggregates below the LCST, while the aggregates formed above are large and compact enough to scatter light efficiently. (2) All of the dendrimers investigated show sharp transitions in both heating and cooling directions (<1 °C), whereby those of the ethoxy-terminated dendrimers (<0.8 °C) are even sharper than methoxy-terminated ones ($<1^{\circ}$). (3) Very small hystereses are observed between heating and cooling, which suggests sensitive and fully reversible hydration and dehydration processes. (4) The LCSTs are tunable in the range of 27-65 °C which includes the highly relevant transition of Et-G2 at 36 °C. These different temperatures reflect the differences in hydrophilicity. It is reasonable to assume that the second generation and methoxy terminated dendrimers are more hydrophilic than the first generation and ethoxy terminated ones, respectively.¹⁶ The concentration dependence of the LCST was also investigated in the range of 0.05-2.0 wt% and the results are plotted in Fig. 2b. Obviously, the lower the dendrimer concentration, the higher the LCST and the broader the phase transition (see Fig. S1 in the ESI[†]). This may be related to an aggregation kinetic effect, *i.e.*, the lower the dendrimer concentration, the longer the time needed for the formation of aggregates. If one compares the thermoresponsive behavior of the present dendrimers with the reported dendronized polymers Me-PG1 and Me-PG2,¹⁶ two differences become evident: Both the concentration and generation dependence of the LCST are lower for the latter than for the former.

The aggregation process of dendrimers in aqueous solution was followed by dynamic light scattering (DLS). Large aggregates with sizes in the range of 2-5 micrometres were observed above the LCST for all G1 and G2 dendrimers (see Fig. S2 in the ESI[†]). The aggregates were also directly observed in aqueous solutions with optical microscopy. As shown in Fig. 3a, Et-G2 formed spherical aggregates with sizes around 2-4 micrometres, which is in good agreement with the results from DLS. Its thermally induced aggregation and segregation process followed by optical microscopy (OM) was video-taped in time intervals of one second (for the movie, see the ESI[†]). As shown in the movie, below the LCST, no aggregates were visible and above, large spherical aggregates appeared suddenly, and their size remained nearly unchanged. They disappeared again when the solution temperature decreased below the LCST. This process was repeated numerous times and is fully reversible. The aggregates were also observed



Fig. 3 (a) Optical micrograph of the aggregates in 0.25 wt% aqueous solutions of **Et-G2**. Note that the white features are the ones in focus. The inset: four-times enlarged feature. (b) Tapping mode AFM image of the aggregates on HOPG from 0.25 wt% aqueous solutions of **Et-G2**. (c) Cross-sectional profile of AFM image in (b).



Fig. 4 Cytotoxicity of dendrimers to B16F1 (a) and HaCat cells (b) by CCK-8 assay. Data are mean values plus/minus standard deviation of four samples/cultures.

in dry state on solid substrates (HOPG) by AFM. For sample preparation, an aqueous solution of **Et-G2** was pre-heated above its LCST (~50 °C) and then spin-coated (2000 rpm) onto the pre-heated HOPG substrate. Fig. 3b shows the tapping-mode image of the aggregates, which very much like in OM appeared spherical. The cross-sectional profile (Fig. 3c) indicates the aggregates on the substrate with an average size of roughly around 30 nm (height) ×1 μ m (width), which suggests the aggregates in dry state to be smaller than those observed in aqueous solution. Further studies are on the way to elucidate the mechanism of aggregate formation aiming, besides others, at an understanding of the origin of this unique behavior.

Because of the rather attractive thermoresponsive characteristics of the dendrimers reported here, their cytotoxicity¹⁷ was investigated using B16F1 and HaCat cell lines, and the results were compared with linear PEG ($M_n = 5600$). These cell lines were incubated with aqueous solutions of all dendrimers (concentrations: 1–100 µg mL⁻¹) for 48 h at 37 °C and treated with a CCK-8 assay for another 3 h. The cytotoxicity results are plotted in Fig. 4. The cell viability fell to <50% for B16F1 and <80% for HaCat, respectively, for both G1 dendrimers (above concentration 50 µg mL⁻¹), with **Et-G1** being slightly more toxic than **Me-G1**. In contrast, for both G2 dendrimers the cell viability was around 100% up to a concentration of 100 μ g mL⁻¹. These latter results compare favorably with linear PEG. Unlike the G1 dendrimers, the cell morphologies after exposure to the G2 dendrimer solutions remained virtually unchanged as compared with these of the control cells (Fig. S3 in the ESI†). Both results indicate a rather low toxicity of the G2 dendrimers.

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