Fluorescent dendrimers with a peptide cathepsin B cleavage site for drug delivery applications†

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The synthesis of a multifunctionally equipped first generation (G1) dendrimer carrying a pentapeptide with a cathepsin B cleavage site, chelating ligands for Pt^{2+}-complexation, and a dansyl fluorescence marker is described and an investigation of its cellular uptake as well as intracellular localization by confocal fluorescence microscopy reported.

In recent years dendrimers have attracted more and more attention in biomedical applications,[1] especially as transfection agents for DNA transfer into eukaryotic cells,[2] as contrast agents for magnetic resonance imaging (MRI),[3] in boron neutron capture therapy (BNCT) for cancer treatment,[1a,4] and most recently as potentially selective drug delivery vehicles.[5] Regarding their use as carrier molecules for biologically active compounds, various methods have been employed: cargo molecules were transported, *e.g.* by entrapping them in the interior of dendrimers by using hydrophobic interactions[6] or by covalently attaching them to the dendrimers' peripheral groups.[7] In the latter case, selective delivery of the cargo molecules can be achieved most effectively by using an attachment that allows the cargo molecules (drugs) to be cleaved off whenever the site of action is reached. For biocompatible HPMA polymers, selective delivery *via* cleavable spacer molecules has been reported.[8]

With this in mind we set out to develop a G1 dendrimer (Scheme 1) which carries dansyl fluorescence tags, an enzymatically cleavable oligopeptide spacer and is designed such that a variety of antitumor agents can be attached easily. As a spacer we chose the H-Gly-Arg-Phe-Gly-OH sequence to ensure a selective cleavage by the endopeptidase activity of cathepsin B.[9] Cathepsin B, a lysosomal cysteine protease, is often overexpressed in malignant cells[10] and seems to play an important role in the process of tumor invasion and metastasis.[11] Initiated by our recent work on the synthesis and biological evaluation of a set of water-soluble, surface-functionalized poly(amidoamine) dendrimers,[12] some of which were equipped with ligands for the complexation

† Electronic supplementary information (ESI) available: synthesis and spectroscopic data of compounds **1**, **3** and **4**. See http://www.rsc.org/suppdata/cc/b4/b415651j/

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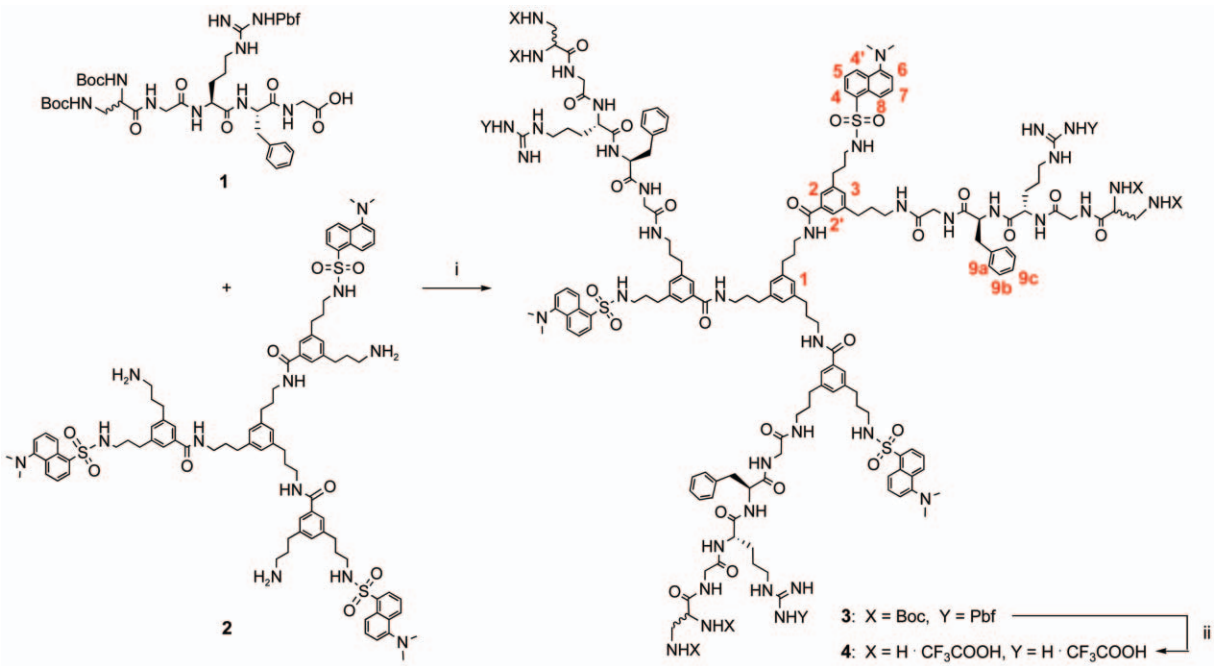

Scheme 1 Synthesis of the partially dansylated, peptide and 2,3-D/L-diaminopropionic acid modified dendrimer **4**. *Reagents and conditions*: (i) (a) **1** (4.8 eq.), HATU (5.3 eq.), DIPEA (15.6 eq.), DMF, 0 °C, 5 min, (b) **2** (as CF_3CO_2H salt 1 eq.), DMF, −20 °C/1 h, r. t. 12 h, 69%; (ii) aq. TFA (95%), r. t., 3 h, 100%. DIPEA = *N*,*N*′-diisopropyl ethylamine, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, TFA = trifluoroacetic acid.

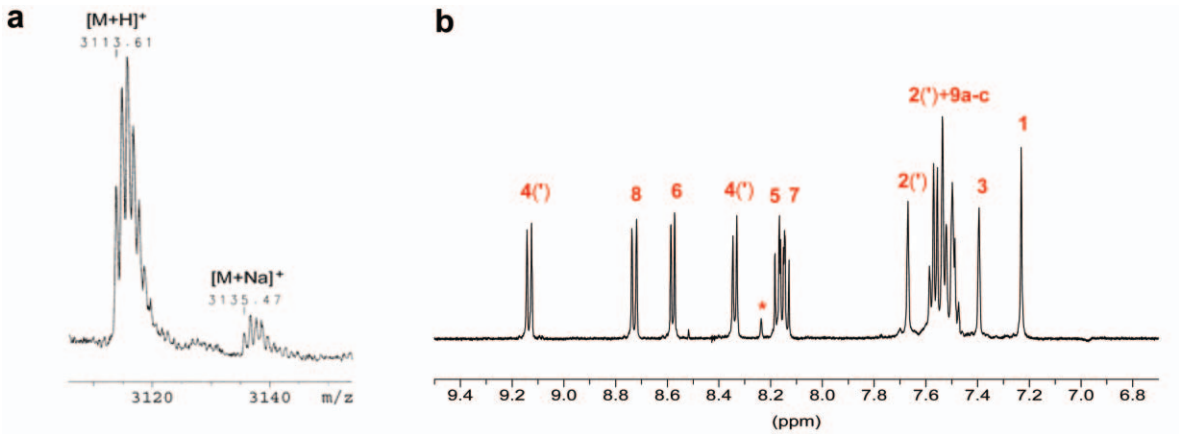

Fig. 1 Section of the (a) MALDI-ToF mass spectrum (reflector mode; *matrix*: dithranol) and (b) ^{1}H-NMR spectrum (500 MHz, *solvent*: [D_2] water + [D_3] acetonitrile, {*}: DMF) of the deprotected, peptide-functionalized and dansylated dendrimer **4** with terminal ethylene diamine ligands.

of anticancer-active Pt^{2+}, we attached the 2,3-D/L-diaminopropionic acid (Dpa) ligand to the present oligopeptide's N-terminus.[13] However, in light of Duncan's work[14] the attachment of antitumor agents with a different mode of action (*e.g.* doxorubicin) could reveal the oligopeptide's potential to a greater extent.[15]

Synthesis of the protected pentapeptide building block **1** was performed using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis techniques on the highly acid-sensitive *o*-chlorotrityl resin (Barlos resin).[16] Details of the peptide synthesis are given in the electronic supplementary information (ESI). The protected pentapeptide **1** was then reacted with the peripheral primary amines of the partially dansylated dendrimer **2**[12] by activation with the coupling reagent HATU in DMF in the presence of DIPEA (Scheme 1). Activation of the peptide's carboxylic acid function was achieved within 5 min at 0 °C. The subsequent amidation reaction with **2** was performed at −20 °C over a period of 12 hours to achieve the protected, peptide-functionalized dendrimer **3** in 69% yield after aqueous workup and standard column chromatography purification.[17] In the final step, dendrimer **3** was deprotected by reaction with aqueous trifluoroacetic acid (TFA; 95%) at room temperature. During the procedure the included water acts as a cation scavenger and prevents undesired alkylation of the peptide dendrimer.[18] Carpino *et al.* reported half-lives of 8 minutes for the rate of acidolytic

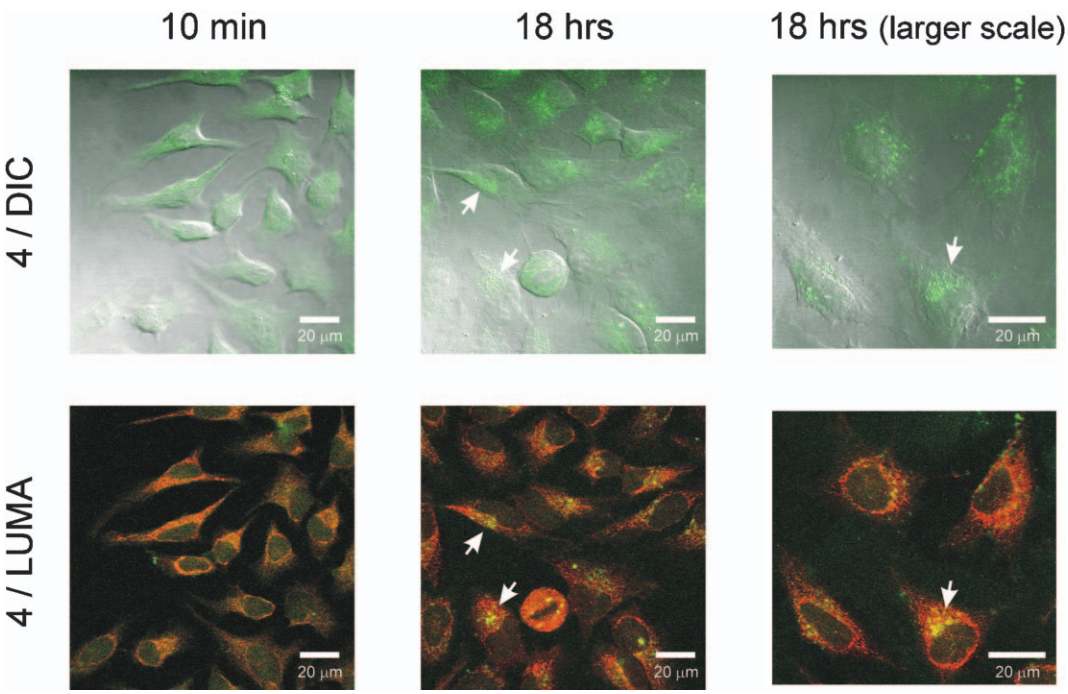

Fig. 2 HeLa cells were incubated with 5 µM dendrimer **4** in growth medium for the times indicated. The cells were then fixed and stained with appropriate antibodies for LUMA, a protein present at the inner nuclear membrane and to some extent in the endoplasmic reticulum. The resulting fluorescence patterns were then visualized by confocal laser-scanning fluorescence microscopy. **4**/DIC: superimposition of the differential interference contrast image and the dendrimer **4** fluorescence (green); **4**/LUMA: superimposition of the fluorescent LUMA image (red) and the dendrimer **4** fluorescence (green). Arrows indicate clusters of **4** that are already visible within the cells after 4 hours. Bars: 20 µm.

cleavage of the Pbf protecting group.[19] In the case of peptide dendrimer **3**, the reaction time was extended to 3 hours to ensure a clean and complete removal of all nine protecting groups (see Scheme 1). Fig. 1 shows sections of the MALDI-ToF mass spectrum and the ^{1}H-NMR spectrum of the completely deprotected, water-soluble peptide dendrimer **4**.

Cellular uptake and intracellular distribution of dendrimers has already been reported.[12,20] In some cases they were complexed with oligonucleotides or DNA for transfection purposes.[21] In order to contribute to this important aspect, dendrimer **4** was incubated with human HeLa cells over a period of 24 hours in a final concentration of 5 μM in cell-culture medium. At different times, the intracellular distribution of **4** was investigated by confocal fluorescence microscopy. Remarkably, more than 90% of all cells show a diffuse staining throughout the cell within 10 minutes, which includes the cell nucleus (Fig. 2). After 4 hours, this staining changes to a punctate pattern. Thereafter, in many of the cells, the dendrimers concentrate in a cluster near the cell nucleus. This may indicate an enrichment of the dendrimer particles at the Golgi apparatus and corresponds with findings recently reported for other fluorescence-labelled dendrimers.[12]

In summary, a multifunctional dendrimer with an enzymatically cleavable peptide spacer was synthesized by standard organic and peptide synthesis techniques. Additionally, it carries dansyl fluorescence markers for intracellular detection. The dendrimer is considered a prototype dendritic carrier where the antitumor agent is connected *via* an enzymatically cleavable oligopeptide spacer. In the initial phase of this work a ligand for Pt^{2+}-complexation was chosen though other options are also promising. Confocal fluorescence microscopy proved that human HeLa cells rapidly internalize the dendrimers which finally concentrate in clusters next to the cell nucleus after 18 hours of incubation. Further co-localization studies will determine the compartments involved in dendrimer trafficking and their final destination in the cell.

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