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PAMAM Structure-Based Multifunctional Fluorescent Conjugates for Improved Fluorescent Labelling of Biomacromolecules

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Abstract: Fluorescent probes are of increasing interest in medicinal and biological applications for the elucidation of the structures and functions of healthy as well as tumour cells. The quality of these investigations is determined by the intensity of the fluorescence signal. High dye/carrier ratios give strong signals. However, these are achieved by the occupation of a high number of derivatisation sites and therefore are accompanied by strong structural alterations of the carrier. Hence, polyvalent substances containing a high number of fluorescent dyes would be favourable because they would allow the introduction of many dyes at one position of the compound to be labelled.

A large number of different dyes have been investigated to determine the efficiency of coupling to a dendrimer scaffold and the fluorescence properties of the oligomeric dyes, but compounds that fulfil the requirements of both strong fluorescence signals and reactivities are rare. Herein we describe the synthesis and characterisation of dye oligomers containing dansyl-, 7-nitro-2,1,3-benzoxadiazol-4-yl- (NBD), coumarin-343, 5(6)-carboxyfluorescein and sulforhodamine B2 moieties based on polyamidoamine (PAMAM) dendrimers. The PAMAM dendrimers were synthesised by an improved protocol that yielded highly homogeneous scaffolds with up to 128 conjugation sites. When comparing the fluorescent properties of the dye oligomers it was found that only the dansylated dendrimers met the requirements of enhanced fluorescence signals. The dendrimer containing 16 fluorescent dyes was conjugated to the anti-epidermal-growth-factor receptor (EGFR) antibody hMAb425 as a model compound to show the applicability of the dye multimer compounds. This conjugate revealed a preserved immunoreactivity of 54%.

We demonstrate the applicability of the dye oligomers to the efficient and applicable labelling of proteins and other large molecules that enables high dye concentrations and therefore high contrasts in fluorescence applications.

Keywords: biomacromolecules · dansyl chloride · dendrimers · fluorescence · fluorescent probes

Introduction

In the past decades, fluorescent dyes have increasingly gained importance for various applications in biology and medicine. They are widely used for non-invasive visualisation on the anatomic as well as on the cellular level.

Their numerous applications in vitro include the identification of genotypes (mutations, expression, spatial processing, localisation, transport of arbitrary genes),[1] interactions between proteins,[2] the influence, activity and function of tumour-suppressor genes,[3,4] imaging of apoptosis by the determination of caspase activity,[5] determination of chromatin elimination,[6] dynamics of chromatin interactions[7] and the activation of genes.[8] In oncology, in vitro fluorescence labelling techniques have become increasingly interesting because they allow the determination of tumour malignancy,[9] chromosomal abnormalities,[10] metastatic disposition of tumours,[11] tumour oxygenation and pH,[12] tumour-induced angiogenesis,[13] gene deletions, duplications and amplifications[14] and also apoptosis versus necrosis imaging in cancer cell lines.[15]

An emerging application of fluorescent compounds is diagnostic imaging in vivo, which allows an estimation of the metastatic disposition of tumours,[16] the detection of tumour-associated enzymes[17] and single cells in living or-

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ganisms,[18,19] the imaging of angiogenesis,[20] intra-operative sentinel lymph node mapping,[21–23] the determination of protein kinase activity,[24] apoptosis imaging,[25] the functional state of cells and the detection of tumour cells.[26]

A disadvantage of fluorescent labels over radioactive labels is their relatively low sensitivity. Effective imaging relies on fluorescent labels that produce strong signals and thus high signal-to-noise ratios. Their applications are limited by the fluorescent properties of the particular dye. To further improve the potential of fluorescent labels, it would be advantageous to develop dyes that produce strong fluorescence signals but do not lead to strongly altered properties of the compound to be labelled. For this purpose polyfluorescent dyes based on polymeric structures would be suitable. These compounds should allow a facilitated introduction into the compound to be labelled without severe structural alteration of the carrier. The multimeric fluorescent substances synthesised so far are merely used as chemosensors for H^+ or metal ions,[27–31] optical brighteners[32,33] or potential compounds for organic light-emitting diodes[34,35] because they are either insoluble in water-containing systems or cannot be introduced into a biocompatible carrier molecule.

Dendrimers, which represent a class of homogeneous molecules with a symmetrically branching structural motif, have been shown to be suitable for the linkage of several effectors. They have, for example, been shown to be effective drug delivery vehicles for oligonucleotides,[36] methotrexate,[37] folic acid,[38] taxol,[38] methylprednisolone,[39] doxorubicin[40] and boron-rich compounds for boron neutron capture therapy (BNCT)[41–43] and are even the basis for approved pharmaceuticals such as the microbicide Vivagel[44] and the diagnostic cardiac marker Stratus.[45]

Thus, well-characterised dendritic substances containing a high number of fluorescent dyes and a functional group for selective coupling to a carrier molecule should be advantageous to the introduction of a high number of dyes concentrated at one position of the compound to be labelled. Therefore, high dye concentrations that produce strong fluorescence signals should become assessable for improved fluorescence labelling.

Herein we describe the synthesis and characterisation of polyfluorescent dendrimers containing a conjugation site for the labelling of macromolecules. Their suitability as fluorescent-labelling probes is shown by the conjugation of an exemplary dansylated dendrimer to the anti-epidermal-growth-factor receptor (anti-EGFR) antibody hMAb425.

Results and Discussion

Improvement of polyamidoamine (PAMAM) dendrimer synthesis: In general, a major advantage of dendrimers over polymers is the low dispersity of the products. However, the homogeneity of commercially available dendrimers was found to be low. Similar results have recently been described for commercially available PAMAM dendrimers, which were analysed by HPLC and MALDI-TOF mass spectrometry and showed high polydispersities of the compounds.[46]

Therefore, these compounds were not chosen as building blocks for the syntheses. Attempts to synthesise PAMAM scaffolds according to literature methods[47–49] resulted in products with higher quality but they also did not meet the homogeneity requirements.

Therefore, improved synthesis protocols were developed to achieve the low dispersity required. As reported before,[50] a substantial modification of the reaction temperatures had to be made to obtain highly homogeneous products: when synthesising the half and full generations by reaction with ethylenediamine special care had to be taken that the reaction temperature did not rise above room temperature and 4 °C, respectively. Reactions that were not controlled showed a high degree of fragmentation, elimination and intramolecular ring formation and as a consequence a high heterogeneity of the products. In contrast, the dendrimers synthesised by the modified protocol were analysed by HPLC and mass spectrometry, which showed they were obtained in good yields and high purity (Figure 1).

A second crucial point that has to be considered to ensure the quality of the products is the purification of the den-

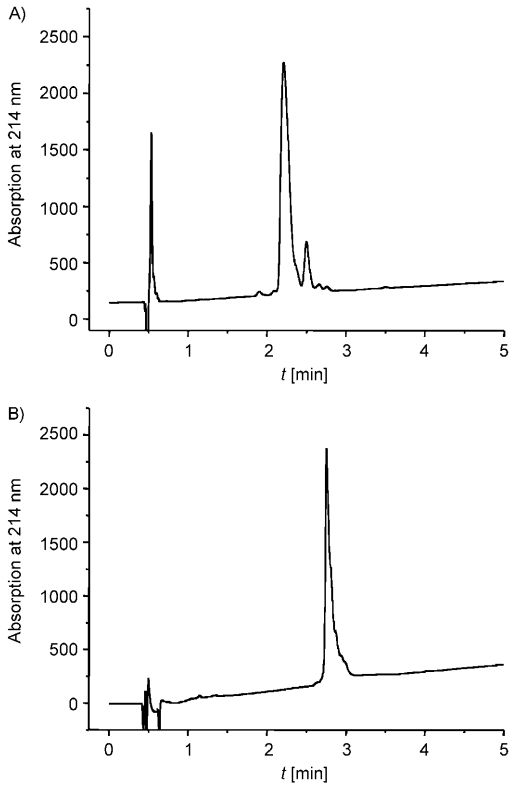
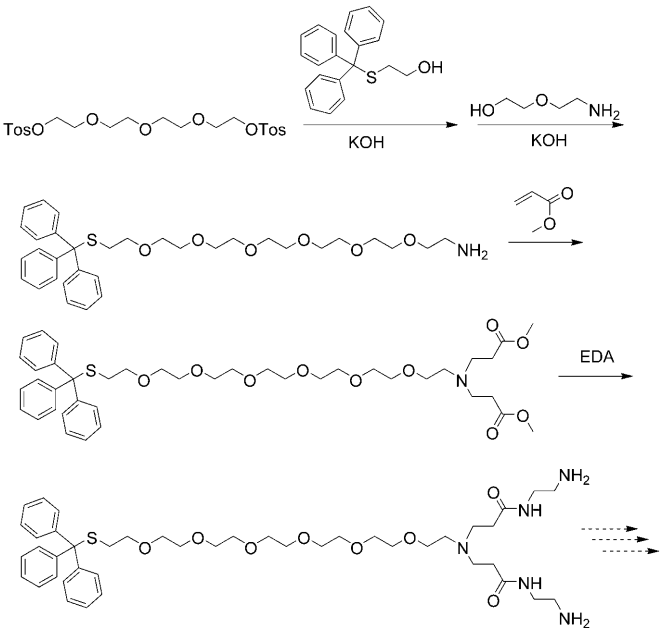


Figure 1. HPLC chromatograms of the G_3 and G_4 dendrimers containing 8 (A) and 16 (B) amino functions synthesised on an *S*-trityl-mercaptoethyl-pentaethylene-glycol-ethylamine core, as described in the Experimental Section. Chromatograms were obtained by using a Chromolith Performance column and a gradient from 20–45 and 0–60% acetonitrile in 5 min, respectively.

drimers. Attempts to purify the reaction mixtures of the higher generation dendrimers by column chromatography, ultrafiltration or dialysis resulted in low yields or impure products. In contrast to this, HPLC purification provided the best results, yielding products of high homogeneity.

To be suitable for selective introduction into proteins and other macromolecules that are to be conjugated with high efficiency, the polyfluorescent dendrimers have to contain a reactive site for selective coupling reactions. Therefore the PAMAM dendrimers were synthesised on a pentaethylene glycol linker containing a trityl-protected thiol. The linker was introduced to increase the accessibility of the free thiol during the coupling reaction. The PAMAM dendrimers were grown on the terminal amino function of the linker by alternating the reaction with methyl acrylate and ethylenediamine to give half and full generations of the dendrimer (Scheme 1).



Scheme 1. Synthesis of the pentaethylene glycol linker and the PAMAM dendrimers.

To synthesise the linker, tetraethylene glycol ditosylate[51] was first treated with *S*-tritylmercaptoethanol[52] and subsequently with 2-(2-aminoethoxy)ethanol. The PAMAM dendrimers were subsequently built by alternating the reaction with methyl acrylate and ethylenediamine.

By these methods, we were able to synthesise PAMAM dendrimers of up to 128 amino functions on a pentaethylene glycol linker that contains a thiol for selective coupling to antibodies or other macromolecules. The dendrimers obtained were found to be of high homogeneity and are therefore suitable as building blocks for the synthesis of the fluorescent dye oligomers.

Derivatisation of the PAMAM dendrimers with fluorescent dyes: The synthesis of the dendrimer was followed by the derivatisation of the amino functions with fluorescent dyes. A large number of different fluorescent dyes were tested with respect to their reactivity with the terminal amino functions of the PAMAM dendrimer. When introducing two or more dyes, a high variation of the coupling efficiencies could be observed. This low reactivity can be attributed to steric hindrance because it has been observed that smaller fluorescent dyes can be coupled with higher efficiencies and give more homogeneous products than bulky ones. Poor coupling yields were observed for sulforhodamine Q4 fluoride, sulforhodamine Q5 fluoride, sulforhodamine B chloride, rhodamine B isothiocyanate, rhodamine 101, rhodamine 101 aminohexanoic acid, fluorescein isothiocyanate, 5-carboxyfluorescein, naphthofluorescein, 6-[fluorescein-5(6)-carbamido]hexanoic acid, NIR 820 chloride,[53] NIR 820 isothiocyanate, NIR 820 thiol, NIR 797 chloride,[54] NIR 797 aminohexanoic acid, Py-5[55] and Py-5 aminohexanoic acid. The reactions with these dyes led to dendritic structures that were not fully derivatised or heterogeneous mixtures that could not be purified (Table 1).

Table 1. Yields from the coupling of the fluorescent dyes to dendrimers containing one (G_0), two (G_1) and four (G_2) amino groups.

Fluorescent dye	Yield[a] [%]		
	G_0	G_1	G_2
sulforhodamine Q4 fluoride	63 (45)[b]	2	0
sulforhodamine Q5 fluoride	2	2	0
sulforhodamine B chloride	15	3	0
rhodamine B isothiocyanate	45 (55)[b]	2	0
rhodamine 101	32 (39)[b]	4	0
rhodamine 101 aminohexanoic acid	70	56	0
fluorescein isothiocyanate	68 (49)[b]	3	0
5-carboxyfluorescein	45 (60)[b]	6	0
naphthofluorescein	13	5	0
6-[fluorescein-5(6)-carbamido]hexanoic acid	73 (79)[b]	52	27
NIR 820 chloride	42 (20)[b]	3	0
NIR 820 isothiocyanate	74	2	0
NIR 820 thiol	12	8	0
NIR 797 chloride	12	5	0
NIR 797 aminohexanoic acid	52	7	0
Py-5	39 (44)[b]	56	0
Py-5 aminohexanoic acid	67 (26)[b]	49	40
dansyl chloride	63[c]	58[c]	40[c]
NBD chloride	43[c]	63[c]	46[c]
coumarin-343	48[c]	54[c]	56[c]
5(6)-carboxyfluorescein pentafluorophenyl ester	89[b]	89[c]	81[c]
sulforhodamine B2 acid fluoride	71[c]	76[c]	46[c]

[a] Yields [%] for the coupling reaction with dendrimers G_0, G_1 and G_2. [b] Monosubstituted. [c] Yields of isolated products.

Five fluorescent dyes, dansyl chloride, 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) chloride, coumarin-343, 5(6)-carboxyfluorescein pentafluorophenyl ester and sulforhodamine B2 acid fluoride, could be introduced into G_0, G_1 and G_2 dendrimers containing one, two and four amino functions, respectively. Comparison of the fluorescence properties of the dendrimers derivatised with these dyes should reveal the

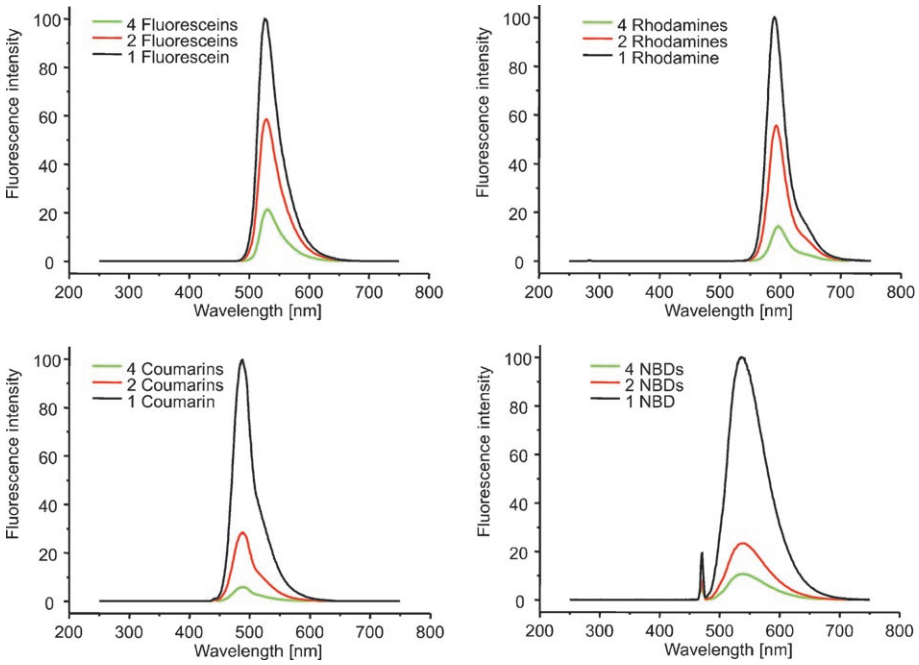


Figure 2. Fluorescence spectra of the compounds containing one, two and four amino functions derivatised with NBD chloride, coumarin-343, 5(6)-carboxyfluorescein pentafluorophenyl ester and sulforhodamine B2 acid fluoride.

most suitable compounds for fluorescence imaging applications.

The fluorescence spectra of G_0, G_1 and G_2 derivatised with NBD chloride, coumarin-343, 5(6)-carboxyfluorescein pentafluorophenyl ester and sulforhodamine B2 acid fluoride unambiguously revealed that the fluorescence intensities decreased with increasing number of coupled dyes (Figure 2). This has been attributed to the self-quenching of the fluorescence, which is due to the relatively small Stokes shift of the compounds and their small distance from each other within the molecules.[56]

In contrast, the compounds derivatised with dansyl chloride showed only relatively low self-quenching. This is due to the large Stokes shift of the dansyl dye of approximately 195 nm. As dansyl chloride is the only fluorescent dye presently available that shows a sufficiently large Stokes shift and because the dansylated dendrimers show an increase in fluorescence with higher numbers of dyes, larger generations of dendrimers were synthesised with an increasing number of dyes. The fluorescence intensities of the dye monomer and the multivalent dyes containing 1–128 dansyl moieties are shown in Figure 3. In these dendrimers the fluorescence intensity varies roughly as a function of the number of dyes per dendrimer.

To enable a dansyl quantisation that cannot be influenced by self-quenching effects, the number of dyes per dendrimer was determined by MALDI-TOF MS and NMR spectroscopy. The results of these measurements are listed in Table 2.

The accuracy of the MS and NMR data for the largest multimeric system containing 128 dansyl moieties was relatively low. Thus, the results of these analyses are not given.

By using dansyl chloride as the fluorescent dye, the amino functions of the dendritic systems could be derivatised quantitatively to yield highly homogeneous products. The compounds obtained produce fluorescence signals that vary roughly as a function of the number of dyes per dendrimer with only a minor self-quenching of the fluorescence.

Conjugation of the dansyl$_{16}$-PAMAM dendrimer to hMAb425: To show the applicability of the synthesised molecules for fluorescence labelling of antibodies and other macromolecules, the dansyl$_{16}$-SH dendrimer was conjugated to the anti-EGFR antibody hMAb425 (Matuzumab) as a model compound.

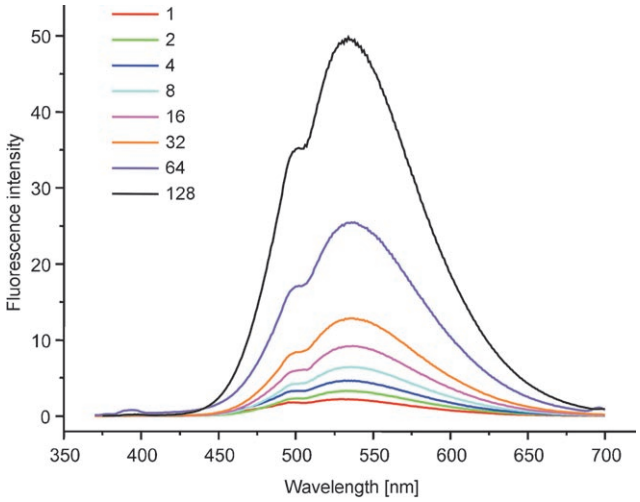


Figure 3. Fluorescence spectra of the dye monomer and the multivalent dyes containing 1, 2, 4, 8, 16, 32, 64 and 128 dansyl moieties.

Therefore, the antibody was derivatised with sulfo-SMCC (sulfo-SMCC = sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) to introduce maleimide functions. The conjugation reaction was performed in phosphate buffer with a seven-fold excess of the maleimide-*N*-hydroxysuccinimide ester following the protocol of Samoszuk et al.[57] with minor modifications.

The number of maleimides per antibody molecule was determined by treating the maleimides with an excess of MESNA (MESNA = sodium 2-mercaptoethanesulfonate).

Table 2. Characterisation of the dansylated dendrimers by MS and NMR spectroscopy.

Generation (number of amine functions)	Theoretical mass of the dansylated compound	Number of dansyl moieties determined by MS	Number of dansyl moieties determined by NMR
G_0 (1)	816	1.0	1.0
G_1 (2)	1278	2.0	2.0
G_2 (4)	1958	4.0	3.9
G_3 (8)	4048	8.0	7.8
G_4 (16)	7736	14.9	15.9
G_5 (32)	15111	28.4	31.2
G_6 (64)	28967	–	59.7

The amount of reacted thiol corresponds to the amount of maleimide and was found to be between 1.4 and 1.7 per antibody. This low number of maleimides represents a minor derivatisation of the antibody molecule, and therefore, it should not influence the integrity of the protein. Despite this low number of reactive sites, an efficient coupling reaction with the thiol-containing dendrimer was possible when the maleimide was allowed to react with a 25-fold excess of the dendritic thiol. The size-exclusion chromatogram of the reaction mixture of the maleimide-derivatised antibody with dansyl$_{16}$-SH is given in Figure 4. The derivatised antibody

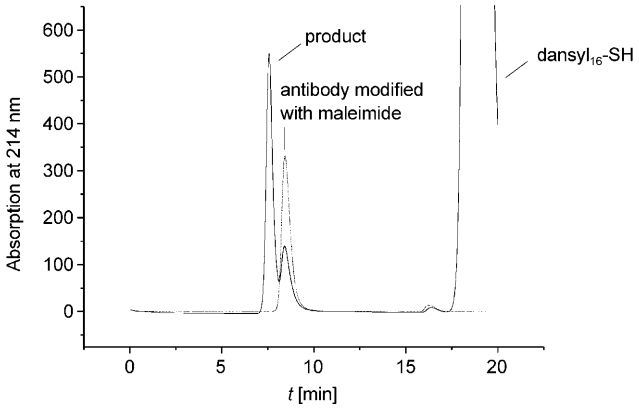


Figure 4. Size-exclusion chromatogram of the reaction mixture of sulfo-SMCC-derivatised hMAB425 with an excess of dansyl$_{16}$-SH obtained by using a Superdex 75 10/300 GL column with 0.1 M phosphate buffer (pH 7.0) as eluent at a flow rate of 1 mL min^{-1}. The FPLC analysis was performed after a reaction time of 5 min.

was isolated by size-exclusion chromatography and desalted by using a NAP-5 column.

The molar extinction coefficient of the antibody containing 16 dansyl moieties was determined and compared with a conjugate containing only one dansyl dye. A molar extinction coefficient (ε) of (31874.94 ± 644.16) L mol^{-1} cm^{-1} was found. In the case of the conjugate containing one dansyl dye, ε was determined to be (3092.95 ± 92.82) L mol^{-1} cm^{-1}, which is comparable to other dansyl-labelled proteins.[58] Consequently, the antibody labelled with the dendrimer containing 16 dansyl moieties should give much stronger fluorescence signals when used for optical imaging by exhibiting only a minor structural alteration compared with a polysubstituted antibody molecule.

To prove the exact ratio of one fluorescent dendritic structure per protein, the conjugate was characterised by mass spectrometry (Figure 5).

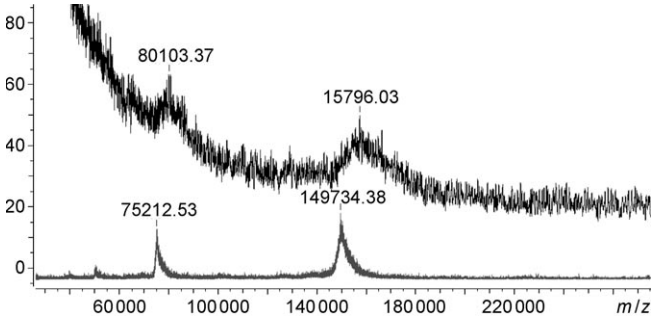


Figure 5. Mass spectra of underivatised hMAb425 and hMAb425 covalently linked to dansyl$_{16}$-SH. The shift of 7661.6 Da of the derivatised antibody molecule with one and two charges corresponds to the introduction of exactly one fluorescent dendrimer into each protein.

To prove that the functionality of the antibody is preserved, its immunoreactivity was determined. HT29 cells were incubated for 1 h with the untreated antibody or the dansyl$_{16}$-antibody conjugate at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 μg mL^{-1}. The amount of antibody or antibody conjugate bound to the cells was quantified by incubating the cells with a secondary fluorescein isothiocyanate (FITC)-labelled F(ab)′$_2$ goat-anti-human antibody for 1 h. The fluorescence of the secondary antibody was quantified by fluorescence-activated cell sorting (FACS) analysis.

By plotting the mean fluorescence intensity (MFI) versus the antibody concentration, binding curves could be obtained for the untreated antibody as well as for the dansyl$_{16}$-antibody conjugate. From these curves, the immunoreactivity of the dansylated antibody conjugate and the untreated antibody could be determined.

It was found that the immunoreactivity of the dansyl$_{16}$-antibody conjugate was 54% of that of the non-derivatised protein, which shows that the dendritic system exerts a minor modification. The dansyl$_1$-antibody conjugate synthesised for comparison showed a preserved immunoreactivity of 61%. An antibody containing 16 isolated dansyl moieties could not be obtained because the protein lost its integrity upon derivatisation.

The relatively highly preserved immunoreactivities of the dansyl$_1$- and dansyl$_{16}$-antibody conjugates show that the dansyl multimers exert only a minor effect on the biological properties of the antibody molecule. In contrast, the introduction of 16 isolated dansyl moieties leads to a loss of integrity of the antibody, which shows the superiority of the approach of introducing multimers into an antibody mole-

cule, which leads to only a minor structural modification and therefore to a minor decrease in immunoreactivity.

To show the applicability of the antibody-multimer conjugates and the multimerisation concept, fluorescence microscopy studies of the dansyl$_1$- and dansyl$_{16}$-antibody conjugates were carried out by using A431 cells. The results of these studies are shown in Figure 6. The images show a significant increase in the fluorescence signal produced by the dansyl$_{16}$-antibody conjugate (bottom) relative to the dansyl$_1$-antibody conjugate (top). This demonstrates that even though dansyl has no optimal fluorescence properties, the concept of dye multimerisation can be exploited to obtain strongly enhanced fluorescence signals of labelled biomacromolecules.

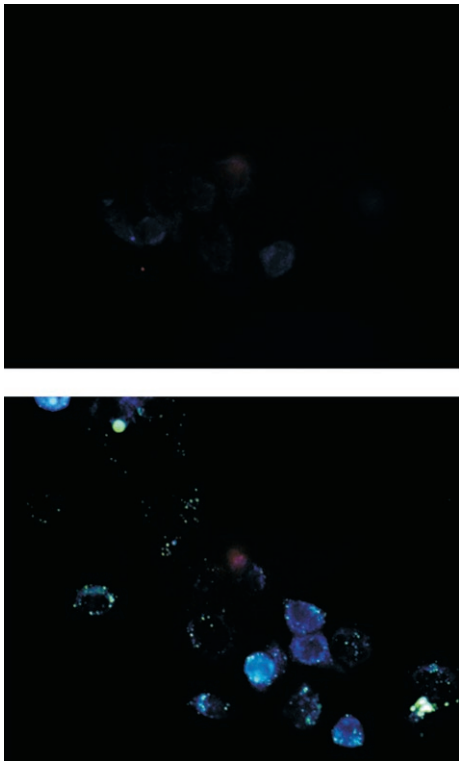


Figure 6. Fluorescence microscopy images of A431 cells treated with the dansyl$_1$-antibody conjugate (top) and the dansyl$_{16}$-antibody conjugate (bottom).

Conclusion

Fluorescent dyes are highly important for various applications in biology and medicine. Due to the fact that imaging is only possible with dyes that produce strong fluorescence signals, these applications are limited by the fluorescent properties of the particular dye. Therefore, the development of new oligomeric fluorescent labelling compounds that produce strong fluorescence signals is desirable.

The majority of the dyes studied were found to be unsuitable for this purpose. However, we have demonstrated the conjugation of dansyl chloride, NBD chloride, coumarin-343, 5(6)-carboxyfluorescein pentafluorophenyl ester and sulforhodamine B2 acid fluoride to dendritic structures containing up to 128 conjugation sites. Analysis of the fluorescence properties of these substances showed that the synthesis of fluorescent oligomers producing favourably strong fluorescence signals is possible. As shown here, dansyl-oligomers based on dendritic structures are highly effective intensive fluorescent probes and can be coupled efficiently to biomacromolecules, for example, antibodies, with a highly preserved functionality of the carrier that enables high dye concentrations and the production of strong fluorescence signals for optical imaging applications.

Because of the favourable signal amplification that can be achieved, oligomeric dyes are a valuable tool for macromolecule labelling. However, as the dansyl moiety shows suboptimal fluorescence properties, this approach could be further improved by the development of a monomeric near-infrared-emitting fluorescent dye that has a higher molar extinction coefficient and a larger quantum yield than dansyl but a similarly high reactivity.

Experimental Section

General: All commercially available chemicals were of analytical grade and used without further purification.

The analytical HPLC system used was an Agilent 1100 system and a Chromolith Performance (RP-18e, 100×4.6 mm, Merck, Germany) column. Semipreparative HPLC purifications were performed with a Gyncotech P-580 system (Germering, Germany) equipped with a variable SPD 6A UV detector and a C-R5A integrator (both Shimadzu, Duisburg, Germany). The column used was a Chromolith (RP-18e, 100× 10 mm, Merck, Germany).

Fluorescence spectra were obtained by using an AMINCO-Bowman Series 2 Luminescence Spectrometer (Thermo Electron Corporation). MALDI-TOF-TOF mass spectrometry was performed by using a Kratos Analytical Compact Maldi III system or a Bruker-Daltonik Reflex II time-of-flight instrument (Bremen, Germany). ESI mass spectra were obtained by using a Triple-Quadrupole TSQ 7000 mass spectrometer (Thermo Fisher Scientific, Bremen). NMR spectra were recorded by using Varian Mercury Plus 300 MHz and Varian NMR System 500 MHz spectrometers.

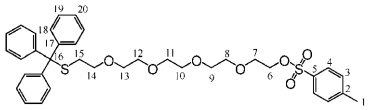
Thin-layer chromatography was performed by using Polygram SIL G/UV$_{254}$ TLC plates (Macherey-Nagel, Düren, Germany).

Size-exclusion gel chromatography was carried out by using NAP-5 columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and size-exclusion fast protein liquid chromatography (FPLC) was performed on a Superdex 200 10/30 GL column (Amersham Biosciences AB, Uppsala, Sweden).

Fluorescence microscopy images were acquired by using an Olympus IX70 inverted fluorescence microscope equipped with a digital image acquisition and processing system (analySIS 3.2, Soft Imaging System, Münster, Germany). The appropriate fluorescence filter set for DAPI was used for the detection of dansyl-labelled antibodies.

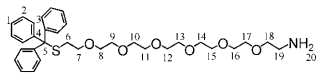
***S*-Trityl-mercaptoethyl-tetraethylene glycol tosylate**: Powdered KOH (≈3 g) was added to a solution of tetraethylene glycol ditosylate (22 g, 45 mmol), *S*-tritylmercaptoethanol (10 g, 31 mmol) and tetrabutylammonium bromide (TBAB; 1.5 g, 4.5 mmol) in toluene (200 mL) After 2 h the mixture was filtered and the resulting solution evaporated. The re-

maining oil was purified by chromatography on silica gel with *n*-hexane/acetone 2:1 as eluent (R_f=0.4). The product was obtained as a light-yellow oil (11 g, 17 mmol, 55%).



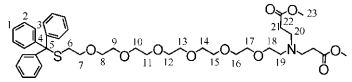
^{1}H NMR ([D$_6$]DMSO): δ=7.80–7.75 (m, 2H; 4-H), 7.49–7.44 (m, 2H; 3-H), 7.33–7.20 (m, 15H; 18-H to 20-H), 4.12–4.08 (m, 2H; 6-H), 3.57–3.53 (m, 2H; 7-H), 3.45 (brs, 4H; 10-H+11-H), 3.42 (brs, 4H; 8-H+9-H), 3.35–3.26 (m, 4H; 12-H+13-H), 3.23 (t, ^3J=6.6 Hz, 2H; 14-H), 2.40 (s, 3H; 1-H), 2.28 ppm (t, ^3J=6.6 Hz, 2H; 15-H); ^{13}C NMR ([D$_6$]DMSO): δ=150.37 (C-2), 138.09 (C-5), 135.64 (C-3), 134.67 (C-19), 133.53 (C-4), 133.12 (C-18), 132.25 (C-17), 75.94, 75.48, 75.31, 75.28, 75.20, 75.12, 75.06, 74.13, 73.44, 71.63 (C-6 to C-14), 36.97 (C-15), 26.62 ppm (C-1); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 651.2; found: 650.2.

***S*-Trityl-mercaptoethyl-pentaethylene glycol-ethylamine (G_0 dendrimer)**: Powdered KOH (≈5 g) was added to a solution of *S*-trityl-mercaptoethyl-tetraethylene glycol-tosylate (28.5 g, 59 mmol) and 2-(2-aminoethoxy)ethanol (31 g, 296 mmol) in toluene (100 mL) and DMF (10 mL). After 5 h the mixture was filtered and the resulting solution evaporated. The remaining oil was purified by chromatography on silica gel with $CHCl_3$/ethanol (5:2 as eluent (R_f=0.37)). The product was obtained as a yellow oil (15.5 g, 27 mmol, 45%).



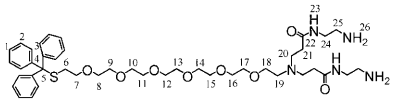
^{1}H NMR ([D$_6$]DMSO): δ=7.32 (d, ^3J=4.4 Hz, 12H; 2,3-H), 7.24 (m, 3H; 1-H), 3.49–3.32 (m, 24H; 8-H to 18-H+20-H); 3.22 (t, ^3J=6.6 Hz, 2H; 7-H), 2.69 (t, ^3J=5.7 Hz, 2H; 19-H), 2.27 ppm (t, ^3J=6.5 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=145.13 (C-4), 129.76 (C-2), 128.66 (C-3), 127.35 (C-1), 70.47, 70.44, 70.31, 70.29, 70.19, 69.38, 69.24, 66.73 (C-7 to C-18), 41.49 (C-19), 32.08 ppm (C-6); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 584.3; found: 582.8, 605.3, 621.2.

$G_{0.5}$ dendrimer: A solution of the G_0 Dendrimer (14.5 g, 25 mmol) in methanol (20 mL) was added to methyl acrylate (450 mL, 5.0 mol) and stirred for 3 d at room temperature. The volatile components were evaporated and the crude product was purified by chromatography on silica gel with *n*-hexane/acetone (4:5) as eluent (R_f=0.55). The product was obtained as a light-yellow oil (12 g, 16 mmol, 64%).



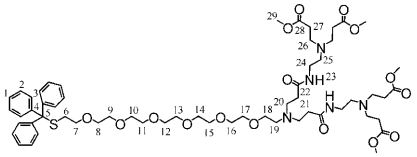
^{1}H NMR ([D$_6$]DMSO): δ=7.33–7.30 (m, 12H; 2-H+3-H), 7.27–7.21 (m, 3H; 1-H), 3.55 (s, 6H; 23-H), 3.48–3.31 (m, 22H; 8-H to 18-H), 3.22 (t, ^3J=6.6 Hz, 2H; 7-H), 2.69 (t, ^3J=6.9 Hz, 4H; 20-H), 2.54 (t, 2H; ^3J=6.1 Hz, 2H; 19-H), 2.38 (t, ^3J=6.9 Hz, 4H; 21-H), 2.27 ppm (t, 2H; ^3J=6.6 Hz, 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=172.20 (C-22), 145.10 (C-4), 129.75 (C-2), 128.68 (C-3), 127.34 (C-1), 70.48, 70.45, 70.31, 70.16, 69.38, 69.17 (C-7 to C-18), 52.17 (C-20), 50.97 (C-23), 36.50 (C-19), 33.14 (C-21), 32.12 ppm (C-6); MS (ESI): *m*/*z* calcd for [*M*+H]$^+$ 756.4; found: 756.4.

G_1 dendrimer: A solution of the $G_{0.5}$ dendrimer (13 g, 17.2 mmol) in methanol (20 mL) was added dropwise to a cooled solution of ethylenediamine (918 mL, 13.8 mol) over 0.5 h and stirred for 5 d at room temperature. Subsequently the ethylenediamine was removed by evaporation. The product was clean enough for further use and was obtained as a yellow oil (13.9 g, 17.2 mmol, 100%).



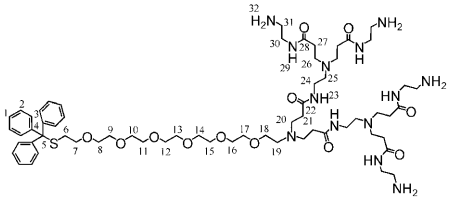
^{1}H NMR ([D$_6$]DMSO): δ=7.31 (m, 12H; 2-H+3-H), 7.24 (m, 3H; 1-H), 3.47–3.33 (m, 24H; 7-H to 18-H), 3.21 (t, ^3J=6.7 Hz, 4H; 20-H), 3.03 (q, ^3J=6.1 Hz, 4H; 19-H), 2.64 (t, ^3J=7.2 Hz, 4H; 23-H), 2.54 (m, 10H; 21-H, 23-H, 26-H), 2.27 (t, ^3J=6.7 Hz, 2H; 6-H), 2.17 ppm (t, ^3J=7.2 Hz, 4H; 25-H); ^{13}C NMR ([D$_6$]DMSO): δ=172.30 (C-22), 144.34 (C-4), 129.06 (C-2), 127.89 (C-3), 126.59 (C-1), 69.69, 69.66, 69.64, 69.53, 69.50, 69.37, 68.60, 68.44, 65.91 (C-7 to C-18), 52.20 (C-20), 48.80 (C-24), 48.56 (C-25), 36.51 (C-19), 33.13 (C-21), 31.25 ppm (C-6); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 812.5; found: 810.8.

$G_{1.5}$ dendrimer: A solution of the G_1 dendrimer (12.5 g, 15.4 mmol) in methanol (30 mL) was added to methyl acrylate (280 mL, 3.1 mol) and stirred for 3 d at room temperature. The volatile components were evaporated and the crude product was purified by chromatography on silica gel with ethanol/acetone (5:6) as eluent (R_f=0.4). The product was obtained as a light-yellow oil (6.5 g, 5.6 mmol, 37%).



^{1}H NMR ([D$_6$]DMSO): δ=7.33–7.31 (m, 12H; 2-H+3-H), 7.26–7.21 (m, 3H; 1-H), 3.56 (s, 12H; 29-H), 3.48–3.39 (m, 22H; 8-H to 18-H), 3.21 (t, ^3J=6.6 Hz, 2H; 7-H), 3.07–3.01 (q, ^3J=6.3 Hz, 4H; 24-H), 2.69–2.62 (m, 16H; 20-H+25-H+26-H), 2.54 (t, ^3J=6.1 Hz, 2H; 19-H), 2.43–2.36 (m, 12H; 21-H+27-H), 2.27 ppm (t, ^3J=6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=172.35 (C-22), 171.02 (C-28), 144.37 (C-4), 129.08 (C-2), 127.91 (C-3), 126.61 (C-1), 69.70, 69.68, 69.65, 69.59, 69.52, 69.40, 68.61, 68.45, 65.95 (C-7 to C-18), 55.93 (C-25), 52.21 (C-20), 51.09 (C-29), 49.85 (C-26), 48.80 (C-24), 36.53 (C-19), 33.16 (C-21), 31.93 (C-27), 31.28 ppm (C-6); MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 1156.6; found: 1156.8.

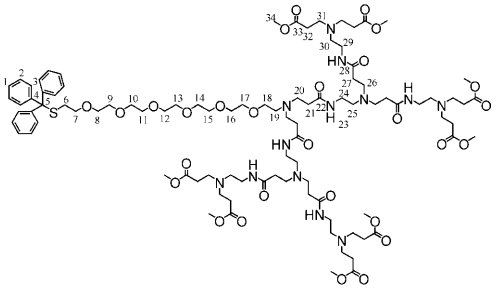
G_2 dendrimer: A solution of the $G_{1.5}$ dendrimer (6.5 g, 5.6 mmol) in methanol (20 mL) was added dropwise to a cooled solution of ethylenediamine (600 mL, 9 mol) over 0.5 h and stirred for 5 d at 4°C. Subsequently the ethylenediamine was evaporated. The product was clean enough for further use and was obtained as a yellow oil (6.7 g, 5.3 mmol, 95%).



^{1}H NMR ([D$_6$]DMSO): δ=7.33–7.31 (m, 12H; 2-H+3-H), 7.26–7.21 (m, 3H; 1-H), 3.47–3.39 (m, 22H; 8-H to 18-H), 3.34–3.31 (m, 2H; 7-H), 3.21 (t, ^3J=6.7 Hz, 2H; 23-H), 3.10–3.00 (m, 12H; 21-H+27-H), 2.70–2.50

(m, 36H; 20-H+26-H+29-H+30-H+32-H), 2.41 (t, ^3J=6.7 Hz, 4H; 24-H), 2.27 (t, ^3J=6.6 Hz, 2H; 6-H), 2.18 ppm (t, ^3J=6.9 Hz, 12H; 25-H+31-H); ^{13}C NMR ([D$_6$]DMSO): δ=171.33 (C-27), 171.08 (C-22), 144.36 (C-4), 128.98 (C-2), 127.89 (C-3), 126.60 (C-1), 69.67, 69.58, 69.52, 69.39, 68.65, 68.45 (C-7 to C-18), 52.17 (C-5), 49.89 (C-30), 49.60 (C-31), 44.35 (C-26), 42.05 (C-19), 41.18 (C-25), 36.79 (C-21+C-27), 33.23 ppm (C-6); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 1268.8; found: 1267.6, 1290.1, 1306.2.

G$_{2.5}$ dendrimer: A solution of the G$_2$ dendrimer (6.7 g, 5.3 mmol) in methanol (30 mL) was added to methyl acrylate (95 mL, 1.1 mol) and stirred for 5 d at room temperature. The volatile components were evaporated and the crude product was purified by chromatography on silica gel with ethanol/acetone (5:2) followed by 20% MeOH in CH_2Cl_2 as eluent (R_f=0.2). The product was obtained as an orange oil (3.1 g, 1.6 mmol, 30%).



^{1}H NMR ([D$_6$]DMSO): δ=7.33–7.30 (m, 12H; 2-H+3-H), 7.27–7.19 (m, 3H; 1-H), 3.65 (s, 24H; 33-H), 3.60–3.24 (m, 30H; 7-H to 18-H+24-H+29-H), 2.80 (brt, ^3J=6.7 Hz, 8H; 26-H), 2.73 (t, ^3J=6.7 Hz, 16H; 31-H), 2.61–2.51 (m, 16H; 20-H+25-H+30-H), 2.43–2.33 (m, 28H; 21-H+27-H+32-H), 2.26 ppm (t, ^3J=6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=173.01 (C-22+C-28), 172.26 (C-33), 144.78 (C-4), 129.54 (C-2), 127.80 (C-3), 126.54 (C-1), 70.50, 70.46, 70.37, 70.26, 70.08, 69.54 (C-7 to C-18), 53.79 (C-25), 52.90 (C-30), 52.53 (C-20), 51.60 (C-34), 50.16 (C-31), 49.86 (C-26), 49.21 (C-24+C-29), 37.41 (C-32), 37.15 (C-19), 33.74 (C-21), 32.64 (C-27), 31.28 ppm (C-6); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 1957.07; found: 1957.08.

G$_3$ dendrimer: A solution of the G$_{2.5}$ dendrimer (3.1 g, 1.6 mmol) in methanol (15 mL) was added dropwise to a cooled solution of ethylenediamine (340 mL, 5.1 mol) over 0.5 h and stirred for 9 d at 4°C. Subsequently the ethylenediamine was evaporated. The product was clean enough for further use and was obtained as a yellow oil (3.5 g, 1.6 mmol, 99%). MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 2181.4; found: 2183.2.

G$_{3.5}$ dendrimer: A solution of the G$_3$ dendrimer (3.5 g, 1.6 mmol) in methanol (15 mL) was added to methyl acrylate (462 mL, 5.1 mol) and stirred for 7 d at room temperature. The volatile components were evaporated and the crude product was purified by chromatography on silica gel with 55% MeCN in water containing 0.1% trifluoroacetic acid (TFA) as eluent (R_f=0.25). The product was obtained as an orange oil (4.8 g, 1.3 mmol, 83%) after lyophilisation. MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 3558.0; found: 3559.7.

G$_4$ dendrimer: A solution of the G$_{3.5}$ dendrimer (4.8 g, 1.3 mmol) in methanol (15 mL) was added dropwise to a cooled solution of ethylenediamine (570 mL, 8.5 mol) over 0.5 h and stirred for 13 d at 4°C. Subsequently the ethylenediamine was evaporated. The product was clean enough for further use and was obtained as a yellow oil (5.2 g, 1.3 mmol, 97%). MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 4006.7; found: 4007.4.

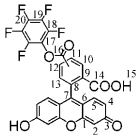
G$_{4.5}$ dendrimer: A solution of the G$_4$ dendrimer (5.5 g, 1.1 mmol) in methanol (15 mL) was added to methyl acrylate (1.3 L, 14.4 mol) and stirred for 17 d at room temperature. The volatile components were evaporated and the crude product was purified by chromatography on silica gel with MeCN containing 0.1% TFA followed by 75% MeCN in water containing 0.1% TFA and 50% MeCN in water also containing 0.1% TFA as eluent (R_f=0.1). The product was obtained as a dark-yellow oil (3.8 g, 568 μmol, 52%) after lyophilisation.

G$_5$ dendrimer: A solution of the G$_{4.5}$ dendrimer (3.8 g, 568 μmol) in methanol (15 mL) was added dropwise to a cooled solution of ethylenediamine (485 mL, 7.3 mol) over 0.5 h and stirred for 16 d at 4°C. Subsequently the ethylenediamine was evaporated. The product was clean enough for further use and was obtained as an orange oil (4 g, 522 μmol, 92%).

G$_{5.5}$ dendrimer: A solution of the G$_5$ dendrimer (4 g, 522 μmol) in methanol (15 mL) was added to methyl acrylate (602 mL, 6.7 mol) and stirred for 24 d at room temperature. The volatile components were evaporated and the crude product was purified by semipreparative HPLC with 0–100% MeCN+0.1% TFA in 7 min as gradient (R_t=2.6 min). The product was obtained as an orange oil (3.2 g, 263 μmol, 50%) after lyophilisation.

G$_6$ dendrimer: A solution of the G$_{5.5}$ dendrimer (2.6 g, 214 μmol) in methanol (15 mL) was added dropwise to a cooled solution of ethylenediamine (365 mL, 5.5 mol) over 0.5 h and stirred for 34 d at 4°C. Subsequently the ethylenediamine was evaporated. The crude product was purified by semipreparative HPLC with 0–100% MeCN+0.1% TFA in 7 min as gradient (R_t=2.1 min). The product was obtained as an orange oil (2.1 g, 150 μmol, 70%) after lyophilisation.

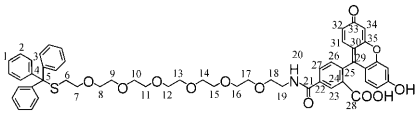
5(6)-Carboxyfluorescein pentafluorophenyl ester: A solution of *N*,*N*-dicyclohexylcarbodiimide (DCC) (603 mg, 2.93 mmol) in pyridine (5 mL) was added to a solution of 5(6)-carboxyfluorescein (1 g, 2.66 mmol) and pentafluorophenol (538 mg, 2.93 mmol) in DMF (10 mL) over 10 min and stirred for 1 h. After removal of urea and the solvent, the crude product was purified by chromatography on silica gel with water/MeCN (1:1) and 0.1% TFA as eluent (R_f=0.45 and 0.55; isomers). The product was obtained as an orange powder after lyophilisation (977 mg, 1.8 mmol, 68%).



^{1}H NMR ([D$_6$]DMSO): δ=10.05 (brs, 0.7H; 15-H), 8.64–8.63 (m, 0.5H; 10-H), 8.49 (dd, ^3J=8.1, ^4J=1.6 Hz, 0.5H; 12-H), 8.44 (dd, ^3J=8.0, ^4J=1.4 Hz, 0.3H; 11-H), 8.23 (d, ^3J=8.1, 0.5H; 12-H), 7.98 (m, 0.3H; 11-H), 7.54 (d, 0.5H; ^3J=8.1 Hz, 13-H), 6.70–6.65 (m, 4H; 4-H+5-H), 6.58–6.50 ppm (m, 2H; 2-H); ^{13}C NMR ([D$_6$]DMSO): δ=168.53 (C-3), 168.50 (C-16), 167.91 (C-14), 166.69 (C-1), 161.79 (C-3), 160.50 (C-18), 160.36 (C-18), 158.70 (C-1), 156.73 (C-20), 153.35 (C-8), 152.57 (C-19), 152.52 (C-19), 152.49 (C-7), 137.96 (C-12), 137.58 (C-5), 133.53 (C-11), 131.54 (C-4), 130.09 (C-10), 129.88 (C-10), 129.84 (C-12), 128.61 (C-9), 128.17 (C-6), 127.74 (C-11), 127.48 (C-6), 126.20 (C-5), 125.26 (C-13), 125.09 (C-13), 113.37 (C-4), 109.66 (C-17), 102.96 ppm (C-2); MS (MALDI-TOF): *m*/*z* calcd for [*M*+H]$^+$: 543.1; found: 542.5; MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 543.1; found: 543.1.

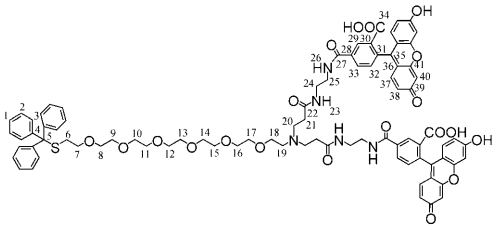
G$_0$ 5(6)-carboxyfluorescein-1: A solution of the G$_0$ dendrimer (20 mg, 34 μmol) in DMF (200 μL) was added to a solution of 5(6)-carboxyfluorescein pentafluorophenyl ester (37 mg, 69 μmol) in DMF (200 μL) and allowed to react overnight. MeCN (1 mL) and 1M NaOH (300 μL) were added to this mixture and the solvents were evaporated. After acidification with 1M HCl (500 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient (R_t=3.3 min). The product was isolated as a yellow powder (28 mg, 30 μmol, 89%) after lyophilisation.

^{1}H NMR ([D$_6$]DMSO): δ=8.88 (t, ^3J=5.3 Hz, 0.5H; 26-H), 8.73 (t, ^3J=5.4 Hz, 0.5H; 26-H), 8.46 (brs, 0.5H; 23-H), 8.23 (dd, ^3J=8.1, ^4J=1.5 Hz,



0.5H; 27-H), 8.16 (dd, $^3J=8.0$, $^4J=1.4$ Hz, 0.5H; 27-H), 7.68 (brs, 0.5H; 23-H), 7.36–7.28 (m, 12H; 2-H+3-H), 7.25–7.18 (m, 3H; 1-H), 6.69 (t, $^4J=1.8$ Hz, 2H; 34-H), 6.59–6.51 (m, 4H; 31-H+32-H), 3.58–3.29 (m, 24H; 8-H to 19-H), 3.19 (t, $^3J=6.6$ Hz, 2H; 7-H), 2.25 ppm (t, $^3J=6.6$ Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): $\delta=168.84$ (C-28), 168.72 (C-21), 165.38 (C-33), 165.26 (C-33), 160.35 (C-35), 155.25 (C-35), 153.28 (C-30), 152.54 (C-30), 145.12 (C-4), 141.24 (C-25), 136.85 (C-25), 135.30 (C-31), 130.09 (C-27), 129.90 (C-32), 129.78 (C-23), 128.66 (C-23), 127.37 (C-31), 125.53 (C-22), 124.89 (C-26), 124.02 (C-26), 122.99 (C-32), 113.39 (C-34), 109.86 (C-29), 109.80 (C-24), 102.92 (C-34), 70.42, 70.28, 70.21, 70.16, 69.43, 69.30, 69.22, 66.72 (C-7 to C-18), 32.05 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$: 942.3; found: 942.2.

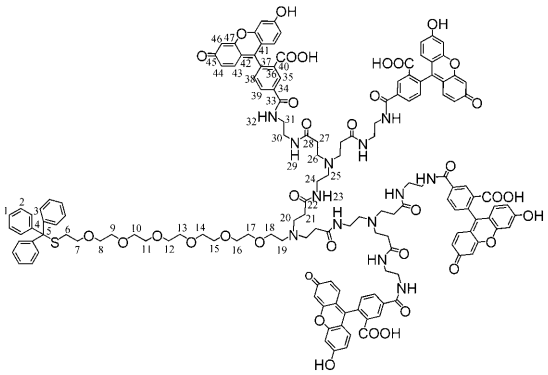
G$_1$ 5(6)-carboxyfluorescein-2: A solution of the G$_1$ dendrimer (20 mg, 25 μmol) in DMF (200 μL) was added to a solution of 5(6)-carboxyfluorescein pentafluorophenyl ester (67 mg, 124 μmol) in DMF (200 μL) and allowed to react overnight. MeCN (1 mL) and 1 M NaOH (300 μL) were added to this mixture and the solvents were evaporated. After acidification with 1 M HCl (500 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient ($R_t=$ 2.3 min). The product was isolated as a yellow powder after lyophilisation (34 mg, 22 μmol, 89%).



^{1}H NMR ([D$_6$]DMSO): $\delta=8.86$ (t, $^3J=5.1$ Hz, 1H; 32-H), 8.72 (t, $^3J=$ 5.0 Hz, 1H; 32-H), 8.44 (brs, 1H; 29-H), 8.24–8.20 (m, 1H; 33-H), 8.16–8.13 (m, 1H; 33-H), 7.64 (brs, 1H; 29-H), 7.37–7.28 (m, 12H; 2-H+3-H), 7.25–7.18 (m, 3H; 1-H), 6.69–6.68 (m, 4H; 40-H), 6.59–6.51 (m, 8H; 37-H+38-H), 3.73–3.17 (m, 42H; 7-H to 21-H+24-H+25-H), 2.25 ppm (t, $^3J=6.6$ Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): $\delta=170.25$ (C-22), 168.87 (C-34), 168.72 (C-27), 165.61 (C-39), 165.50 (C-39), 160.35 (C-41), 155.37 (C-41), 153.40 (C-36), 152.50 (C-36), 145.12 (C-4), 141.33 (C-31), 136.94 (C-31), 135.36 (C-38), 130.05 (C-33), 129.90 (C-38), 129.77 (C-29), 128.67 (C-29), 127.37 (C-37), 125.54 (C-28), 124.89 (C-32), 124.01 (C-32), 122.93 (C-38), 113.42 (C-40), 109.77 (C-35), 109.71 (C-30), 102.93 (C-40), 70.42, 70.36, 70.26, 70.13, 69.21, 66.72, 64.91 (C-7 to C-18), 52.67 (C-5), 50.16 (C-20), 32.05 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$: 1528.6; found: 1528.6.

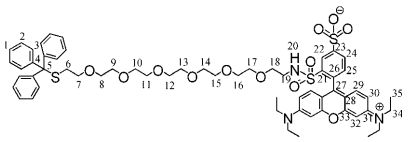
G$_2$ 5(6)-carboxyfluorescein-4: A solution of the G$_2$ dendrimer (20 mg, 16 μmol) in DMF (200 μL) was added to a solution of 5(6)-carboxyfluorescein pentafluorophenyl ester (85 mg, 158 μmol) in DMF (200 μL) and allowed to react overnight. MeCN (1 mL) and 1 M NaOH (300 μL) were added to this mixture and the solvents were evaporated. After acidification with 1 M HCl (500 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient ($R_t=$ 2.0 min). The product was isolated as a yellow powder after lyophilisation (35 mg, 13 μmol, 81%).

^{1}H NMR ([D$_6$]DMSO): $\delta=8.89$ (t, $^3J=4.8$ Hz, 2H; 38-H), 8.74 (t, $^3J=$ 4.8 Hz, 2H; 38-H), 8.44 (brs, 2H; 35-H), 8.24–7.96 (m, 4H; 39-H), 7.65 (brs, 2H; 39-H), 7.38–7.29 (m, 12H; 2-H+3-H), 7.25–7.18 (m, 3H; 1-H), 6.70 (brs, 8H; 46-H), 6.60–6.51 (m, 16H; 43-H+44-H), 3.73 (brs, 2H; 23-H), 3.52–2.52 (m, 74H; H-7 to 21-H+24-H to 27-H+30-H+31-H), 2.25 ppm (t, $^3J=6.6$ Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): $\delta=170.75$ (C-22), 170.21 (C-28), 168.87 (C-40), 168.73 (C-33), 165.66 (C-45), 165.53 (C-45), 160.37 (C-47), 155.39 (C-47), 152.51 (C-42), 145.11 (C-4), 141.30 (C-37), 136.92 (C-37), 135.33 (C-44), 130.03 (C-39), 129.88 (C-44), 129.78 (C-35), 128.66 (C-35), 127.36 (C-43), 125.51 (C-34), 124.89 (C-38), 124.05 (C-38), 113.38 (C-46), 109.75 (C-41), 109.62 (C-36), 102.97 (C-46), 70.38,



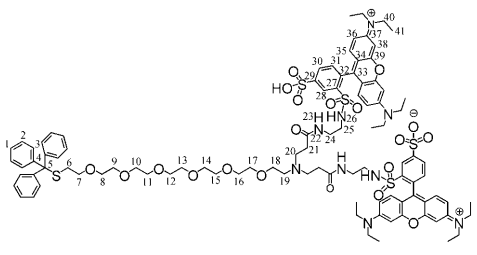
70.24, 70.13, 69.21, 66.72, 64.89 (C-7 to C-18), 49.97 (C-20), 32.05 ppm (C-6); MS (MALDI-TOF): m/z calcd for $[M+H]^+$: 2701.0; found: 2704.2; MS (ESI): m/z calcd for $[M+H]^+$: 2701.0; found: 2701.0.

G$_0$ sulforhodamine B2 acid fluoride-1: A solution of the G$_0$ dendrimer (20 mg, 34 μmol) in DMF (200 μL) was added to a solution of sulforhodamine B2 acid fluoride (58 mg, 103 μmol) and *N*,*N*-diisopropylethylamine (DIPEA) (10 μL) in DMF (200 μL) and allowed to react overnight. After acidification with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 0–100% MeCN+0.1% TFA in 10 min as gradient ($R_t=7.1$ min). The product was isolated as a violet oil after lyophilisation (27 mg, 24 μmol, 71%).



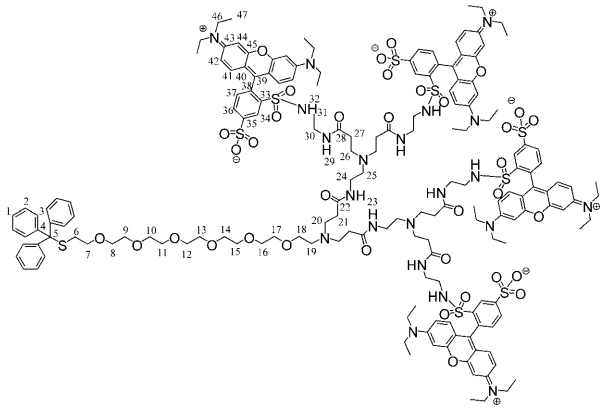
^{1}H NMR ([D$_6$]DMSO): $\delta=8.41$ (d, $^4J=1.5$ Hz, 1H; 22-H), 8.00 (dd, $^3J=$ 7.9, $^4J=1.6$ Hz, 1H; 24-H), 7.42 (d, $^3J=7.9$ Hz, 1H; 25-H), 7.31–7.28 (m, 12H; 2-H+3-H), 7.25–7.18 (m, 3H; 1-H), 7.07–6.99 (m, 4H; 29-H+30-H), 6.93 (d, $^4J=2.0$ Hz, 2H; 32-H), 3.62 (q, $^3J=7.0$ Hz, 8H; 34-H), 3.43–3.25 (m, 22H; 8-H to 18-H), 3.19 (t, $^3J=6.6$ Hz, 2H; 7-H), 2.78 (m, 2H; 19-H), 2.25 (t, $^3J=6.6$ Hz, 2H; 6-H), 1.18 ppm (t, $^3J=7.0$ Hz, 12H; 35-H); ^{13}C NMR ([D$_6$]DMSO): $\delta=157.85$ (C-26), 155.96 (C-31), 155.82 (C-33), 151.08 (C-23), 145.11 (C-4), 140.81 (C-21), 132.41 (C-29), 131.57 (C-25), 131.00 (C-27), 129.75 (C-2), 128.68 (C-3), 127.38 (C-1), 126.42 (C-24), 114.87 (C-30), 114.22 (C-28), 96.35 (C-32), 70.40, 70.27, 70.20, 70.16, 69.75, 69.20, 66.72 (C-7 to (C-18), 46.01 (C-34), 42.84 (C-19), 32.06 (C-6), 13.12 ppm (C-35); MS (ESI): m/z calcd for $[M+H]^+$: 1124.4; found: 1124.4.

G$_1$ sulforhodamine B2 acid fluoride-2: A solution of the G$_1$ dendrimer (20 mg, 25 μmol) in DMF (200 μL) was added to a solution of sulforhodamine B2 acid fluoride (83 mg, 148 μmol) and DIPEA (20 μL) in DMF (200 μL) and allowed to react overnight. After acidification with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient ($R_t=4.0$ min). The product was isolated as a violet powder (36 mg, 19 μmol, 76%) after lyophilisation.



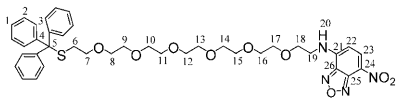
^{1}H NMR ([D$_6$]DMSO): δ=8.39 (d, ^4J=1.4 Hz, 2H; 28-H), 8.01 (dd, ^3J=7.9, ^4J=1.4 Hz, 2H; 30-H), 7.45 (d, ^3J=7.9 Hz, 2H; 31-H), 7.31–7.28 (m, 12H; 2-H+3-H), 7.26–7.18 (m, 3H; 1-H), 7.08–6.94 (m, 12H; 35-H, 36-H, 38-H), 3.72 (t, ^3J=4.7 Hz, 2H; 26-H), 3.62 (q, ^3J=7.0 Hz, 16H; 40-H), 3.54–3.29 (m, 30H; 8-H to 18-H+20-H+21-H), 3.19 (t, ^3J=6.6 Hz, 2H; 7-H), 3.01 (q, ^3J=5.7 Hz, 4H; 25-H), 2.76 (q, ^3J=5.8 Hz, 4H; 24-H), 2.57 (t, ^3J=6.3 Hz, 2H; 19-H), 2.25 (t, ^3J=6.6 Hz, 2H; 6-H), 1.19 ppm (t, ^3J=7.0 Hz, 24H; 41-H); ^{13}C NMR ([D$_6$]DMSO): δ=170.12 (C-22), 157.82 (C-32), 155.84 (C-37), 155.67 (C-39), 150.97 (C-29), 145.11 (C-4), 140.51 (C-27), 132.31 (C-35), 131.89 (C-31), 131.24 (C-33), 130.03 (C-28), 129.76 (C-2), 128.69 (C-3), 127.40 (C-1), 126.10 (C-30), 114.98 (C-36), 114.17 (C-34), 96.41 (C-38), 70.42, 70.27, 70.16, 69.21, 66.72, 64.92 (C-7 to C-18), 52.73 (C-5), 50.03 (C-20), 46.04 (C-40), 42.44 (C-19), 32.06 (C-6), 13.12 ppm (C-41); MS (ESI): m/z calcd for $[M+H]^+$: 1892.7; found: 1893.8.

G$_2$ sulforhodamine B2 acid fluoride-4: A solution of the G$_2$ dendrimer (20 mg, 16 μmol) in DMF (200 μL) was added to a solution of sulforhodamine B2 acid fluoride (88 mg, 158 μmol) and DIPEA (20 μL) in DMF (200 μL) and allowed to react overnight. After acidification with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient (R_t=3.8 min). The product was isolated as a violet powder after lyophilisation (25 mg, 7.3 μmol, 46%).



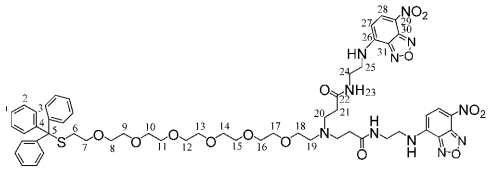
^{1}H NMR ([D$_6$]DMSO): δ=8.38 (d, ^4J=1.2 Hz, 4H; 34-H), 8.02 (dd, ^3J=7.9, ^4J=1.3 Hz, 4H; 36-H), 7.46 (d, ^3J=7.9 Hz, 4H; 37-H), 7.31–7.27 (m, 12H; 2-H+3-H), 7.24–7.19 (m, 3H; 1-H), 7.07–6.94 (m, 24H; 41-H, 42-H, 44-H), 3.75 (brs, 2H; 23-H), 3.62 (q, ^3J=6.5 Hz, 32H; 46-H), 3.56–2.60 (m, 72H; 8-H to 21-H+24-H to 27-H+30-H+31-H), 2.24 (t, ^3J=6.6 Hz, 2H; 6-H), 1.18 ppm (t, ^3J=6.7 Hz, 48H; 47-H); ^{13}C NMR ([D$_6$]DMSO): δ=170.16 (C-22+C-28), 157.82 (C-38), 155.84 (C-43), 155.54 (C-45), 150.63 (C-35), 145.10 (C-4), 140.55 (C-33), 132.30 (C-41), 131.99 (C-37), 131.39 (C-39), 129.76 (C-2), 128.69 (C-3), 127.39 (C-1), 126.09 (C-36), 114.96 (C-42), 114.15 (C-40), 96.43 (C-44), 70.41, 70.26, 70.15, 69.21 (C-7 to C-18), 46.04 (C-47), 13.12 ppm (C-48); MS (ESI): m/z calcd for $[M+H]^+$: 3429.3; found: 3430.3.

G$_0$ NBD-1: A solution of the G$_0$ dendrimer (20 mg, 34 μmol) in DMF (250 μL) was added to a solution of NBD chloride (17 mg, 86 μmol) and DIPEA (100 μL) in DMF (250 μL) and allowed to react overnight. After acidification with 1 M HCl (250 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient (R_t=3.9 min). The product was isolated as a brown oil after lyophilisation (11 mg, 15 μmol, 43%).



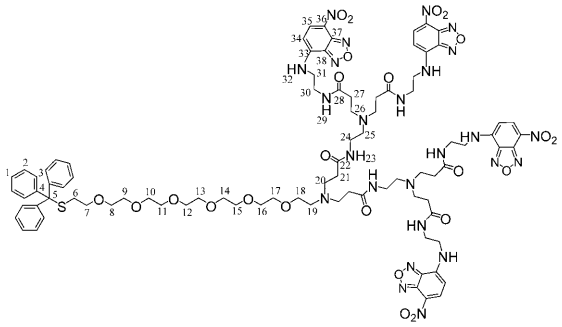
^{1}H NMR ([D$_6$]DMSO): δ=8.48 (d, ^3J=8.9 Hz, 1H; 23-H), 7.34–7.19 (m, 15H; 1-H to 3-H), 6.46 (d, ^3J=9.0 Hz, 1H; 22-H), 3.56–3.30 (m, 24H; 8-H to 18-H), 3.20 (t, ^3J=6.6 Hz, 2H; 7-H), 2.25 ppm (t, ^3J=6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=145.11 (C-4), 129.77 (C-2), 128.68 (C-3), 127.38 (C-1), 70.53, 70.42, 70.28, 70.16, 69.21, 68.56, 66.72 (C-7 to C-18), 44.10 (C-19), 32.05 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$: 747.3; found: 747.3.

G$_1$ NBD-2: A solution of the G$_1$ dendrimer (20 mg, 25 μmol) in DMF (250 μL) was added to a solution of NBD chloride (20 mg, 99 μmol) and DIPEA (100 μL) in DMF (250 μL) and allowed to react overnight. After acidification with 1 M HCl (250 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient (R_t=3.7 min). The product was isolated as a brown oil after lyophilisation (18 mg, 16 μmol, 63%).



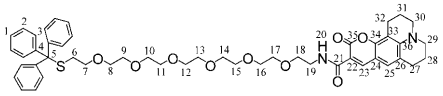
^{1}H NMR ([D$_6$]DMSO): δ=8.48 (d, ^3J=8.9 Hz, 2H; 28-H), 7.32–7.29 (m, 12H; 2-H+3-H), 7.26–7.21 (m, 3H; 1-H), 6.40 (d, ^3J=8.9 Hz, 2H; 27-H), 3.57–3.13 (m, 38H; 8-H to 18-H+20-H+21-H+24-H+25-H), 3.21 (t, ^3J=6.6 Hz, 2H; 7-H), 2.59 (t, ^3J=6.9 Hz, 2H; 19-H), 2.26 ppm (t, ^3J=6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=168.91 (C-22), 149.77 (C-30), 144.36 (C-4), 129.01 (C-2), 127.92 (C-3), 126.62 (C-1), 69.65, 69.51, 69.40, 68.46, 67.86, 64.13 (C-7 to C-18), 31.29 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$: 1138.5; found: 1138.7.

G$_2$ NBD-4: A solution of the G$_2$ dendrimer (20 mg, 16 μmol) in DMF (250 μL) was added to a solution of NBD chloride (38 mg, 190 μmol) and DIPEA (100 μL) in DMF (250 μL) and allowed to react overnight. After acidification with 1 M HCl (250 μL) the product was purified by semipreparative HPLC with 40–100% MeCN+0.1% TFA in 10 min as gradient (R_t=3.4 min). The product was isolated as a brown oil after lyophilisation (14 mg, 7.3 μmol, 46%).



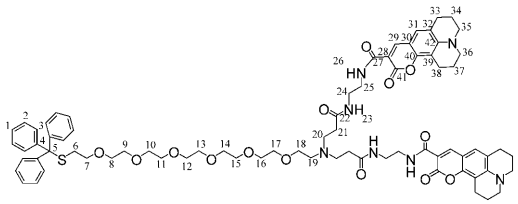
^{1}H NMR ([D$_6$]DMSO): δ=8.49–8.44 (m, 4H; 35-H), 7.32–7.28 (m, 12H; 2-H+3-H), 7.25–7.20 (m, 3H; 1-H), H6.38 (d, ^3J=8.9 Hz, 4H; 34-H), 3.74 (brt, ^3J=4.7 Hz, 2H; 20-H), 3.55–3.30 (m, 62H; 8-H to 18-H+20-H+21-H+24-H to 27-H+30-H), 3.20 (t, ^3J=6.6 Hz, 2H; 7-H), 2.64 (t, ^3J=6.8 Hz, 2H; 19-H), 2.59 (t, ^3J=6.6 Hz, 8H; 31-H), 2.25 ppm (t, ^3J=6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ=169.73 (C-22+C-28), 144.34 (C-4), 129.00 (C-2), 127.91 (C-3), 126.62 (C-1), 69.63, 69.48, 69.37, 68.44, 65.95, 64.17 (C-7 to C-18), 51.09 (C-31), 49.20 (C-20+C-26), 48.80 (C-5), 33.52 (C-27), 31.27 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$: 1920.8; found: 1921.8.

G_0 coumarin-343-1: A solution of benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP; 51 mg, 98 μmol) in DMSO (200 μL) and DIPEA (50 μL) were added to a solution of coumarin-343 (30 mg, 103 μmol) in DMSO (200 μL). After 2 min a solution of the G_0 dendrimer (30 mg, 51 μmol) in DMSO (250 μL) was added and the mixture was allowed to react overnight. After acidification with 1 M HCl (200 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 8.1 min). The product was isolated as a brown oil after lyophilisation (21 mg, 25 μmol, 48 %).



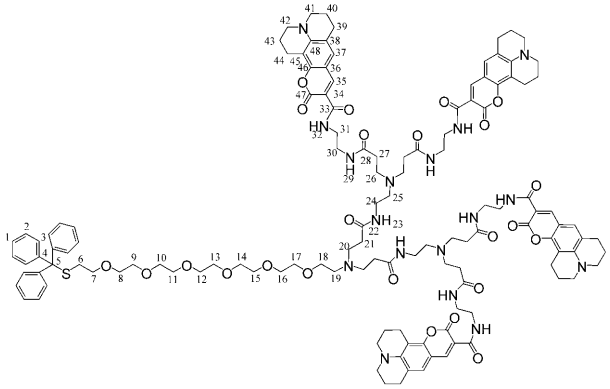
^{1}H NMR ([D$_6$]DMSO): δ = 8.78 (t, ^4J = 5.4 Hz, 1H; 25-H), 8.48 (s, 1H; 23-H), 7.31–7.28 (m, 12H; 2-H, 3-H), 7.24–7.20 (m, 3H; 1-H), 3.51–3.27 (m, 32H; 8-H to 19-H + 29-H + 30-H), 3.19 (t, ^3J = 6.6 Hz, 2H; 7-H), 2.68 (q, ^3J = 6.6 Hz, 4H; 27-H + 32-H), 2.25 (t, ^3J = 6.6 Hz, 2H; 6-H), 1.84 ppm (dt, ^3J = 5.9 Hz, 4H; 28-H + 31-H); ^{13}C NMR ([D$_6$]DMSO): δ = 162.39 (C-21), 161.77 (C-35), 151.99 (C-34), 147.89 (C-36), 147.39 (C-23), 144.37 (C-4), 129.01 (C-2), 127.91 (C-3), 127.04 (C-1), 126.62 (C-25), 119.33 (C-22), 107.68 (C-26), 107.27 (C-24), 105.94 (C-33), 69.74, 69.68, 69.61, 69.53, 69.40, 68.99, 68.46, 65.96 (C-7 to C-18), 49.44 (C-19), 48.91 (C-5), 31.29 (C-6), 26.70 (C-29 + C-30), 20.43 (C-28 + C-31), 19.48 ppm (C-27 + C-32); MS (MALDI-TOF): m/z calcd for $[M+H]^+$: 851.4; found: 849.4; MS (ESI): m/z calcd for $[M+H]^+$: 851.4; found: 851.4.

G_1 coumarin-343-2: A solution of PyBOP (92 mg, 178 μmol) in DMSO (200 μL) and DIPEA (50 μL) were added to a solution of coumarin-343 (56 mg, 197 μmol) in DMSO (200 μL). After 2 min a solution of the G_1 dendrimer (40 mg, 50 μmol) in DMSO (250 μL) was added and the mixture was allowed to react overnight. After acidification with 1 M HCl (200 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 7.6 min). The product was isolated as a brown oil after lyophilisation (37 mg, 27 μmol, 54 %).



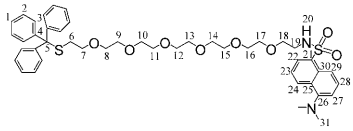
^{1}H NMR ([D$_6$]DMSO): δ = 8.73 (t, ^4J = 5.8 Hz, 2H; 31-H), 8.44 (s, 2H; 29-H), 7.32–7.29 (m, 12H; 2-H + 3-H), 7.25–7.21 (m, 3H; 1-H), 3.75 (t, ^3J = 4.7 Hz, 2H; 23-H), 3.56–3.18 (m, 48H; 7-H to 18-H + 21-H + 22-H + 24-H + 25-H + 35-H + 36-H), 2.69–2.60 (m, 10H; 19-H + 33-H + 38-H), 2.26 (t, ^3J = 6.6 Hz, 2H; 6-H), 1.89–1.82 ppm (m, 8H; 34-H + 37-H); ^{13}C NMR ([D$_6$]DMSO): δ = 169.54 (C-27), 162.80 (C-22), 161.72 (C-41), 151.97 (C-40), 147.90 (C-42), 147.31 (C-29), 144.39 (C-4), 129.02 (C-2), 127.91 (C-3), 127.02 (C-1), 126.65 (C-31), 119.33 (C-28), 107.72 (C-32), 107.27 (C-30), 104.50 (C-39), 69.68, 69.53, 69.45, 69.42, 68.48, 65.99, 64.19 (C-7 to C-18), 49.49 (C-19), 48.93 (C-5), 31.31 (C-6), 26.71 (C-35 + C-36), 20.44 (C-34 + C-37), 19.49 ppm (C-33 + C-38); MS (MALDI-TOF): m/z calcd for $[M+H]^+$: 1346.6; found: 1347.6; MS (ESI): m/z calcd for $[M+H]^+$: 1346.6; found: 1346.9.

G_2 coumarin-343-4: A solution of PyBOP (62 mg, 120 μmol) in DMSO (200 μL) and DIPEA (50 μL) were added to a solution of coumarin-343 (36 mg, 126 μmol) in DMSO (200 μL). After 2 min a solution of the G_2 dendrimer (20 mg, 16 μmol) in DMSO (250 μL) was added and the mixture was allowed to react overnight. After acidification with 1 M HCl (200 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 6.3 min). The product was isolated as a brown solid after lyophilisation (21 mg, 9 μmol, 56 %).



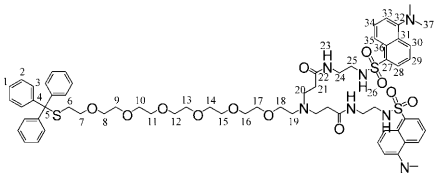
^{1}H NMR ([D$_6$]DMSO): δ = 8.73 (t, ^4J = 5.6 Hz, 4H; 37-H), 8.41 (s, 4H; 35-H), 7.32–7.30 (m, 12H; 2-H + 3-H), 7.26–7.21 (m, 3H; 1-H), 3.54–3.18 (m, 88H; 7-H to 18-H + 20-H + 21-H + 24-H to 27-H + 30-H + 31-H + 41-H + 42-H), 2.68–2.60 (m, 28H; 19-H + 39-H + 44-H), 2.25 (t, ^3J = 6.6 Hz, 2H; 6-H), 1.89–1.79 ppm (m, 16H; 40-H + 43-H); ^{13}C NMR ([D$_6$]DMSO): δ = 169.57 (C-33), 162.77 (C-22 + C-28), 161.67 (C-47), 151.91 (C-46), 147.84 (C-48) 147.25 (C-35), 144.34 (C-4), 129.00 (C-2), 127.91 (C-3), 126.98 (C-1), 126.63 (C-37), 119.26 (C-34), 107.63 (C-38), 107.24 (C-36), 104.44 (C-45), 69.63, 69.49, 69.39, 68.46 (C-7 to C-18), 49.45 (C-19), 48.89 (C-5), 26.70 (C-41 + C-42), 20.43 (C-40 + C-43), 19.48 ppm (C-39 + C-44); MS (MALDI-TOF): m/z calcd for $[M+H]^+$: 2337.1; found: 2339.9.

G_0 dansyl-1: A solution of the G_0 dendrimer (30 mg, 51 μmol) and DIPEA (50 μL) in MeCN (200 μL) was added to a solution of dansyl chloride (56 mg, 206 μmol) in MeCN (300 μL). After 0.5 h and acidification of the mixture with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 6.8 min). The product was isolated as a yellow oil after lyophilisation (26 mg, 32 μmol, 63 %).



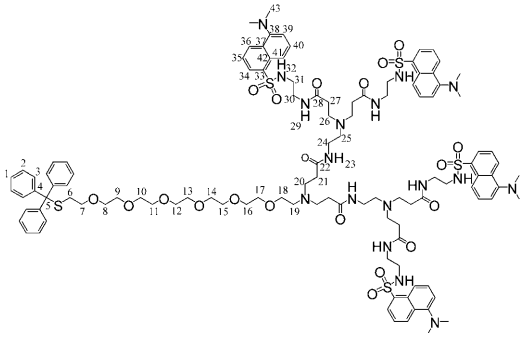
^{1}H NMR ([D$_6$]DMSO): δ = 8.45 (d, ^3J = 8.5 Hz, 1H; 24-H), 8.32 (d, ^3J = 8.7 Hz, 1H; 29-H), 8.11 (m, 1H; 22-H), 7.59 (m, 2H; 23-H + 28-H), 7.30 (d, ^3J = 4.2 Hz; 12H; 2-H + 3-H), 7.22 (m, 4H; 1-H + 27-H), 3.44–3.17 (m, 25H; 7-H to 18-H + 20-H), 2.93 (q, ^3J = 5.9 Hz, 2H; 19-H), 2.84 (s, 6H; 31-H), 2.25 ppm (t, ^3J = 6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 151.00 (C-26), 145.12 (C-4), 137.11 (C-21), 129.76 (C-2), 129.45 (C-25 + C-30), 128.77 (C-24 + C-28), 128.68 (C-3), 128.40 (C-22), 127.39 (C-1), 124.45 (C-23), 120.55 (C-29), 116.13 (C-27), 70.43, 70.40, 70.28, 70.20, 70.16, 69.64, 69.21, 66.72 (C-7 to C-18), 45.86 (C-31), 42.92 (C-19), 32.06 ppm (C-6); MS (ESI): m/z calcd for $[M+H]^+$ 817.4; found: 817.4.

G_1 dansyl-2: A solution of the G_1 dendrimer (25 mg, 31 μmol) and DIPEA (50 μL) in MeCN (200 μL) was added to a solution of dansyl chloride (67 mg, 247 μmol) in MeCN (300 μL). After 0.5 h and acidification of the mixture with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 5.8 min). The product was isolated as a yellow solid after lyophilisation (23 mg, 18 μmol, 58 %).



^{1}H NMR ([D$_6$]DMSO): δ = 8.44 (d, ^3J = 8.5 Hz, 2H; 30-H), 8.27 (d, ^3J = 8.7 Hz, 2H; 35-H), 8.16–8.01 (m, 2H; 28-H), 7.59 (m, 4H; 29-H + 34-H), 7.30 (d, ^3J = 4,3 Hz, 12H; 2-H + 3-H), 7.23 (m, 5H; 1-H + 33-H), 3.68 (t, ^3J = 4.5 Hz, 2H; 26-H), 3.50–3.22 (m, 24H; 7-H to 18-H), 3.19 (t, ^3J = 6.6 Hz, 4H; 20-H), 3.06 (q, ^3J = 6.4 Hz, 4H; 19-H), 2.81 (s, 12H; 37-H), 2.25 ppm (t, ^3J = 6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 170.10 (C-22), 151.57 (C-32), 145.11 (C-4), 136.47 (C-27), 130.03 (C-30), 129.77 (C-2), 129.60 (C-31 + C-36), 128.92 (C-34), 128.68 (C-3), 128.57 (C-28), 127.37 (C-1), 124.35 (C-29), 119.91 (C-35), 116.01 (C-33), 70.38, 70.25, 70.14, 69.20, 66.72, 64.83 (C-7 to C-18), 52.72 (C-5), 45.71 (C-37), 42.27 (C-19), 32.05 ppm (C-6); MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 1278.6; found: 1278.7.

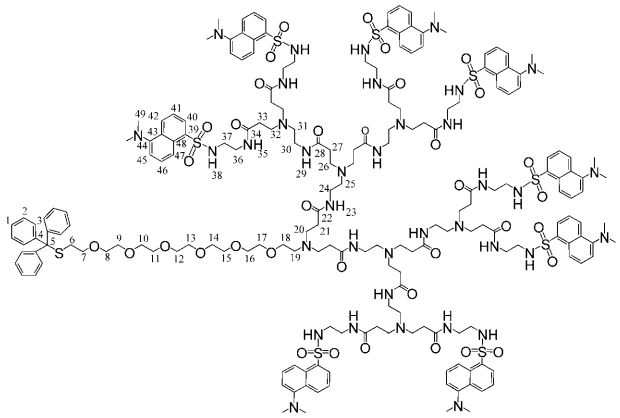
G$_2$ dansyl-4: A solution of the G$_2$ dendrimer (30 mg, 24 μmol) and DIPEA (50 μL) in MeCN (200 μL) was added to a solution of dansyl chloride (102 mg, 379 μmol) in MeCN (300 μL). After 0.5 h and acidification of the mixture with 1 M HCl (100 μL) the product was purified by semipreparative HPLC with 30–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 3.4 min). The product was isolated as a yellow solid after lyophilisation (21 mg, 9.5 μmol, 40 %).



^{1}H NMR ([D$_6$]DMSO): δ = 8.43 (d, ^3J = 8.5 Hz, 4H; 36-H), 8.26 (d, ^3J = 8.7 Hz, 4H; 41-H), 8.19–8.01 (m, 4H; 34-H), 7.58 (m, 8H; 35-H + 40-H), 7.30 (d, ^3J = 4.1 Hz, 12H; 2-H + 3-H), 7.24 (m, 6H; 1-H + 39-H), 3.70 (br s, 4H; 32-H), 3.51–2.91 (m, 52H; 7-H to 18-H + 20-H + 21-H, + 23-H to 27-H + 30-H + 31-H), 2.80 (s, 23H; 42-H), 2.61 (t, ^3J = 7.1 Hz, 2H; 19-H), 2.25 ppm (t, ^3J = 6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 170.67 (C-22), 169.98 (C-28), 151.81 (C-38), 145.11 (C-4), 136.47 (C-33), 130.11 (C-36), 129.74 (C-2), 129.67 (C-37 + C-42), 128.84 (C-40), 128.68 (C-3), 128.58 (C-34), 127.30 (C-1), 124.28 (C-35), 119.79 (C-41), 115.91 (C-39), 70.38, 70.24, 70.13, 69.20, 66.72, 64.88 (C-7 to C-18), 52.67 (C-5), 51.82, 49.95, 49.67 (C-20 + C-24 to C-26 + C-30 + C-31), 45.72 (C-43), 42.29 (C-19), 32.05 ppm (C-6); MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 2201.0; found: 2202.5.

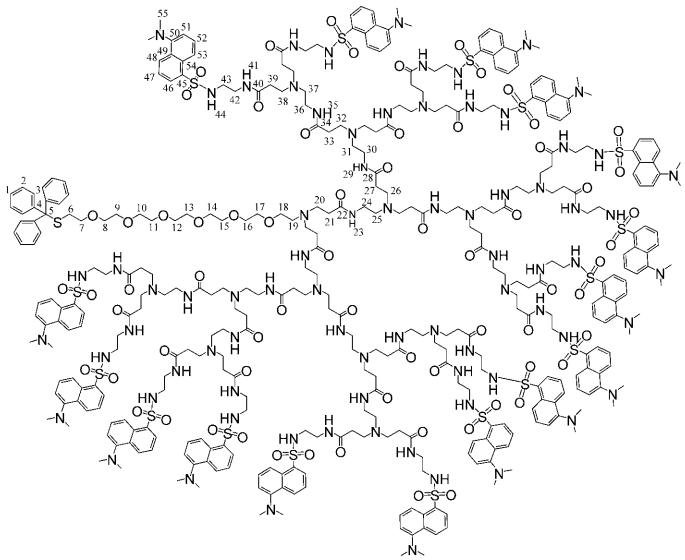
G$_3$ dansyl-8: A solution of the G$_3$ dendrimer (20 mg, 9.2 μmol) and DIPEA (50 μL) in DMF (200 μL) was added to a solution of dansyl chloride (50 mg, 183 μmol) in DMF (300 μL). After 30 min 1 M NaOH (200 μL) was added and after acidification of the mixture with 1 M HCl (150 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 6.0 min). The product was isolated as a yellow solid after lyophilisation (22 mg, 5.4 μmol, 59 %).

^{1}H NMR ([D$_6$]DMSO): δ = 8.47–8.42 (m, 8H; 42-H), 8.28–8.18 (m, 9H; 47-H), 8.08–8.00 (m, 9H; 40-H), 7.61–7.54 (m, 15H; 41-H + 46-H), 7.30–



7.26 (m, 12H; 2-H + 3-H), 7.24–7.19 (m, 8H; 1-H + 45-H), 3.52–3.04 (m, 64H; 7-H to 18-H + 20-H + 21-H + 24-H to 27-H + 30-H to 33-H + 36-H + 37-H), 2.80 (s, 47H; 49-H), 2.67–2.51 (m, 2H; 19-H), 2.24 ppm (t, ^3J = 6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 170.38 (C-22), 169.92 (C-28), 151.74 (C-44), 145.10 (C-4), 136.46 (C-39), 130.08 (C-42), 129.77 (C-2), 129.65 (C-43 + C-48), 128.87 (C-46), 128.67 (C-3), 128.56 (C-40), 127.37 (C-1), 124.29 (C-41), 119.84 (C-47), 115.94 (C-45), 70.36, 70.23, 70.12, 69.20 (C-7 to C-18), 51.80 (C-5), 49.60, 49.56, 49.46 (C-20 + C-24 to C-26 + C-30 to C-32 + C-36 + C-37), 45.72 (C-49), 42.28 (C-19), 32.04 ppm (C-6); MS (ESI): *m*/*z* calcd for [*M*+H]$^+$: 4045.8; found: 4048.0.

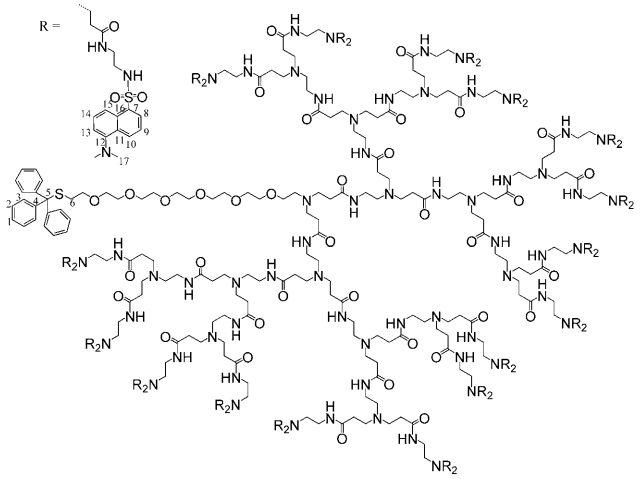
G$_4$ dansyl-16: A solution of the G$_4$ dendrimer (15 mg, 3.7 μmol) and DIPEA (50 μL) in DMF (200 μL) was added to a solution of dansyl chloride (49 mg, 180 μmol) in DMF (300 μL). After 30 min 1 M NaOH (200 μL) was added and after acidification of the mixture with 1 M HCl (150 μL) the product was purified by semipreparative HPLC with 0–100 % MeCN + 0.1 % TFA in 10 min as gradient (R_t = 5.7 min). The product was isolated as a brown solid after lyophilisation (14 mg, 1.9 μmol, 51 %).



^{1}H NMR ([D$_6$]DMSO): δ = 8.55–8.41 (m, 17H; 48-H), 8.28–8.19 (m, 16H; 53-H), 8.08–8.01 (m, 18H; 46-H), 7.61–7.54 (m, 32H; 47-H + 52-H), 7.30–7.27 (m, 12H; 2-H + 3-H), 7.23–7.18 (m, 19H; 1-H + 51-H), 3.50–3.03 (m, 133H; 7-H to 18-H + 20-H + 21-H + 24-H to 27-H + 30-H to 33-H + 36-H to 39-H + 42-H + 43-H), 2.79 (s, 95H; 55-H), 2.69–2.51 (m, 2H; 19-H), 2.24 ppm (t, ^3J = 6.6 Hz, 2H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 170.34 (C-22), 169.91 (C-28), 151.68 (C-50), 145.09 (C-4), 136.46 (C-45), 130.06 (C-

48), 129.76 (C-2), 129.65 (C-49+C-54), 128.89 (C-52), 128.64 (C-3), 128.56 (C-46), 127.36 (C-1), 124.28 (C-47), 119.87 (C-53), 115.95 (C-51), 70.35, 70.21, 70.10 (C-7 to C-18), 51.79 (C-5), 49.59, 49.54, 49.42 (C-20+C-24 to C-26+C-30 to C-32+C-36 to C-38+C-42+C-43), 45.70 (C-55), 42.28 (C-19), 32.04 ppm (C-6); MS (ESI): *m*/*z* calcd for $[M+H]^+$: 7741.8; found: 7741.0.

G_5 dansyl-32: A solution of the G_5 dendrimer (30 mg, 3.9 μmol) and DIPEA (50 μL) in DMF (200 μL) was added to a solution of dansyl chloride (85 mg, 314 μmol) in DMF (300 μL). After 30 min 1 M NaOH (200 μL) was added and after acidification of the mixture with 1 M HCl (150 μL) the product was purified by semipreparative HPLC with 20–100 % MeCN+0.1 % TFA in 10 min as gradient (R_t = 5.0 min). The product was isolated as a brown solid after lyophilisation (17 mg, 1.1 μmol, 29 %).

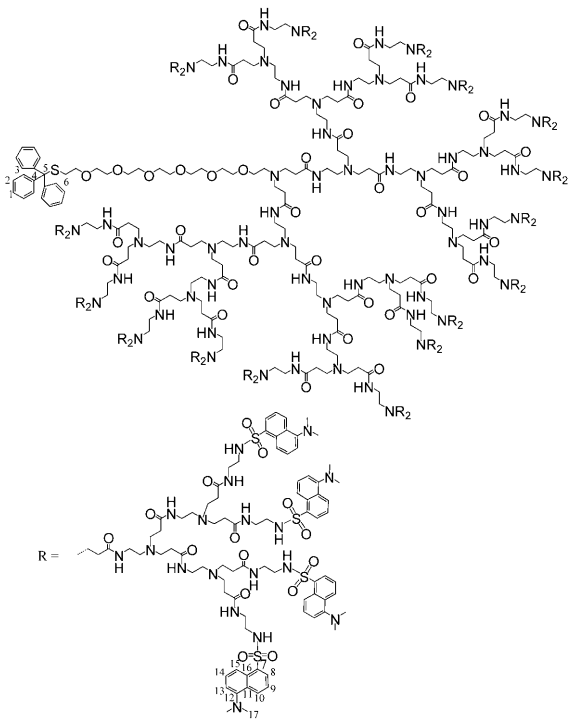


^{1}H NMR ([D$_6$]DMSO): δ = 8.55–8.40 (m, 25 H; 10-H), 8.26–8.18 (m, 29 H; 15-H), 8.06–8.00 (m, 33 H; 8-H), 7.59–7.52 (m, 62 H; 9-H+14-H), 7.31–7.20 (m, 47 H; 1-H to 3-H+13-H), 3.52–2.99 (m, 139 H), 2.79 (s, 187 H; 17-H), 2.68–2.51 (m, 2 H), 2.22 ppm (t, ^3J = 6.6 Hz, 2 H; 6-H); ^{13}C NMR ([D$_6$]DMSO): δ = 169.88; 151.92 (C-12), 136.43 (C-7), 130.05 (C-10), 129.76 (C-2), 129.53 (C-11+C-16), 128.85 (C-14), 128.63 (C-3), 128.56 (C-8), 124.22 (C-9), 119.70 (C-15), 115.83 (C-13), 70.33, 51.74 (C-5), 50.21, 49.55, 49.52, 49.31, 45.67 (C-17), 42.28 ppm; MS (MALDI-TOF): *m*/*z* calcd for $[M+H]^+$: 15 112.4; found: ≈13 950.

G_6 dansyl-64: A solution of the G_6 dendrimer (48 mg, 3.4 μmol) and DIPEA (50 μL) in DMF (200 μL) was added to a solution of dansyl chloride (119 mg, 439 μmol) in DMF (300 μL). After 30 min 1 M NaOH (200 μL) was added and after acidification of the mixture with 1 M HCl (150 μL) the product was purified by semipreparative HPLC with 25–100 % MeCN+0.1 % TFA in 10 min as gradient (R_t = 4.1 min). The product was isolated as a brown solid after lyophilisation (30 mg, 1.0 μmol, 30 %).

^{1}H NMR ([D$_6$]DMSO): δ = 8.55–8.39 (m, 60 H; 10-H), 8.27–8.18 (m, 61 H; 15-H), 8.06–8.00 (m, 63 H; 8-H), 7.58–7.52 (m, 125 H; 9-H+14-H), 7.26–7.16 (m, 79 H; 1-H to 3-H+13-H), 3.48–2.93 (m, 207 H), 2.79 (s, 359 H; 17-H), 2.61 ppm (br s, 12 H); ^{13}C NMR ([D$_6$]DMSO): δ = 170.31, 169.88, 151.78 (C-12), 136.44 (C-7), 130.20 (C-10), 129.66 (C-2), 128.87 (C-14), 128.54 (C-3), 124.22 (C-9), 119.79 (C-15), 115.87 (C-13), 51.79 (C-5), 49.53, 45.65 (C-17), 42.28 ppm (C-19).

Deprotection of G_4 dansyl-16 to dansyl$_{16}$-SH: G_4 dansyl-16 (41 mg, 5.3 μmol) was dissolved in triisopropylsilane (TIS; 200 μL) and TFA (2 mL). After 2 min the volatile components of the mixture were evaporated and the crude product was purified by semipreparative HPLC with 20–100 % MeCN+0.1 % TFA in 7 min as gradient (R_t = 2.7 min). The product was isolated as a brownish solid after lyophilisation (39 mg,



5.2 μmol, 98 %). MS (MALDI-TOF): *m*/*z* calcd for $[M+H]^+$: 7495.2; found: 7490.4.

Derivatisation of hMAb425 (Matuzumab) with sulfo-SMCC: A freshly prepared solution of sulfo-SMCC (42 μg, 9.7×10^{-8} mol) in DMF/water (1:1, 10 μL) was added to a fresh solution of hMAb425 (2 mg, 1.39×10^{-8} mol) in phosphate buffer (PB; 0.05 M, pH 7.2, 500 mL). After 1 h of incubation, the derivatised antibody was isolated and desalted by size-exclusion chromatography using NAP-5 columns (GE Healthcare Bio-Sciences AB, Uppsala). The derivatised antibody was obtained in high yields (95–98 %). The number of maleimide functions per antibody molecule was determined by reaction of the maleimides with a known excess of MESNA (sodium 2-mercaptoethanesulfonate) and subsequent quantification of the remaining thiol by Ellman's assay. The number of maleimides per antibody molecule was found to be between 1.4 and 1.7.

hMAb425-dansyl$_{16}$: A freshly prepared solution of dansyl$_{16}$-SH (4.0 mg, 2.78×10^{-7} mol) in DMSO (100 μL) was added to a solution of hMAb425 derivatised with sulfo-SMCC (1.6 mg, 1.11×10^{-8} mol) in PB (0.05 M, pH 7.2, 1 mL). The reaction was complete after 2 min and the product was purified by size-exclusion FPLC with phosphate buffer saline (PBS; 0.05 M, 0.15 M NaCl, pH 7.0) as eluent (R_t = 7.6 min) and desalted on a NAP-5 sephadex column. The product was obtained in moderate yield (23–31 %) and characterised by MALDI-TOF MS and gel electrophoresis. MS (MALDI-TOF): *m*/*z* calcd for $[M+H]^+$: 157 449.6; found: 157 396.0.

Immunoreactivities of the antibody–dendrimer conjugates

Cells: The human colon adenocarcinoma cell line HT29 expressing considerable amounts of epidermal growth factor receptor was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM pyruvate and 10 % heat-inactivated foetal calf serum. Prior to use, cells were detached from the surface of the tissue culture flask by treatment with 0.25 % trypsin plus 0.25 % ethylenediaminetetraacetic acid (EDTA) in PBS and counted.

Flow cytometry: Serial dilutions of dendrimer-conjugated Matuzumab were compared with unmodified Matuzumab for binding to HT29 cells. For this, antibody dilutions ranging from 5 μg mL^{-1} to 1 ng mL^{-1} were prepared in FACS buffer (Dulbecco's PBS with 2 % foetal calf serum and 0.1 % sodium azide). Staining was performed in a 96-well microtitre plate (U bottom). Antibody (100 μL per well) was mixed with a 50 μL

cell suspension containing 5×10^5 HT-29 cells and incubated for 1 h on ice. Cells were washed twice with FACS buffer by centrifuging the plate and siphoning off the supernatant. Subsequently, $F(ab)'_2$ goat-anti-human IgG (H+L)-FITC (100 μL per well; Jackson ImmunoResearch, West Grove, PA) diluted by 1.100 in FACS buffer was added as the second step reagent. The plate was incubated for 1 h on ice in the dark and cells were washed again twice as above. Dead cells were discriminated by staining with propidium iodide. Analysis was carried out on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany) using the CELLQUEST software.

For each dendrimer conjugate as well as for unconjugated Matuzumab a binding curve was obtained by plotting the mean fluorescence intensity (MFI) versus the antibody concentration.

Fluorescence microscopy studies of dansyl-labelled antibodies: A431 cells expressing considerable amounts of epidermal growth factor receptor were used for the experiments. 5×10^4 cells were applied on cover slips and incubated for 24 h. After washing, the cells were incubated with solutions of the labelled antibodies (0.3 nmol mL^{-1}) for 30 min at room temperature. The cells were washed and subsequently fixed with paraformaldehyde (4%) for 15 min. Fluorescence microscopy was carried out after 12 h of desiccation.

Acknowledgements

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